## 新潟大学脳研究所年報 2015



アクアポリン分子イメージング

新潟大学脳研究所で独自に開発したリガンド TGN020 を用いて、世界に先駆けて PET によるヒト脳 におけるアクアポリンの画像化に成功した。図 (a) は、TGN020 のオートラジオグラフィー (ARG) の結果で、AQP4 ノックアウトマウス (AQP4(-/-)) では野生型マウス (WT) と異なり集積を認めない。 図 (b) は、脳腫瘍 (Glioblastoma) 患者のアクアポリン PET 画像 (左:水平断、右:矢状断)で、腫 瘍本体に強い集積 (赤) を認め、悪性腫瘍におけるアクアポリンの分布特性が解析できる。 目 次

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### 1. 各分野の研究活動

I 研究組織(構成員 平成28年3月31日現在)

教授:	那波宏之	技術職員:	荒木一明
准教授:	武井延之	博士課程大学院生:	湯川尊行
助教:	難波寿明		古川和郎
助教:	岩倉百合子		斎藤摩美
特任助教:	外山英和	修士課程大学院生:	小林雄太郎

#### Ⅱ 研究活動

- 統合失調症の病因病態解明の為、動物モデルの開発解析や遺伝子解析を行っている。
  EGFモデル動物を開発し、マウス、ラット、マーモセットを用い、行動、細胞、分子レベルでの解析を行っている。
- 2) EGFファミリー分子とErbBsのリガンド-受容体相互作用、EGFファミリー分子のシェディング 機構の詳細な検討、及び神経細胞に対する作用について解析を行っている。
- 3) 霊長類のジンクフィンガー遺伝子に着目し、各霊長類の比較ゲノムから、進化による機能的変 化を検討している。
- 4) 神経細胞における翻訳機構のメカニズムを解析している。
- 5) mTORシグナル系の解析を行っている。

Ⅲ 論文(原著、総説、症例報告を区別しない)

1) Giridharan VV, Thandavarayan RA, Arumugam S, Mizuno M, Nawa H, Suzuki K, Ko KM, Krishnamurthy P, Watanabe K, Konishi T. Schisandrin B Ameliorates ICV-Infused Amyloid  $\beta$  Induced Oxidative Stress and Neuronal Dysfunction through Inhibiting RAGE/NF-  $\kappa$  B/MAPK and Up-Regulating HSP/Beclin Expression. *PLoS One.* 2015 Nov 10;10(11):e0142483. doi: 10.1371/journal.pone.0142483. eCollection 2015.

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3) Sakai M, Watanabe Y, Someya T, Araki K, Shibuya M, Niizato K, Oshima K, Kunii Y, Yabe H, Matsumoto J, Wada A, Hino M, Hashimoto T, Hishimoto A, Kitamura N, Iritani S, Shirakawa O, Maeda K, Miyashita A, Niwa S, Takahashi H, Kakita A, Kuwano R, Nawa H. Assessment of copy number variations in the brain genome of schizophrenia patients. *Mol Cytogenet*. 2015 Jul 1;8:46. doi: 10.1186/s13039-015-0144-5. eCollection 2015.

4) Kato T, Abe Y, Hirokawa S, Iwakura Y, Mizuno M, Namba H, Nawa H. Neurobehavioral Differences Between Mice Receiving Distinct Neuregulin Variants as Neonates; Impact on Sensitivity to MK-801. *Curr Mol Med.* 2015;15(3):222-36. 5) Nakashima M, Saitsu H, Takei N, Tohyama J, Kato M, Kitaura H, Shiina M, Shirozu H, Masuda H, Watanabe K, Ohba C, Tsurusaki Y, Miyake N, Zheng Y, Sato T, Takebayashi H, Ogata K, Kameyama S, Kakita A, Matsumoto N. Somatic Mutations in the MTOR gene cause focal cortical dysplasia type IIb. *Ann Neurol.* 2015 Sep;78(3):375-86. doi: 10.1002/ana.24444.

#### IV 共同研究

- (1)研究題目:「ヒトを特徴づける脳比較トランスクリプトーム・比較メチローム解析」
  研究内容:ヒト及び霊長類でジンクフィンガー遺伝子群に着目し、発現比較及びゲノムメチル化比較 を行い、ヒトを特徴づける遺伝子を探索する。
   参加機関:京都大学霊長類研究所 郷 康広
- (2)研究題目:「統合失調症におけるドパミンシグナルの変調」
  研究内容:死後脳を用いて統合失調症におけるドパミン関連分子のゲノム解析及び発現解析を行う。
  参加機関:福島県立医科大学 國井泰人
- (3)研究題目:「患者 iPS 細胞からのドパミン神経分化能力の比較」 研究内容:統合失調症患者から樹立した iPS 細胞のドパミン神経細胞への分化能力を比較する。 参加機関:理化学研究所 理化学研究所脳科学総合研究センター 吉川武男
- (4)研究題目:「霊長類をもちいた統合失調症モデル動物の作成」
  研究内容:マーモセットを用いた統合失調症モデルの樹立を目指す。
  参加機関:京都大学霊長類研究所 中村克樹

Ι	研究組織	(構成員	平成28年3月31日現在	:)

教授	﨑村	建司	実験補助	大堀	千洋
准教授	阿部	学	実験補助	望月	雪絵
助教	中務	胞	実験補助	井澤	聡子
助教	内田	仁司	実験補助	番場	彩子
特任助教	川村	名子	実験補助	石本	菜穂子
特任助教	周	麗	秘書	野澤	佳世
技術職員	櫛谷	悦子	大学院生(博士)	長澤	寿磨
技術職員	夏目	里恵	大学院生 (博士)	鈴木	康浩
研究員	鈴木	哲世	大学院生(博士)	中本	千尋
研究員	飯田	和泉	大学院生(修士)	高田	華子
実験補助	矢部	恵稚子	外国人客員研究員	彭菲	
実験補助	石川	裕利子			

Ⅱ 研究活動

本分野では脳高次機能の分子機構解明を目的として、シナプス伝達、可塑性に重要な役割を果たす 分子を標的とした遺伝子改変マウスをC57BL/6N純系胚性幹細胞を用いて作製し、その分子の生理機能 を解析している。当該年度では、科学研究費補助金「シナプスにおける逆行性シグナルによる機能的 神経回路形成の機構解明」、「高次脳領域におけるシナプス伝達制御機構の分子形態学的研究」等を受 けて研究を遂行し、グルタミン酸受容体の生理機能や、内因性カンナビノイドによるシナプス伝達制 御機構の一端を明らかにするなど、主としてシナプス伝達に関わる学術論文22本の発表に貢献した。

また「新潟大学脳研究所共同利用・共同研究」として8件の共同研究を行い、「包括型脳科学研究推進支援ネットワーク リソース・技術支援」として19件のマウス作製支援を行った。さらに、科学研究費補助金「ヒト脳機能解明への道程としての遺伝子改変ラット作製法の開発」を受け、生殖伝達系列可能なラット胚性幹細胞の開発を行った。

Ⅲ 論文(原著、総説、症例報告を区別しない)

- Hori K, Nagai T, Shan W, Sakamoto A, Abe M, Yamazaki M, Sakimura K, Yamada K, Hoshino M. : Heterozygous Disruption of Autism susceptibility candidate 2 Causes Impaired Emotional Control and Cognitive Memory. *PLoS One*. 2015 Dec 30;10(12):e0145979. doi: 10.1371/journal.pone.0145979. eCollection 2015.
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#### IV 共同研究

- (1)研究題目 「新潟大学脳研究所 共同利用・共同研究」
  研究内容 C57BL/6系統ES細胞を用いた遺伝子改変マウスの作製支援
  参加機関 自然科学研究機構、東北大学、東京大学、関西医科大学
- (2)研究題目 「包括型脳科学研究推進支援ネットワーク」研究内容 独創性の高い脳モデル動物、モデルマウス脳機能解析に特化した遺伝子改変
  - 参加機関 自然科学研究機構、京都大学、東京大学、新潟大学ほか

マウス作製支援

(3)研究題目 「シナプスにおける逆行性シグナルによる機能的神経回路形成の機構解明」
 研究内容 内因性カンナビノイド合成及び分解に関与する分子群の遺伝子改変マウス作
 製、解析、供与

参加機関 東京大学、徳島文理大学、新潟大学

- (4)研究題目 「高次脳領域におけるシナプス伝達制御機構の分子形態学的研究」
  研究内容 シナプス伝達制御に関与する分子群の遺伝子改変マウス作製、解析、供与
  参加機関 北海道大学、新潟大学
- (5)研究題目 「C57BL/6系統由来ES細胞を用いた各種遺伝子改変動物の設計と作成」
  研究内容 シナプス形成に関与する分子群の遺伝子改変マウスの作製、解析、供与
  参加機関 慶應大学、北海道大学、新潟大学
- (6)研究題目 「遺伝子改変動物の作製に有用なES細胞の作成・評価」
  研究内容 C57BL/6由来ES細胞RENKAを用いた、遺伝子改変マウス作製方法に関する新規 技術開発
  - 参加機関 株式会社トランスジェニック、新潟大学
- (7)研究題目 「mES細胞株RENKAを用いた遺伝子導入細胞の構築とそのTGマウス細胞作製能評価」
  - 研究内容 C57BL/6由来ES細胞RENKAを用いた、遺伝子改変マウス作製方法に関する新規 技術開発とその評価
  - 参加機関 タカラバイオ株式会社、新潟大学

システム脳生理学分野

I 研究組織(構成員 平成28年3月31日現在)

教授	澁木	克栄			
准教授	菱田	竜一			
助教	塚野	浩明			
助教	吉武	講平			
技術職員	丸山	佐英子			
技術職員	松島	綾乃			
博士課程大学院生					

間庭	圭一	(整形外科)
大西	毅	(麻酔科)
小木	学	(耳鼻科)

#### Ⅱ 研究活動

我々は、経頭蓋フラビン蛋白蛍光イメージングを主要な研究手段として、マウス大脳皮質感覚 野(聴覚野・視覚野・体性感覚野)の性質を解析している。また聴覚・視覚・体性感覚の個別の 感覚情報を取り扱う一次感覚野だけでなく、より高次に位置する感覚連合野の機能も解析してい る。視覚系連合野は、空間情報を取り扱う背側経路と図形情報を取り扱う腹側経路に大別される。 霊長類で知られたこの構造は、マウスにも存在し、背側経路は後部頭頂連合野を終着点とする。 後部頭頂連合野の機能としては、空間情報の短期記憶と視覚情報と体性感覚情報の統合などの具 体的な働きが判ってきた。一方、図形情報を取り扱う腹側経路連合野は、視覚野と聴覚野の境界 領域に存在し、図形情報の短期記憶と視覚情報と聴覚情報の統合などの働きを持つことが判って きた。非常に興味深いことに、この4種類の機能のいずれもが、クラスター型プロトカドへリン (cPcdh)遺伝子の多様性に依存する。即ち、12種類あるα型cPcdhクラスターのうち、α2からα11 までが欠損し、α1かα12のどちらかだけが発現するマウス(cPcdhα1,12)の解析を行ったところ、 空間情報の短期記憶、図形情報の短期記憶、視覚情報と体性感覚情報の統合、視覚情報と聴覚情 報の統合がすべて障害されることが判った。

cPcdha1,12マウスは、一見したところ異常がないように見えるが、よく観察すると4種類もの 多彩な障害が存在する。この知見をどのように解釈すべきであろうか?現在の神経科学では、そ もそも意識とは複雑な神経回路に必然的に生じる現象なのか、意識を支える特異的神経機構が存 在するのか、定説はない。意識の主要な性質を考察した統合情報理論によれば、意識とは何らか の脳内の動的短期記憶が複雑に統合されたものであると推定されている。もし意識を特異的に支 える神経機構が存在するとしたら、この神経機構を支える機能分子も存在するはずであり、この ような機能分子が障害されたら、動的短期記憶と情報統合の両者が同時に障害されることが予想 される。cPcdha1,12マウスに見られる複雑な症状は、意識を支える特異的な神経機構が存在し、 それがcPcdha1,12マウスでは障害されていると考えるとよく説明できる。この仮説については、 今後も検討を重ねる必要があるが、今まで有効な攻略法が存在しなかった意識の特異的神経機構 について、分子・細胞レベルで研究する手掛かりが得られつつあるように思える。 (1) Horie M, Tsukano H, Takebayashi H, Shibuki K, Specific distribution of non-phosphorylated neurofilaments characterizing each subfield in the mouse auditory cortex. Neurosci Lett. 606, 182-187, 2015.

(2) Watanabe T, Sasaki M, Komagata S, Tsukano H, Hishida R, Kohno T, Baba H, Shibuki K, Spinal mechanisms underlying potentiation of hindpaw responses observed after transient hindpaw ischemia in mice. Scientific Reports 5, 11191, 2015.

(3) Tsukano H, Horie M, Bo T, Uchimura A, Hishida R, Kudoh M, Takahashi K, Takebayashi H, Shibuki K, Delineation of a frequency-organized region isolated from the mouse primary auditory cortex. J .Neurophysiol. 113, 2900-2920, 2015.

(4) Meguro R, Hishida R, Tsukano H, Yoshitake K, Imamura R, Tohmi M, Kitsukawa T, Hirabayashi T, Yagi T, Takebayashi H, Shibuki K, Impaired clustered protocadherin- $\alpha$  (cPcdh- $\alpha$ ) leads to aggregated retinogeniculate terminals and impaired visual acuity in mice. J. Neurochem. 133, 66-72, 2015.

IV 共同研究

- (1)研究題目 「プロトカドヘリンの脳機能」
- 研究内容 神経特異的かつ多様性を有する細胞接着因子のプロトカドヘリンがどのような 脳機能に関与するかを解析する。

参加機関 大阪大学

- (2) 研究題目 「大脳皮質NMDA受容体の機能」
- 研究内容 大脳皮質特異的にNMDA受容体機能が半減している遺伝子改変マウスを用い、 大脳皮質NMDA受容体がどのような経験依存的可塑性や脳機能に関わるのか を解析する。

参加機関 遺伝学研究所

(3) 研究題目 「大脳皮質抑制ニューロンの機能」

研究内容 抑制性ニューロンに特異的にGFPを発現するマウスを用い、大脳皮質の抑制
 性ニューロンがどのような経験依存的可塑性や脳機能に関わるのかを解析する。
 参加機関 群馬大学

病理学分野

デジタル医学分野(統合脳機能研究センター) 脳疾患標本資源解析学分野(生命科学リソース研究センター)

I 研究組織(構成員 平成28年3月31日現在)

I-1 病理学分野

教	授	(兼)	高橋	均	技術職員	丹田智恵子
准教	女授		豊島	靖子		濁川 慎吾
助	教		他田	真理		高崎 順子
助	教		清水	宏		斎藤 春美
						藤田 知子
					事務職員	町田三保子
						上田 洋子
					大学院博士課程	竹内 亮子
						(神経内科)
						田中 英智
						齋藤 理恵
						(神経内科)
						佐藤 朋江
						(神経内科)
						清家 尚彦
						(神戸大学・神経内科)
						伊藤 絢子
						張 璐 (留学生)

I-2 デジタル医学分野(統合脳機能研究センター)
 教授柿田明美
 脳疾患標本資源解析学分野(生命科学リソース研究センター)
 教授(兼)柿田明美

#### Ⅱ 研究活動

病理学分野と脳疾患標本資源解析学分野は、共同で基礎と臨床の融合の下、生検・剖検に立脚した「人体神経病理学」を実践している。病理解剖は、24時間365日体制で行なっており、ヒト脳科学の研究発展に資する脳神経疾患標本リソースの量的、かつ質的な充実に努めている。

研究対象には、各種神経変性疾患、脳の発生のメカニズムとその異常、脳腫瘍、脳血管障害、脱 髄性疾患、さらに中毒・代謝・炎症性疾患などがある。脳神経疾患の多様性に応じた検索を基盤に 臨床病理学的研究を行うとともに、原因・機序の解明を指向した主導的、支援的共同研究に取り組 んでいる。

平成27年度における研究成果については、以下の発表論文を発表した。

Ⅲ 論文(原著、総説、症例報告を区別しない)

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- 2. Mitsui J, Matsukawa T, Sasaki H, Yabe I, Matsushima M, Dürr A, Brice A, Takashima H, Kikuchi A, Aoki M, Ishiura H, Yasuda T, Date H, Ahsan B, Iwata A, Goto J, Ichikawa Y, Nakahara Y, Momose Y, Takahashi Y, Hara K, Kakita A, Yamada M, Takahashi H, Onodera O, Nishizawa M, Watanabe H, Ito M, Sobue G, Ishikawa K, Mizusawa H, Kanai K, Hattori T, Kuwabara S, Arai K, Koyano S, Kuroiwa Y, Hasegawa K, Yuasa T, Yasui K, Nakashima K, Ito H, Izumi Y, Kaji R, Kato T, Kusunoki S, Osaki Y, Horiuchi M, Kondo T, Murayama S, Hattori N, Yamamoto M, Murata M, Satake W, Toda T, Filla A, Klockgether T, Wüllner U, Nicholson G, Gilman S, Tanner CM, Kukull WA, Stern MB, Lee VM, Trojanowski JQ, Masliah E, Low PA, Sandroni P, Ozelius LJ, Foroud T, Tsuji S. Variants associated with Gaucher disease in multiple system atrophy. Ann Clin Transl Neurol 2015; 2 (4): 417-426.
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- 4. 清水 宏,柿田明美. 頭蓋咽頭腫 Craniopharyngioma. 病理診断クイックリファレンス.病理と臨床,文光堂,東京,p. 332.
- 5. 豊島 靖子. ALS (ブニナ小体) Amyotrophic lateral sclerosis (Bunina bodies). 病理診断クイッ クリファレンス.病理と臨床,文光堂,東京, p. 341.
- 6. 豊島 靖子. 多発性硬化症 Multiple sclerosis (MS). 病理診断クイックリファレンス. 病理 と臨床,文光堂,東京, p. 342.
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  - IV 共同研究

病理学分野・脳疾患標本資源解析学分野は、文部科学省認定の新潟大学脳研究所「脳神経病理標本資源活用の先端的共同研究拠点」の中核分野として、ヒト脳科学に関するプロジェクト型および 連携資源利用型の国内共同研究を推進している。

- (1) 研究題目 「神経変性疾患に関する神経病理学的研究」
  - 研究内容 神経変性疾患、とくに進行性核上性麻痺(タウオパチー)、パーキンソン病 (シヌクレイノパチー)や筋萎縮性側索硬化症(TDP-43プロテイノパチーの臨 床病理や病因解明に連なる共同研究を行なっている。

参加機関 弘前大学、東京都医学総合研究所、岐阜大学・岐阜薬科大学 信州大学、東京女子医科大学、愛知医科大学、京都大学

(2) 研究題目 「難治てんかん原性病巣に関する外科病理標本の解析」

研究内容 難治てんかん原性病巣の病態形成機序の解明を目的に、各種病態(限局性皮 質形成異常、結節性硬化症など)の切除脳組織を用いた病理組織学的、生化 学的、生理学的解析を進めている。

- 参加機関 国立病院機構西新潟中央病院、国立精神・神経医療研究センター病院、 広島大学、国立成育医療センター病院
- (3) 研究題目 「精神神経疾患の分子病理学的解析」
  - 研究内容 精神神経疾患の剖検脳を対象とした臨床病理、及び分子病理学的病態解析のための凍結脳標本資源を提供することで、精神神経疾患、とくに統合失調症の 病態形成機序の解析を進めている。
  - 参加機関 筑波大学、福島県立医科大学、理化学研究所

分子病態学(客員)分野

I 研究組織(構成員 平成28年3月31日現在)

教 授(併) 若林 孝一

准教授(併) 森 文秋

#### Ⅱ 研究活動

当分野では、神経難病の病態解明を目標に、病理形態学、分子生物学、病態生化学などの手法を用い研究を進めている。神経変性疾患の多くはタンパク質蓄積病であることから、「タンパク質の結合・ 修飾・分解」の観点からアプローチを行っている。さらに、「封入体形成」や「神経細胞死」だけでな く、神経症状の発現に重要な部位として「シナプス」の変化にも焦点を当てている。

現在の研究テーマは、1)神経変性疾患における封入体形成メカニズム、2)グリア細胞の機能と 各種病態における変化、3)遺伝子改変モデル動物を用いた病態解析である。特に、シヌクレイノパ チー(パーキンソン病、レビー小体型認知症、多系統萎縮症)やポリグルタミン病の剖検脳組織を用 いた研究は病理学分野や脳疾患標本資源解析学分野と共同で進めている。

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#### Ⅳ 共同研究

(1)	研究題目	「神経変性疾患におけるオートファジーの異常」		
	研究内容	神経変性疾患、特にレビー小体病や多系統萎縮症におけるオートファジーの異常		
について、剖検脳組織やモデル動物を用い研究を進めている。				
	参加機関	弘前大学脳神経病態研究施設脳神経病理学講座、高度先進医学研究センター、		
		ジョージア医科大学分子シャペロンセンター		

#### 脳神経外科学分野

I 研究組織 (構成員 平成28年3月31日現在)

教授	藤井幸彦			
准教授	岡本浩一郎	ß		
助教	青木 洋			
博士課程大学院生	岡田正康,	鈴木倫明,	藤原秀元,	塚本佳広,
	中山瑶子,	野村俊春,	佐藤圭輔	

#### Ⅱ 研究活動

[基礎研究]

- 1. オートファジーに注目した悪性神経膠腫に対する新たな治療法の展開.
- 2. MGMT活性, IDH1 mutation, p53など, 脳腫瘍の遺伝子解析に基づいた悪性神経膠腫の病態研究.
- 3. 近赤外分光法を用いたヒト大脳皮質間の伝播現象に関する研究
- 4. Multi-parametric MRIを用いた虚血性脳血管障害の病態解析
- 5. Neuromodulationを用いた運動感覚野皮質における機能代償機転の解明(fMRI, TMSを用いた研究)
- 6. ラット脳幹腫瘍モデルに対するconvection-enhanced delivery法に関する研究.
- 7. 先進的3次元工学を活用した脳神経外科手術シミュレーション・トレーニングシステムの開発.

[臨床研究]

- 1. 遺伝子解析情報を基盤とした悪性神経膠腫に対する化学療法効果および予後の推定
- 2. リツキシマブを使用した中枢神経原発悪性リンパ腫に対する新たな化学療法プロトコールの確立
- 3. 高齢者脳腫瘍患者に対する少分割照射法の確立とその効果判定
- 4. 小児奇形としての水頭症に対する神経内視鏡下第3脳室底開窓術の長期成績判定
- 5. 新規デバイスを導入した新世代血管内治療法の確立、その効果と合併症および安全性の評価
- 6. 頭蓋底部腫瘍の積極的摘出に必要な微小解剖・生理解剖・脈管解剖に関する超高解像度画像診断 を基調とした生体的研究

Ⅲ 論文(原著,総説,症例報告を区別しない)

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Ⅳ 共同研究

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- てんかん原性獲得の機序解明に関する研究 新潟大学脳研究所 国立病院機構西新潟中央病院

I 研究組織(構成員 平成28年3月31日現在)

教授 西澤 正豊 准教授 下畑 享良 河内 泉 講師 助教 高橋 哲哉 助教 赤岩 靖久 助教 他田 正義 助教 金澤 雅人 助教 堅田 慎一 特任助教 徳武 孝允 佐治 越爾 特任助教 特任助教 中野 仁美 救急部 特任助教 二宮 格 技術職員 金子 三津子、川口 さやか、小山 美咲, 大野 萌

博士課程大学院生 樋口 真也、會田 泉、遠藤 寿子、竹内 亮子、伊藤 岳、 穂苅 万李子、鳥谷部 真史、 目崎 直実、三浦 健、下畑 敬子、佐藤 朋江、齋藤 理恵、宇津見 宏太、上村 昌寛、 柳村 文寛、畠山 公大、飛永 雅信、竹島 明、堅田 直子、小池 佑佳、石黒 敬信

修士課程大学院生

藤田 菜摘、高山 幹大

Ⅱ 研究活動

【脳梗塞に対する血管保護療法の開発】

1) 研究の概要

下畑享良を中心とする研究グループ(高橋哲哉、金澤雅人ら)は、血管保護作用に加え、神経細胞 保護作用、炎症抑制作用を合わせもつ従来にない脳保護薬の候補分子としてプログラニュリンを同定 し(Brain 2015;138:1932-1948)、国際特許出願(PCT出願)を行った。新聞報道(新潟日報1面)さ れた。

2) 研究の成果

(論文)

- Kanazawa M, Kawamura K, Takahashi T, Miura M, Tanaka Y, Koyama M, Toriyabe M, Igarashi H, Nakada T, Nishihara M, Nishizawa M, Shimohata T\*. Multiple therapeutic effects of progranulin on experimental acute ischaemic stroke. Brain 2015;138:1932-1948.
- 2. 下畑享良,金澤雅人,川村邦雄,高橋哲哉,西澤正豊. tPA 療法後の脳出血防止を目指したト ランスレーショナルリサーチ.脳循環代謝2015;26:93-97.
- 3. 高橋哲哉, 下畑享良, 西澤正豊. 虚血耐性現象の神経保護メカニズムと治療への応用. 脳循環 代謝2015;26:197-202.

(特許出願) 虚血後の再灌流に起因する出血を予防するための薬剤

【多系統萎縮症の突然死予防を目的としたチーム医療モデルの構築】

研究の概要

多系統萎縮症患者を対象に睡眠呼吸障害の機序の解明、および睡眠呼吸障害に伴う突然死の予防を目 的とした臨床研究。とくに神経内科、呼吸器内科、耳鼻咽喉科、循環器内科、摂食・嚥下機能回復部 がチームを組織しMSA患者の診療に当たるという新しいチーム医療モデルを提唱した。本年度は本疾 患では嚥下障害を認める場合には高率に食道病変を合併し、突然死の原因になりうることを初めて明 らかにした(Dysphagia 2015;30:669-673.に発表)。

2) 研究の成果

2001年から進めている研究で、14年間で16の英文原著論文を報告した。

(論文)

- 1. Taniguchi H, Nakayama H, Hori K, Nishizawa M, Inoue M, Shimohata T. Esophageal Involvement in Multiple System Atrophy. Dysphagia 2015;30:669-673.
- 下畑享良, 饗場郁子, 西澤正豊. 【内科疾患の診断基準・病型分類・重症度】神経・筋 多系統 萎縮症,進行性核上性麻痺,大脳皮質基底核変性症. 内科 2015;115:1203-1209.

【低血糖脳症に対する治療薬開発に関する研究】

1)研究の概要

低血糖脳症に対する治療薬は、ブドウ糖を除き臨床応用されていない。下畑享良を中心とする研究 グループ(池田哲彦先生ら)は、治療薬スクリーニングを可能とする簡便なげっ歯類モデルを開発し、 さらにアルデヒドデヒドロゲナーゼ2アゴニストであるAlda-1が、低血糖脳症に対する脳保護薬とし て有望であることを初めて明らかにした(PLOS ONE 2015;10:e0128844に発表)。新聞報道された。

#### 2) 研究の成果

(論文)

- Ikeda T, Takahashi T, Tsujita M, Kanazawa M, Toriyabe M, Koyama M, Itoh K, Nakada T, Nishizawa M, Shimohata T. Effects of Alda-1, an Aldehyde Dehydrogenase-2 Agonist, on Hypoglycemic Neuronal Death. PLOS ONE 2015;10:e0128844.
- 2. 下畑享良,池田哲彦,高橋哲哉,金澤雅人,西澤正豊. 低血糖脳症の臨床―病態の理解と創 薬に向けて―. 新潟医学会雑誌 2015;129:350-354.
- 3. 下畑享良,池田哲彦,西澤正豊. 低血糖脳症一重症化の予防を目指して一. 糖尿病診療マス ター 2015;13:653-657.

(特許出願)低血糖脳症モデル及びその製造方法,並びに,神経保護剤のスクリーニング方法, 及び神経保護剤

【多発性硬化症・視神経脊髄炎の病態メカニズムの検討】

1) 研究の概要

多発性硬化症(multiple sclerosis: MS)と視神経脊髄炎(neuromyelitis optica: NMO)は中枢 神経系炎症性自己免疫疾患である。これまでに河内泉を中心とする研究グループは、本邦のNMO症例、 特に限局型NMO症例の臨床免疫学的・病理学的特徴を明らかにしてきた(Neurology 2009;73:1628)。 引き続き、NMOにおける認知機能障害の臨床的心理学的・病理学的特徴を解析し、その発症機序を世 界に先駆けて発表した(Annals of Neurology 2013;73:65)。さらにNMOのミトコンドリア蓄積を伴う 神経変性の詳細を明らかにした(Annals of Neurology 2016;79:605)。またMSに関しては、新規治療 薬フィンゴリモドによる髄腔内免疫細胞動態を可視化し、服用早期におけるMS再発のリスク因子を解 析した (Multiple Sclerosis Journal 2013;19(9):1230-1233)。さらにMSとNMOの免疫現象の詳細を 検討中である。

- 2) 研究の成果
  - Hokari M, Yokoseki A, Arakawa M, Saji E, Yanagawa K, Yanagimura F, Toyoshima Y, Okamoto K, Ueki S, Hatase T, Ohashi R, Fukuchi T, Akazawa K, Yamada M, Kakita A, Takahashi H, Nishizawa M, Kawachi I. Clinicopathological features in anterior visual pathway in neuromyelitis optica. Annals of Neurology 2016;79(4):605-624. doi: 10.1002/ana.24608. PMID: 26836302.
  - 2. Izumi Kawachi, Masatoyo Nishizawa. Cortical gray matter lesions in multiple sclerosis. Brain Nerve 2015;67(4):497-504. doi: 10.11477/mf.1416200166.
  - Kawachi I, Nishizawa M. Significance of gray matter brain lesions in multiple sclerosis and neuromyelitis optica. Neuropathology 2015;35(5):481-486. doi:10.1111/neup.12216.
  - Yamasaki R, Matsushita T, Fukazawa T, Yokoyama K, Fujihara K, Ogino M, Yokota T, 4. Miyamoto K, Niino M, Nomura K, Tomioka R, Tanaka M, Kawachi I, Ohashi T, Kaida KI, Matsui M, Nakatsuji Y, Ochi H, Fukaura H, Kanda T, Nagaishi A, Togo K, Mizusawa H, Murai H, Kira JI. Efficacy of intravenous methylprednisolone pulse therapy in patients with multiple sclerosis and neuromyelitis optica. Mult Scler. 2015 Nov 12. PMID: pii: 1352458515617248. Epub ahead print]. 26564994. of doi: 10.1177/1352458515617248.
  - 5. Ogino M, Kawachi I, Otake K, Ohta H, Otsuka Y, Iwasaki K, Hiroi S. Current treatment status and medical cost for multiple sclerosis based on analysis of a Japanese claims database. Clinical and experimental neuroimmunology 2016 Article first published online: 23 MAR 2016. doi: 10.1111/cen3.12299.
  - Yajima R, Utsumi K, Ishihara T, Kanazawa M, Okamoto K, Kawachi I, Nishizawa M. Varicella-zoster virus encephalitis localized to the bilateral medial temporal lobes. Neurol Neuroimmunol Neuroinflamm. 2015 May 7;2(4):e108. doi: 10.1212/NXI.000000000000108. eCollection 2015.
  - 7. Kuroha Y, Tada M, Kawachi I, Nishizawa M, Matsubara N, Koike R. Effect of sodium pyruvate on exercise intolerance and muscle weakness due to mitochondrial myopathy: a case report. Rinsho Shinkeigaku 2015;55(6):412-416. doi: 10.5692/clinicalneurol.cn-000652.
  - 8. 柳村文寛,河内泉,西澤正豊. NMOの診断と治療-NMOのあらたな診断基準と治療薬. 医学のあ ゆみ 2015;255(5):390-396.
  - 9. 河内泉, 西澤正豊. 「神経変性」の視点から中枢神経系自己免疫疾患を考える. Medical science digest 2015;4(14):541-543.

【免疫介在性肥厚性硬膜炎の臨床像に関する検討】

1) 研究の概要

河内泉を中心とする研究グループは、免疫介在性肥厚性硬膜炎の臨床免疫学的・病理学的特徴を検討し、特にANCA関連疾患群において新たな亜型の存在を明らかにした(Brain 2014;137(2):520-536)。 引き続き、臨床研究を推進している。

#### 2) 研究の成果

 Yajima R, Toyoshima Y, Wada Y, Takahashi T, Arakawa H, Ito, Kobayashi D, Yamada M, Kawachi I, Narita I, Takahashi H, Nishizawa M. A fulminant case of granulomatosis with polyangiitis with meningeal and parenchymal involvement. Case Rep Neurol 2015;7:101-104. DOI: 10.1159/000381942

【NMDA受容体抗体脳炎をはじめとした傍腫瘍性・自己免疫性神経症候群の臨床像に関する検討】

1) 研究の概要

河内泉を中心とする研究グループはペンシルバニア大学のJosep Dalmau教授との共同研究により、 NMDA 受 容 体 抗 体 脳 炎 の 長 期 治 療 予 後 を 解 析 し 、 Lancet Neurology 誌 (Lancet Neurology 2013;12(2):157-65. doi: 10.1016/S1474-4422(12)70310-1) 、 Neurology 誌 (Neurology 2013; 81(12):1058-1063. DOI: 10.1212/WNL.0b013e3182a4a49c.) に報告した。さらに他の傍腫瘍性・自己 免疫性神経症候群を含めて臨床免疫像の解析を進め、新しい標的抗体を発見し、Neurology誌に掲載 が決定している。

【POEMS症候群のサリドマイド治療に関する検討】

1) 研究の概要

河内泉、西澤正豊を中心とする研究グループは千葉大学の桑原聡教授らとの共同研究により、POMS 症候群に対するサリドマイド治療の開発を行っている。

- 2) 研究の成果
  - Katayama K, Misawa S, Sato Y, Sobue G, Yabe I, Watanabe O, Nishizawa M, Kusunoki S, Kikuchi S, Nakashima I, Ikeda S, Kohara N, Kanda T, Kira J, Hanaoka H, Kuwabara S; J-POST Trial study investigators (Aoyagi R, Tanemura N, Inamata C, Sekiguchi Y, Shibuya K, Mitsuma S, Watanabe K, Iwai Y, Kawagashira Y, Koike H, Shirai S, Sasaki H, Futagawa T, Ushinohama K, Kawachi I, Mitsui Y, Kato M, Suzuki H, Niino M, Fujihara K, Tateyama M, Katoh N, Kawamoto M, Koga M, Matsuse D, Yamasaki R). Japanese POEMS syndrome with Thalidomide (J-POST) Trial: study protocol for a phase II/III multicentre, randomised, double-blind, placebo-controlled trial. BMJ Open. 2015 Jan 8;5(1):e007330. doi: 10.1136/bmjopen-2014-007330.

#### Ⅲ 論文(原著、総説、症例報告を区別しない)

- (1) Kanazawa M, Kawamura K, Takahashi T, Miura M, Tanaka Y, Koyama M, Toriyabe M, Igarashi H, Nakada T, Nishihara M, Nishizawa M, Shimohata T\*. Multiple therapeutic effects of progranulin on experimental acute ischaemic stroke. Brain 2015;138:1932-1948.
- (2) 下畑享良,金澤雅人,川村邦雄,高橋哲哉,西澤正豊. tPA 療法後の脳出血防止を目指したトランスレーショナルリサーチ.脳循環代謝2015;26:93-97.
- (3) 高橋哲哉,下畑享良,西澤正豊. 虚血耐性現象の神経保護メカニズムと治療への応用. 脳循環代 謝2015;26:197-202. Taniguchi H, Nakayama H, Hori K, Nishizawa M, Inoue M, Shimohata T. Esophageal Involvement in Multiple System Atrophy. Dysphagia 2015;30:669-673.
- (4) 下畑享良, 饗場郁子, 西澤正豊. 【内科疾患の診断基準・病型分類・重症度】神経・筋 多系統萎縮症,進行性核上性麻痺,大脳皮質基底核変性症. 内科 2015;115:1203-1209.

- (5) Ikeda T, Takahashi T, Tsujita M, Kanazawa M, Toriyabe M, Koyama M, Itoh K, Nakada T, Nishizawa M, Shimohata T. Effects of Alda-1, an Aldehyde Dehydrogenase-2 Agonist, on Hypoglycemic Neuronal Death. PLOS ONE 2015;10:e0128844.
- (6) 下畑享良,池田哲彦,高橋哲哉,金澤雅人,西澤正豊.低血糖脳症の臨床―病態の理解と創薬に 向けて―.新潟医学会雑誌 2015;129:350-354.
- (7) 下畑享良,池田哲彦,西澤正豊.低血糖脳症―重症化の予防を目指して―.糖尿病診療マスター 2015;13:653-657.
- (8) Hokari M, Yokoseki A, Arakawa M, Saji E, Yanagawa K, Yanagimura F, Toyoshima Y, Okamoto K, Ueki S, Hatase T, Ohashi R, Fukuchi T, Akazawa K, Yamada M, Kakita A, Takahashi H, Nishizawa M, Kawachi I. Clinicopathological features in anterior visual pathway in neuromyelitis optica. Annals of Neurology 2016;79(4):605-624. doi: 10.1002/ana.24608. PMID: 26836302.
- (9) Izumi Kawachi, Masatoyo Nishizawa. Cortical gray matter lesions in multiple sclerosis. Brain Nerve 2015;67(4):497-504. doi: 10.11477/mf.1416200166.
- (10) Kawachi I, Nishizawa M. Significance of gray matter brain lesions in multiple sclerosis and neuromyelitis optica. Neuropathology 2015;35(5):481-486. doi:10.1111/neup.12216.
- (11) Yamasaki R, Matsushita T, Fukazawa T, Yokoyama K, Fujihara K, Ogino M, Yokota T, Miyamoto K, Niino M, Nomura K, Tomioka R, Tanaka M, Kawachi I, Ohashi T, Kaida KI, Matsui M, Nakatsuji Y, Ochi H, Fukaura H, Kanda T, Nagaishi A, Togo K, Mizusawa H, Murai H, Kira JI. Efficacy of intravenous methylprednisolone pulse therapy in patients with multiple sclerosis and neuromyelitis optica. Mult Scler. 2015 Nov 12. pii: 1352458515617248. [Epub ahead of print]. PMID: 26564994. doi: 10.1177/1352458515617248.
- (12) Ogino M, Kawachi I, Otake K, Ohta H, Otsuka Y, Iwasaki K, Hiroi S. Current treatment status and medical cost for multiple sclerosis based on analysis of a Japanese claims database. Clinical and experimental neuroimmunology 2016 Article first published online: 23 MAR 2016. doi: 10.1111/cen3.12299.
- (13) Yajima R, Utsumi K, Ishihara T, Kanazawa M, Okamoto K, Kawachi I, Nishizawa M. Varicella-zoster virus encephalitis localized to the bilateral medial temporal lobes. Neurol Neuroimmunol Neuroinflamm. 2015 May 7;2(4):e108. doi: 10.1212/NXI.000000000000108. eCollection 2015.
- (14) Kuroha Y, Tada M, Kawachi I, Nishizawa M, Matsubara N, Koike R. Effect of sodium pyruvate on exercise intolerance and muscle weakness due to mitochondrial myopathy: a case report. Rinsho Shinkeigaku 2015;55(6):412-416. doi: 10.5692/clinicalneurol.cn-000652.
- (15) 柳村文寛,河内泉,西澤正豊.NMOの診断と治療-NMOのあらたな診断基準と治療薬. 医学のあゆ み 2015;255(5):390-396.
- (16)河内泉,西澤正豊.「神経変性」の視点から中枢神経系自己免疫疾患を考える. Medical science digest 2015;4(14):541-543.
- (17) Yajima R, Toyoshima Y, Wada Y, Takahashi T, Arakawa H, Ito, Kobayashi D, Yamada M, Kawachi I, Narita I, Takahashi H, Nishizawa M. A fulminant case of granulomatosis with polyangiitis with meningeal and parenchymal involvement. Case Rep Neurol 2015;7:101-104. DOI: 10.1159/000381942
- (18) Katayama K, Misawa S, Sato Y, Sobue G, Yabe I, Watanabe O, Nishizawa M, Kusunoki S, Kikuchi S, Nakashima I, Ikeda S, Kohara N, Kanda T, Kira J, Hanaoka H, Kuwabara S; J-

POST Trial study investigators (Aoyagi R, Tanemura N, Inamata C, Sekiguchi Y, Shibuya K, Mitsuma S, Watanabe K, Iwai Y, Kawagashira Y, Koike H, Shirai S, Sasaki H, Futagawa T, Ushinohama K, Kawachi I, Mitsui Y, Kato M, Suzuki H, Niino M, Fujihara K, Tateyama M, Katoh N, Kawamoto M, Koga M, Matsuse D, Yamasaki R). Japanese POEMS syndrome with Thalidomide (J-POST) Trial: study protocol for a phase II/III multicentre, randomised, double-blind, placebo-controlled trial. BMJ Open. 2015 Jan 8;5(1):e007330. doi: 10.1136/bmjopen-2014-007330.

#### Ⅳ 共同研究

- (1) Niigata MSA study (院内共同研究)
  - (概要) 2001年に開始し、多系統萎縮症患者を対象に睡眠呼吸障害の機序の解明、および睡眠呼吸障害に伴う突然死の予防を目的とした臨床研究を本年度も継続した。
  - (参加機関)新潟大学脳研究所神経内科、同統合脳機能研究センター、新潟大学医歯学総合病 院呼吸器内科、耳鼻咽喉科、循環器内科、摂食・嚥下機能回復部
  - (発表論文) Shimohata T, Nakayama H, Aizawa N, Nishizawa M. Discontinuation of continuous positive airway pressure treatment in multiple system atrophy. Sleep Med. 2014;15:1147-9.
- (2) 脳梗塞に対する血管保護療法の開発 (学外共同研究)
  - (概要) 金澤雅人、川村邦雄らは、東京大学農学部東京大学大学院農学生命科学研究科の 西原真杉教授、および脳研究所統合脳機能研究センターの中田力教授らとの共同 研究を行い、脳梗塞に対する新規脳梗塞治療薬プログラニュリンに対する研究を 行った。
  - (参加機関)新潟大学脳研究所神経内科、新潟大学脳研究所統合脳機能研究センター、東京大 学大学院農学生命科学研究科獣医生理学研究室
  - (特許) 虚血後の再灌流に起因する出血を予防するための薬剤 (PCT/JP2014/076117)

I 研究組織(構成員 平成28年3月31日現在)

教授	五十嵐	は 博中	センター
教授	柿田	明美	デジタ
特任教授	中田	力	統合脳構
准教授	松澤	等	脳機能角
准教授	鈴木	清隆	生体磁线
准教授	鈴木	雄治	臨床機能
准教授	ビンセ	ント フーバー	臨床機能
准教授	辻田	実加	臨床機能
助教	伊藤	浩介	脳機能的
助教	渡辺	将樹	生体磁线
助教	中村	亨弥	生体磁线
助教	山田	謙一	臨床機能
助教	北浦	弘樹	臨床機能
助教	植木	智志	臨床機能
助教	倉部	聡	臨床機能
実験助手	大湊	詩保	
実験助手	三冨	明夫	
実験助手	五十嵐	し 妙	
大学院生	武田	基秀	
大学院生	松田	将門	
特別研究学生	酒多	穂波	
医局秘書	佐藤	直子	
医局秘書	松崎	玲奈	
医局秘書	遠藤	智代	
医局秘書	岡本	真梨	

センター長・生体磁気共鳴学分野 デジタル医学分野 統合脳機能研究センター 脳機能解析学分野 生体磁気共鳴学分野 臨床機能脳神経科学分野 臨床機能脳神経科学分野 生体磁気共鳴学分野 生体磁気共鳴学分野(超域学術院) 臨床機能脳神経科学分野 臨床機能脳神経科学分野 臨床機能脳神経科学分野 臨床機能脳神経科学分野 臨床機能脳神経科学分野

Ⅱ 主な研究活動

新潟大学脳研究所統合脳機能研究センターでは「こころの科学的解明」を目的とした中核的研究 拠点(COE)形成プログラムから、さらに文部科学省連携融合事業「水分子の脳科学」(平成17年度 ~22年度)と引き継がれた研究活動を推進してきた。このプロジェクトでは水分子の移動に特異的 に関与するタンパク質のチャンネル、アクアポリンの動態的機能解析を行い、生体におけるアクア ポリンの動態を画像化する方法の開発及び実践を目指し、成果を上げてきた。

さらに、このプロジェクトは、特別経費(プロジェクト分)-国際的に卓越した教育研究機能の充 実-として、脳の統合機構の解明を目的とした5年間(平成23年度~27年度)の文部科学省特別経 費「意識の脳科学」へと引き継がれ、今年度はその最終年度であった。

尚、研究活動の成果については、以下の発表論文の報告に委ねることとする。

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- Ikeda T, Takahashi T, Tsujita M, Kanazawa M, Toriyabe M, Koyama M, Itoh K, Nakada T, Nishizawa M, Shimohata T. Effects of Alda-1, an Aldehyde Dehydrogenase-2 Agonist, on Hypoglycemic Neuronal Death. PLoS One. 2015 Jun 17;10(6):e0128844. doi: 10.1371/journal.pone.0128844. eCollection 2015.
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- 17. 照光 真, 瀬尾 憲司, 松澤 等, 稲田 有史, 中村 達雄, 茂野 啓示, 渡辺 将樹, 鈴木 清隆, 中田 力:「三叉神経障害に対する PGA-C tube による生体内再生治療の高分解 能神経 MRI を用いた予後評価」 Peripheral Nerve. 2015 Aug;26(1):71-9.
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#### IV 共同研究

中核的研究拠点(COE)形成プログラム、連携融合事業「水分子の脳科学」からさらに特別経費 「意識の脳科学」プロジェクトとして引き継がれ、ヒトの意識と心を司る脳機構の直接解明を実践 する研究・教育の国際的拠点形成を推進している。

- (1) 研究題目 特別経費(プロジェクト分)「意識の脳科学」(平成23年~27年度)
  国際的に卓越した教育研究拠点機能の充実
  - 研究内容 超高解像度水分子画像とアクアポリン-4分子画像とを駆使した国際研究組 織の有機的統合により、水分子の動態解析を通してヒトの意識を司る脳の統 合機構の解明を目指す。
  - 参加機関 Neurology, University of California, Davis (米国)

# (2) 研究題目 C<sup>11</sup>-AQP4分子画像法の臨床研究(平成23年~27年度) 研究内容 当施設で開発したAQP4リガンドの臨床応用を目指す。 参加機関 ガチョン医科大学 (大韓民国)

- (3) 研究題目 サル類における聴覚事象関連電位の記録(平成25年~27年度)
  - 研究内容 サル類を対象に無麻酔・無侵襲で頭皮上から聴覚誘発電位や事象関連電位を 記録し、脳進化に伴う聴覚処理の種差を検討する。

#### 参加機関 京都大学霊長類研究所

遺伝子機能解析学分野·生命情報工学分野

I 研究組織(構成員 平成28年3月31日現在)

教	授	池内 健	
助	教	春日健作	(超域学術院)

ВН	對	官下折曲	(休職由)
旫	叙	呂「召典	(11小瓶屮丿

- 特任助手 原 範和
- 技術職員 深海晴代
  - 古川英理
    - 佐藤怜奈
      - 見田順子
      - 河合麗子
      - 小林智子
      - 廣瀬美香
      - 平井香織
- 事務職員 高殿恵子
- Ⅱ 研究活動
- (1) 認知症を対象とした国内の多施設共同臨床研究の生化学・遺伝子解析拠点として活動している。 この活動により、認知症に関する生体試料バンクを構築し、バイオマーカー開発を行っている。
- (2) 認知症関連疾患・遺伝子解析センターとして活動しており,特にアルツハイマー病については 国内最大のゲノムDNAを集積している。家族性アルツハイマー病の遺伝子診断を全国の各施設 から依頼を受け,結果を返却するクリニカルシークエンスを行っている。
- (3) 神経変性疾患の中で進行性核上性麻痺,パーキンソン病,前頭側頭葉変性症を対象とした多施 設共同研究の生化学コアとして活動している。神経変性疾患に関する生体試料バンクを構築し, バイオマーカー開発を行っている。
- (4) 全国共同利用・共同研究のプロジェクト,また学内の共同利用施設として、次世代シークエン サーを活用した共同研究を実施している。
- (5) 当分野の個別研究として
  - 4-1) アルツハイマー病を含む認知症のゲノム医学解析
  - 4-2) 認知症の新規バイオマーカーの開発
  - 4-3) 大脳白質変性症の分子遺伝学的病態解析
  - 4-4) アルツハイマー病の後天的因子に着目した病態研究 を行っている。
- Ⅲ 論文(原著、総説、症例報告を区別しない)
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- Suzuki Y, Nakamura Y, Yamada K, Igarashi H, Kasuga K, Yokoyama Y, Ikeuchi T, Nishizawa M, Kwee IL, Nakada T. Reduced CSF water influx in Alzheimer's disease supporting the ß-amyloid clearance hypothesis. *PLoS ONE* 10(5), e0123708, 2015
- IV 共同研究
- (1) 研究題目:「家族性アルツハイマー病に関する縦断的観察コホート研究」
- 研究内容:遺伝子変異が同定された家族性アルツハイマー病の家系員を対象とした縦断的コホー ト研究である。認知症を発症前のバイオマーカーの変化を明らかにするトランスレー ショナル研究。
  - 参加機関:大阪市立大学、弘前大学,東京大学など
- (2) 研究題目:「認知症の根本的な原因の解明を目指したコホート研究と網羅的ゲノム配列解析研究」
  - 研究内容:アルツハイマー病の発症に関与する遺伝的リスクを明らかにする目的で縦断的コホート 研究を行い,次世代シーケンサーを用いた網羅的な遺伝子解析を実施している。 参加機関:東京大学
- (3) 研究題目:「プレクリニカル期におけるアルツハイマー病に対する客観的画像診断・評価法の確立を目指す臨床研究」
  - 研究内容:健康高齢者およぼ軽度認知障害を対象とした縦断的臨床研究である。アルツハイマー病 を発症する超早期の臨床所見,画像所見,バイオマーカー変化などを明らかにする。
  - 参画期間:大阪市立大学,東京大学,東京都健康長寿医療センター,先端医療センター研究所,国 立精神神経研究センター,国立長寿医療センター
- (4) 研究題目:「認知症臨床研究の実施を支援する国内・国際連携体制の構築に関する研究」
  - 研究内容:健康高齢者およぼ軽度認知障害を対象とした縦断的臨床研究である。アルツハイマー病 を発症する超早期の臨床所見,画像所見,バイオマーカー変化などを明らかにする。
    - 参画期間:大阪市立大学,東京大学,先端医療センター研究所,国立精神神経研究センター,国立 長寿医療センター
- (5) 研究題目:「神経軸索スフェロイド形成を伴う遺伝性びまん性白質脳症(HDLS)の診断基準の策 定」
  - 研究内容:神経軸索スフェロイド形成を伴う遺伝性びまん性白質脳症(HDLS)について遺伝子診 断で確定診断された症例を用い、臨床・画像的な特徴を明らかにするとともに、診断基 準を策定する。
  - 参加機関: Mayo Clinic Florida, 信州大学, 京都府立医科大学, 徳島大学
I 研究組織(構成員 平成28年3月31日現在)

教授	笹岡	俊邦
助教	藤澤	信義
助教	前田	宜俊
特任助教	小田	佳奈子
フェロー	横山	峯介
非常勤講師	佐藤	俊哉
非常勤講師	森田	邦恵
技術専門職員	田中	稔
事務職員	加藤	明子
技術職員	那須野	纾 純映
技術職員	中尾	聡宏
事務補佐員	田代	智子
事務補佐員	久住	真由美
派遣技術職員	山本	美丘
派遣技術職員	神保	幸弘
派遣技術職員	坪井	広樹

#### Ⅱ 研究活動

(1) 大脳基底核にあるドーパミンが減少すると、パーキンソン病の「無動」などの重篤な運動障害 が生じることが知られている。ドーパミンは、大脳基底核の線条体神経細胞に発現するD1ドーパミ ン受容体(D1R)とD2ドーパミン受容体を介して情報を伝達する。しかし、これらの受容体を介する情 報がそれぞれ、大脳基底核内の信号伝達をどのように調節するのか、運動の制御にはどのように働 くのか、詳しく解明されていない。

生理学研究所の知見聡美助教と南部篤教授、北里大学の佐藤朝子研究員らと共同して、D1Rを介す る情報伝達は、大脳基底核の神経回路のうち運動を誘発するように働く「直接路」を通る信号の伝 達に不可欠であり、D1Rを介する情報伝達が消失すると運動を起こしにくくなることを、新たに開発 した遺伝子改変マウスを用いた研究によって明らかにした。

(2) 遺伝子改変動物作成の新たな方法として、人工制限酵素技術の先駆けとなったZinc-Finger Nuclease (ZFN) 技術を2011年に導入し、さらに第三世代の改変技術であるCRISPR/Casシステムの導 入にも成功し、利用者からの遺伝子改変マウスの作成の依頼を受けて作成している。本技術は、遺 伝子改変動物作成のハードルを一気に下げ、今後の研究発展に必須であるため、当分野でも自在に 扱える態勢を整えている。

(3) 生殖工学的技術を用い、ドーパミン受容体欠損マウス等の脳・神経系疾患の病態モデルをはじめ、各種遺伝子改変マウスを供給しているが、この技術を用いて生まれたマウスは、体重が増加する傾向にあることを見いだした。そこで生殖工学的操作で必須である体外培養が初期胚の発生およ

び個体発生に影響を及ぼすかを明確にする必要があり、研究を行っている。これまでに体外培養胚 のインプリント遺伝子に発現変化があることを明らかにした。

(4)動物実験施設は、平成25年度の大規模改修工事を経て本格的に稼働して以来、飼育動物数が順調に増加し、また施設利用者数も増加している。さらに、今年度は水生動物飼育室を新設し、メダカを用いた研究が開始した。併せて、標準操作手順書(SOP)の改訂を重ねると同時に、空調監視システムや非常電源回路等の設備の改善を行い、より適正な動物実験の実施に向けた取り組みを推進した。改修工事とその後の稼働の経過について、第49回日本実験動物技術者協会の第48回総会にて発表し、第49回総会にて第18回日本実験動物器材協議会賞を授与された。また、同協会の東北支部・奥羽支部の第1回実験動物技術研究交流大会、及び日本実験動物協会の教育セミナーフォーラム2016において、実験動物技術に関する研究発表を行っている。

- Ⅲ 論文(原著、総説、症例報告を区別しない)
- (1) Chiken S, Sato A, Ohta C, Kurokawa M, Arai S, Maeshima J, Sunayama-Morita T, Sasaoka T, Nambu A. Dopamine D1 receptor-mediated transmission maintains information flow through the cortico-striato-entopeduncular direct pathway to release movements. *Cerebral Cortex* 2015 Dec;25(12):4885-97. doi: 10.1093/cercor/bhv209. Epub 2015 Oct 6.
- (2) Nakamura T, Sato A, Kitsukawa T, Sasaoka T, Yamamori T; Expression pattern of immediate early genes in the cerebellum of D1R KO, D2R KO, and wild type mice under various conditions.

*Frontiers in Cell and Developmental Biology.* Vol. 3, Article 38, June 2015 doi: 10.3389/fcell.2015.00038

- (3) Hayashiji N, Yuasa S, Miyagoe-Suzuki Y, Hara M, Ito N, Hashimoto H, Kusumoto D, Seki T, Tohyama S, Kodaira M, Kunitomi A, Kashimura S, Takei M, Saito Y, Okata S, Egashira T, Endo J, Sasaoka T, Takeda S and Fukuda K; G-CSF supports long-term muscle regeneration in mouse models of muscular dystrophy *Nature Communications* 2015 Apr 13; Vol. 6: Article number 6745. doi: 10.1038/ncomms7745. PMID: 25865621
  - Ⅳ 共同研究

以下の共同研究課題について、胚操作実験技術を利用して研究を推進している。

- (1) 平成25~27年度 基盤研究(A)
  TDP43の調節機能に注目したALSの病態機序の解明
  研究代表者:西澤 正豊 教授
- (2) 平成25~27年度 基盤研究 (B)
  脳小血管病の解明と治療方法の確立: CARASILの病態機序からのアプローチ 研究代表者:小野寺 理 教授

- (3) 平成25~27年度 厚生労働科学研究費補助金(障害者対策総合研究事業) 新評価方法を用いたフォールディング病の分子シャペロン療法の検討 研究代表者:小野寺 理 教授
- (4) 遺伝子改変動物作成脳における系統的遺伝子破壊マウスの作製脳研究所 細胞神経生物学分野 阿部 学 准教授
- (5) 平成27年度 共同利用・共同研究(プロジェクト型)
  PNPLA6遺伝子の脳における機能-有機リン被爆との関連から
  研究代表者:木村 穣 教授(東海大学)
- (6) 平成27年度 共同利用・共同研究 (プロジェクト型)
  BQLN2コンディショナルノックアウトマウスの解析に基づく神経変性機序の解明
  研究代表者:田中 章景 教授(横浜市立大学)
- (7) 平成27年度 共同利用・共同研究(プロジェクト型) 哺乳類中枢神経系における神経回路形成の遺伝学的解析 研究代表者:岩里 琢治 教授(国立遺伝学研究所)
- (8) 平成27年度 共同利用・共同研究 (プロジェクト型) 大脳基底核内情報伝達におけるドーパミン神経伝達の機能の解析 研究代表者:南部 篤 教授(自然科学研究機構生理学研究所)
- (9) 平成27年度 共同利用・共同研究(連携資源利用型) ドーパミン受容体変異マウスを用いた不安様行動発症機序の解明 研究代表者:山森 早織 講師(北里大学)
- (10) 平成27年度 共同利用・共同研究(連携資源利用型) 胎仔期および発達期の脳におけるドーパミン受容体D1Rの機能解析 研究代表者:大久保 直 准教授(北里大学)
- (11) 平成27年度 共同利用・共同研究(連携資源利用型)
  APP細胞内ドメインの神経毒性の解析
  研究代表者:中山 耕造 講師(信州大学)
- (12) 平成27年度 共同利用・共同研究(連携資源利用型) ドパミン-D1Rシグナルが心不全に果たす役割の解明 研究代表者:小室 一成 教授(東京大学)
- (13) 平成27年度 共同利用・共同研究(連携資源利用型)
  筋線維メインテナンスに果たすWWP1ユビキチンリガーゼの機能の解析
  研究代表者: 今村 道博 室長(国立精神・神経医療研究センター神経研究所)
- (14) 平成27年度 共同利用・共同研究(連携資源利用型)
  ゲノム編集技術と生殖工学技術を用いた効率的な遺伝子改変マウス作製
  研究代表者:中潟 直己 教授(熊本大学生命資源研究・支援センター)

分子神経疾患資源解析学分野

I 研究組織(構成員 平成28年3月31日現在)

教授	小野	寺 理
特任准教授	横関	明男
助教	石原	智彦
技術職員	廣川	祥子

#### Ⅱ 研究活動

本教室は神経疾患の分子生物学的解析により,病態機序を明らかにし,最終的には神経疾患の 有効な治療方法の開発を行うこと目的としている.本学脳研究所,神経内科学教室の支援を受け て,臨床との融合拠点として活動を推進している.また病理学教室,動物実験施設,遺伝子実験 施設を中心とする,脳研究所の各教室,および国内,国外の研究室とも共同研究を推進している. 当施設では特に遺伝性脳小血管病,筋萎縮性側索硬化症(ALS),脊髄小脳変性症の各疾患につい て研究を推進している.

遺伝性脳小血管病,特にcerebral autosomal recessive arteriopathy with subcortical infarcts and leukoencephalopathy (CARASIL) について,分子病態機序からの分析・解明を行っている.本年度は,既報 のCARASIL症例のMRI所見の特徴と経時的変化について,定性および半定量的手法によって検討を加えた論文 をneurology誌に発表した.本誌は神経内科学分野の臨床系雑誌としては,annals of neurologyに次いで高 いIF 8.3 (2015 Journal Citation Reports [Thomson Reuters, 2015])を有する権威ある学会誌である.ま た,本論文は同誌のCME course topicとして取り上げられており,その学術的価値が評価されている.

ALSについては、TDP-43 mRNAの代謝,局在,発現量調節に関する研究,機能性RNA代謝に関連 した新たな病態機序に関する研究,ミトコンドリア代謝関連蛋白質 mRNAについての研究を行い, 国内外の学会にて発表を行っている.ALSはTDP-43,FUS,C9orf72 など疾患関連遺伝子,蛋白質 の発見を端緒として,病態機序解明に向けて国際的な競争が行われている.その中で本施設では 疾患感受性蛋白質のRNA代謝に注目してALS病態機序の解析を行っている点に特色がある.

共同研究では、"HtrA1欠損マウスにおける脳小血管の機能解析"を推進し、CARASILの病態研究を進めた.

また研究資金は科学研究費補助金(B)を始めとして,各々の研究者が競争的研究資金を獲得し, 研究活動を推進した.

Ⅲ 論文(原著、総説、症例報告を区別しない)

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- 2. Tada M, Nishizawa M, Onodera O. Redefining cerebellar ataxia in degenerative ataxias:

lessons from recent research on cerebellar systems. Journal of Neurology, Neurosurgery & Psychiatry. 2015:jnnp-2013-307225.

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- Onodera O, Sekine Y, Kato T, Koyama A, Nozaki H, Nishizawa M. Emerging molecular mechanism for cerebral small vessel disease: Lessons from hereditary small vessel disease. Neurology and Clinical Neuroscience. 2015;3(1):7-13.
- 6. 上村昌寛,野崎洋明,西澤正豊,小野寺理.脳の微小循環維持シグナルと血管性認知症CARASIL からのアプローチ. Dementia Japan. 2015;29(4):534-540.
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#### Ⅳ 共同研究

- 1. 研究題目 「HtrA1欠損マウスにおける脳小血管の機能解析」
  - 研究内容 CARASILモデルマウスにおける脳血流の解析
    - 参加機関 国立循環器病循環器病研究センター

プロジェクト研究分野

I 研究組織(構成員 平成28年3月31日現在)

准教授	鷲山	和雄	助	教	棗田	学
技術職員	小林	一雄				
研究員	薄井	宏	研究	記員	小林	徹

Ⅱ 研究活動

当分野では、従来の形態学、免疫組織化学、生化学的分析法などの手法を用いた神経病理学に加 え、各種脳疾患の遺伝子レベルでの解析を行なっている。研究内容としては、脳腫瘍の病因病態の 研究をはじめ、ウィルス疾患、脳虚血、精神神経作動薬などを対象とした研究を行なっている。

脳腫瘍に関しては、近年では、癌抑制遺伝子をCre/loxPシステムを用いて、細胞選択的に破壊す ることによる脳腫瘍モデルマウスの病態観察をおこなっている.コンディショナルノックアウト法 を用いて発生させた脳腫瘍の髄膜播種を解析中である。また、ヒト脳腫瘍の分化にかかわる研究で は、重要ながん抑制関連遺伝子のひとつでもある ARF-BP1 が頭蓋内胚細胞性腫瘍、特に胚腫に高 頻度に発現するだけでなく、培養細胞系でも発現誘導が可能なことを明らかにした。

また、G蛋白質活性型内向き整流性カリウムチャネル(GIRK; Kir3)に対する分子薬理学的研究 も継続している。このチャネルは、神経伝達物質、G蛋白質共役型受容体と機能的な関係を持ち、 神経細胞の興奮性をダイナミックに制御する重要なイオンチャネルである。本年度も、うつ病の治 療薬として用いられている薬剤を中心に、GIRKチャネルが抑制的に働いていることを、アフリカツ メガエル卵母細胞蛋白質発現系を用いて、解析している。

Ⅲ 論文(原著、総説、症例報告を区別しない)

- Washiyama K., Kumanishi T. AEF-BP1 expression in primary intracranial germ cell tumors. 4<sup>th</sup> International CNS Germ cell Tumor Symposium. (suppl): p46, 2015.
- (2) Natsumeda M, Maitani K, Liu Y, Miyahara H, Kaur H, Chu Q, Zhang H, Kahlert U, Eberhart CG. Targeting Notch Signaling and Autophagy Increases Cytotoxicity in Glioblastoma Neurospheres. Brain Pathol. 2015 Nov 27. p 1-p11

IV 共同研究

(1)研究題目 「悪性グリオーマにおけるがん抑制遺伝子に関する研究」
 研究内容 悪性グリオーマ細胞株を用いて、がん抑制遺伝子が腫瘍の増殖や治療効果 にどのように影響を与えているかを研究している
 参加機関 和歌山県立医科大学

## 2. 業績論文(抜粋)

論文は出版元の転載許可を受けています

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## Neurobehavioral Differences Between Mice Receiving Distinct Neuregulin Variants as Neonates; Impact on Sensitivity to MK-801

T. Kato<sup>1</sup>, Y. Abe<sup>1</sup>, S. Hirokawa<sup>2</sup>, Y. Iwakura<sup>1</sup>, M. Mizuno<sup>1</sup>, H. Namba<sup>1</sup> and H. Nawa<sup>\*,1</sup>

<sup>1</sup>Department of Molecular Neurobiology, Brain Research Institute, Niigata University, Niigata 951-8585, Japan

<sup>2</sup>Department of Molecular Genetics, Niigata University Graduate School of Medical and Dental Sciences, Niigata 951-8510, Japan

Abstract: Neuregulin-1 (NRG1) is a well-recognized risk gene for schizophrenia and is often implicated in the neurodevelopmental hypothesis of this illness. Alternative splicing and proteolytic processing of the NRG1 gene produce more than 30 structural variants; however, the neuropathological roles of individual variants remain to be characterized. On the basis of the neurodevelopmental hypothesis of schizophrenia, we administered eNRG1 (0.1~1.0 µg/g), a core epidermal growth factor-like (EGF) domain common for all splicing NRG1 variants, to neonatal mice and compared their behavioral performance with mice challenged with a full mature form of type 1 NRG1 variant. During the neonatal stage, recombinant eNRG1 protein administrated from the periphery passed the blood-brain barrier and activated its receptor (ErbB4) in the brain. In adults, the mice receiving the highest dose exhibited lower locomotor activity and deficits in prepulse inhibition and tonedependent fear learning, although the hearing reduction of the eNRG1-treated mice may explain these behavioral deficits. Neonatal eNRG1 treatment also significantly potentiated MK-801-driven locomotor activity in an eNRG1 dose-dependent manner. In parallel eNRG1 treatment enhanced MK-801-driven c-Fos induction and decreased immunoreactivity for NMDA receptor subunits in adult brain. In contrast, mice that had been treated with the same molar dose of a full mature form of type 1 NRG1 as neonates did not exhibit hypersensitivity to MK-801. However, both animal models exhibited similar hypersensitivity to methamphetamine. Collectively, our findings suggest that aberrant peripheral NRG1 signals during neurodevelopment alter later behavioral traits and auditory functions in the NRG1 subtype-dependent manner.

Keywords: Behavior, MK-801, Neuregulin-1, NMDA receptor, hearing, schizophrenia.

#### INTRODUCTION

Genetic polymorphism of neuregulin-1 (NRG1) is suggested to associate with vulnerability to psychiatric diseases such as schizophrenia and bipolar disorder [1-3]. The genetic association of NRG1 with schizophrenia was first demonstrated with the haplotype block called HAPICE that is located in the 5'end of the gene and consists of three single nucleotide polymorphisms (SNPs) and two microsatellites [1]. This initial finding has been replicated in multiple samples, but not in all populations [1-3]. Alternative splicing or promoter usage of the NRG1 gene produces multiple isoforms of NRG1 precursor proteins (type I-VI) [4-6]. The posttranslational processing of the NRG1 precursors further modifies the protein structures of these isoforms to alter their biological activity [7]. However, the pathological contribution of each NRG1 isoform is not fully understood. Genetic mutant mice that lack the isoform-specific exon(s) or overexpress a specific isoform of NRG1 exhibit distinct behavioral abnormalities relevant to schizophrenia endophenotypes, potentially suggesting the divergence of the isoforms' functions in vivo [8-12]. These experiments were often performed by independent researchers and did not allow us to accurately compare the behavioral consequences of individual the isoforms' manipulations. To test the functional difference of the NRG1 isoforms, here, we investigated and compared the behavioral traits of the mice treated with type1 NRG1 (T1-NRG1) and those with its processing product; an epidermal growth factor (EGF) domain peptide (eNRG1). These NRG1 derivatives are potentially induced or recruited in the prenatal and perinatal immune inflammatory processes that are implicated in schizophrenia etiology [13,14].

We have been testing the neurodevelopmental of schizophrenia, hypothesis assessing the neurobehavioral impact of neonatal exposure to T1-NRG1 [15]. In addition to T1-NRG1, here, we challenged mouse neonates with eNRG1 to compare the neurobehavioral effects of the two NRG1 variants, eNRG1 and T1-NRG1. Although there are multiple isoforms of NRG1 precursor proteins (type I-VI), which are generated by alternative splicing or promoter usage of the NRG1 gene, but all NRG1 isoforms carry this core active domain (i.e., eNRG1) for their receptor binding [5,6]. Moreover, eNRG1 is naturally produced by proteolytic processing of T1-NRG1 with a protease, neuropsin, and suggested to exert a distinct action in

<sup>\*</sup>Address correspondence to this author at the Department of Molecular Neurobiology, Brain Research Institute, Niigata University, 1-757 Asahimachi-dori, Niigata 951-8585, Japan; E-mail: hnawa@bri.niigata-u.ac.jp

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#### Neuregulin-1 Variants and Behavioral Difference

hippocampal synaptic plasticity [7]. We assessed basal behavioral traits to evaluate the relevancy of eNRG1injected mice to an animal model for schizophrenia, comparing the previous results from the mice exposed to T1-NRG1 [15]. As NRG1 signals are known to regulate NMDA receptor expression and function in the brain [16-19]. Thus, we investigated the sensitivity of eNRG1-treated mice to the NMDA receptor antagonist MK-801 as well as the expression of NMDA receptor subunits in these mice [17,18].

#### MATERIALS AND METHODS

#### **Ethics Statement**

All of the animal experiments described were approved by the Animal Use and Care Committee of Niigata University and performed in accordance with the guidelines of NIH (USA).

#### Animals

C57BL/6NCrj mouse dams of late pregnancy were purchased from Nihon Charles River (Yokohama, Japan) and their pups were used for experiments. Mice were housed with their dam in polypropylene cages  $(24L \times 17W \times 12H \text{ cm})$  in temperature-controlled colony room maintained under a 12-h light-dark cycle (light on 8:00 h) with free access to food and water. At postnatal day (PND) 25-28, pups were weaned and separated into cage (three or four animals per cage). Both male and female mice were subjected to behavioral tests during PND 56-70 [15]. Behavioral tests were performed during the light-cycle.

#### Reagents

Recombinant epidermal growth factor domain of human NRG 1 $\beta$ 1/heregulin 1 $\beta$ 1 was purchased from PeproTech EC (London, UK). This NRG1 peptide is sufficient for activation of the ErbB4 receptor tyrosine kinase. We will refer to it simply as "eNRG1". Alternatively, we used the full mature form of recombinant type-1 NRG1 $\beta$ 1 protein (T1-NRG1; MW 25400 Da) [15]. (+)-MK-801 (dizocilpine) was purchased from Tocris Cookson Ltd (Ellisville, MO, USA).

#### NRG1-Treatment and Psychotomimetic Challenge

eNRG1 or T1-NRG1 was administered subcutaneously (s.c.) to individual litters during postnatal days PND 2-10 at the nape of the neck at a dose of 0.1, 0.3, or 1.0  $\mu$ g/g body weight (injection volume 15  $\mu$ l/g). The experimental design and procedures were based on our previous studies of the EGF model for schizophrenia [20]. Control littermates received a phosphate-buffered saline (PBS; vehicle) injection of the same volume. MK-801 (0.3  $\mu$ g/g; 10  $\mu$ l/g) and methamphetamine (1.0  $\mu$ g/g; 10  $\mu$ l/g) dissolved in saline were administered intraperitoneally (i.p.) to adult mice (PND 56-70) [15,21].

#### Immunoprecipitation

Protein extracts were prepared from whole brains of eNRG1-treated or vehicle-treated control mice on PND2 [15]. Each brain was homogenized in a RIPA lysis buffer [50 mM Tris-HCl buffer (pH 7.4) with 1% TritonX-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 150 mM NaCl, 5 mM EDTA, 1 mM Na<sub>3</sub>VO<sub>4</sub>, and 1 mM NaF) plus a protease inhibitor cocktail (Complete Mini; Roche, Mannheim, Germany). After centrifugation, supernatants were harvested and protein content was determined using a Micro BCA kit (Pierce Chemical, Rockland, IL, USA). Each brain lysate (2 mg protein) was then incubated with 2 µg of anti-ErbB4 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight and mixed with Protein G Sepharose beads (20 µl; GE Healthcare Bio-Science AB, Uppsala, Sweden) for 3 h. Sepharose beads were washed with RIPA buffer, and denatured with 100 µl of 2 × SDS sample buffer (125 mM Tris-HCl pH 6.8, 4% SDS, 10% 2-mercaptoethanol). The specificity of the anti-ErbB4 antibody was ascertained by immunoblotting of cell lysates containing individual ErbB1-4 proteins (HN, unpublished data). Immunoprecipitates were then analyzed by gel electrophoresis as described below.

#### Immunoblotting

Brain tissues were homogenized in the sample lysis buffer (62.5 mM Tris-HCl pH 6.8, 2 % SDS, 0.5 % NP-40, 5 mM EDTA) plus the protease inhibitor cocktail (Roche). Protein samples (15-30 µg/lane) were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Membranes were probed with antibodies directed against phosphotyrosine (1:1000, Millipore, Bedford, MA, USA), ErbB4 (1:1000, Santa Cruz Biotechnology). Alter-natively, immunoblots were probed with antibodies directed against excitatory and inhibitory neuronal markers [GluA1 (1:500, Millipore), GluA2/3 (1:1000, Millipore), GluA4 (1:1000, Millipore), GluN1 (1:1000, Millipore), GluN2A (1:500, Millipore), GluN2B (1: 500, Millipore), GluN2C (1:500, Millipore), GluN2D (1:1000, Santa Cruz Biotechnology), GAD65/67 (1:1000, Millipore), parvalbumin (1:3000, Abcam, Cambridge UK), tyrosine hydroxylase (1:1000, (1:300, RandD Systems, Millipore) neuregulin-1 Minneapolis, MN), and  $\beta$ -actin (1:2000, Millipore)]. Immunoreactivity on membranes was detected by peroxidase-conjugated anti-immunoglobulin antibodies followed by chemiluminescence reaction (ECL kit, GE Healthcare) and film exposure. The intensity of an immunoreactive band, whose size matched the authentic molecular weight, was measured by an image processing software, GENETOOLS (Syngene, Cambridge, UK).

#### Auditory Brain-Stem Evoked Response

The lowest level, at which an auditory brain-stem evoked response (ABR) pattern is recognized, was

determined in each mouse [15]. Mice were anesthetized with an intramuscular injection of ketamine (75 mg/kg) and xylazine (7.5 mg/kg), and placed in a close acoustic room [15]. A stainless-steel electrode was inserted subcutaneously into the vertex (positive pole), retroauricular region (negative pole), and opposite retroauricular region (background). Acoustic stimuli, consisting of a tone burst (0.1 ms rise and fall no plateau) at a frequency of 10 kHz, were presented to each mouse with a sound stimulator (DPS-725, Diamedical System, Tokyo, Japan) and speaker (PT-R9, Pioneer, Tokyo, Japan) in an open field. For each time point, 500 responses for each mouse were recorded and filtered for band widths of 100-3000 Hz. The Neuropac µ (Nihonkoden, Tokyo, Japan) was used to analyze the ABR response.

#### c-Fos Immunohistochemistry

Levels of c-Fos expression were assessed by counting the number of c-Fos-immunoreactive cells [22]. Mice were deeply anesthetized with halothane (Takeda Pharmaceutical Co, Osaka, Japan) 3 h after the single injection of MK-801 (0.3 µg/g) and perfused PBS, followed intracardiacally with by 4% paraformaldehyde in 0.1 M phosphate-buffer (pH 7.2). Coronal sections (40 µm) were made by a cryostat and incubated with a blocking solution containing PBS, 5% nonfat milk and 0.25% TritonX-100 for 2 h and incubated with rabbit anti-c-Fos antibody (1:20000; PC-38, Calbiochem, La Jolla, CA, USA) in the blocking solution 48 h at 4°C, followed by biotinylated anti-rabbit immunoglobulin antibody (1:200; BA-1000, Vector Laboratories). Sections were washed and processed with the avidin-biotinylated horseradish peroxidase complex (Vector ABC kit Vector Laboratories, Immunoreactivity Burlingame, CA. USA). was visualized with the chromogen 3, 3'- diaminobenzidine. To quantify the number of c-Fos positive cells in the brain, we used an all-in-one microscope (BZ-9000, Keyence, Osaka, Japan) and a BZ-Analyzer (Keyence) to scan the sections, and quantified the cell numbers from the digital images using NIH Image (ver. 1.61) cell-counting system [23].

#### **Behavioral Testing**

Acoustic startle response and prepulse inhibition (PPI) were measured as fully described previously [10,15]. Test paradigm of context- and tone-dependent fear learning, locomotor measurement, residentintruder assessment [24] and further procedural details of individual behavioral tests, see Supplementary Information. Three independent cohorts of mice were made for each NRG1 subtypes and each doses and assigned to the following three types of experiments; basal behavioral tests (locomotor assessment, sound startle test, and fear conditioning in the less stressful order), the MK-801 experiments, and the residentintruder test (see below).

#### **Statistical Analysis**

Data are presented as mean ± SEM. Computation was carried out using the SPSS 11.0 for Windows (Tokyo, Japan). Behavioral or immunohistochemical data were first subjected to the Shapiro-Wilk test (for the fitting to Gesian distribution) and Levene's test (for the equality of standard deviations) and then either one-way analysis of variance (ANOVA) or Student's ttest. ANOVA adopts NRG1-treatment as a betweensubject factor, NRG1-treatment as a between-subject factor and time as a within-subject factor, or eNRG1/ T1-NRG1 treatment and drug injection as betweensubject factors. ABR data were subjected to two-way ANOVA with eNRG1-treatment as a between-subject factor and frequency of auditory stimulus as a withinsubject factor. Other data stem from experimental groups were subjected to Student's t-test. Some of the data in the frequency of c-fos, startle responses and PPI scores had showed biased distributions or difference in deviations among groups, suggesting the risk of type 1 errors in ANOVA (Supplemental Table S1). Subsequently, a Tukey HSD test was applied to the data carrying equal deviations as a post hoc test. Alternatively, a Scheffe's test was applied to the data having non-equal deviations to validate the initial ANOVA assessment. When there was significant interaction between eNRG1-treatment and drug, data were analyzed separately to avoid the interaction for the final analysis. A p value < 0.05 was regarded as statistically significant.

#### RESULTS

#### eNRG1 Penetration into the Brain

Since the blood-brain barrier of neonatal mice is immature, peripherally administered cytokines can reach the brain [15,25]. To confirm that eNRG1 in circulation penetrates the blood-brain barrier and activates intracerebral NRG1 receptors, we subcutaneously administered eNRG1 (1.0 µg/g) to mouse pups (PND2) and examined eNRG1 immunoreactivity and the phosphorylation of ErbB4 in the cerebrum. Immunoblotting revealed that there was eNRG1 immunoreactivity in the cerebrum of the eNRG1-injeced mice but not in that of saline-injected controls (Fig. 1A). Peripheral injection of eNRG1 also significantly increased phosphorylation levels of ErbB4 [F(4,11) = 11.4, p < 0.01]. The increase persisted between 1-6 h after the injection with a peak of ErbB4 phosphorylation at 3 h (Fig. 1B, C). Thus, a peripheral injection of eNRG1 to mouse pups can produce hypersignaling of NRG1/ErbB4 in the neonatal mouse brain.

#### Exploratory Activity in Novel Environment

We administered various concentrations (vehicle alone, 0.1, 0.3, and 1.0  $\mu$ g/g) of eNRG1 repeatedly to mouse neonates. We monitored several parameters of

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Fig. (1). ErbB4 activation and eNRG1 immunoreactivity in the brain following peripheral eNRG1 injection. (A) Neonatal mice (PND2) were subcutaneously challenged with eNRG1 (1.0 µg/g) and intracardiacally perfused with PBS 3 h after eNRG1 injection. Brain lysates (200 µg/lane) were subjected to SDS polyacrylamide electrophoresis with the authentic eNRG1 protein (1 ng; Posi). The presence of eNRG1 protein in the brain was examined by immunoblotting (n = 4 mice per)group). (B) Whole brain lysates were prepared 0, 1, 3, 6, and 12 h after subcutaneous injection of eNRG1 (1.0 µg/g) to neonatal mice (PND2). ErbB4 phosphorylation levels were examined using immunoblotting for phosphotyrosine combined with ErbB4 immunoprecipitation. (C) Relative levels of ErbB4 phosphorylation in the brains of eNRG1injected mice are presented (% of 0 time; mean  $\pm$  SEM, n = 3per each time points). p < 0.05 compared with 0 time by Tukey HSD.

p < 0.001] in a dose-dependent manner. These results verified the physiological activity of injected eNRG1 *in vivo*. In addition, injections with higher doses of eNRG1 (i.e. 0.3 µg/g and 1.0 µg/g), produced a 10-20% decrease in body weight gain that persisted until adulthood (Table **1**).

At the young adult stage (PND54-70), we assessed the locomotor activity of eNRG1-treated mice in a novel environment. A one-way ANOVA yielded a significant difference among eNRG1 doses [F(3,36) = 4.6, p <0.01]. Post hoc comparisons revealed that the mice treated with the highest dose (1.0 µg/g) of eNRG1 displayed significantly fewer horizontal movements compared with the other three groups (p < 0.05) (Fig. 2A), whereas stereotypic behaviors of these mice were similar among the groups [dose: F(3,36) = 0.7, p =0.54] (Fig. 2B). There were no significant differences in the above indices between males and females [gender  $\times$  dose: F(3,32) = 1.23, p = 0.31 for horizontal movements; gender × dose: F(3,32) = 0.27, p = 0.85for stereotypic movements], although stereotypic movements fundamentally differed between males and females [gender: F(3,32) = 1.23, p < 0.01].

## Effects of Neonatal eNRG1-Treatment on Other Behavioral Traits

Using the mice treated with 1.0 and 0.3  $\mu$ g/g doses of eNRG1, we further analyzed other behaviors of these animals in adulthood (PND56-70) such as fear learning ability, sensorimotor gating and social behavioral traits. The mice treated with the 1.0 µg/g dose of eNRG1 as neonates exhibited a marked reduction of PPI [eNRG1: F(1,49) = 61.3, p < 0.001, repeated ANOVA for 4 prepulses] (Table 2). There were no significant differences in eNRG1 effects between males and females [gender  $\times$  eNRG1: *F*(1,47) = 2.26, p = 0.14]. These mice also showed a reduction in tone-dependent learning scores but did not alter context-dependent learning scores [F(1,39) = 0.004, p = 0.95 for context-dependent learning; F(1,39) = 23.8, p < 0.001 for tone-dependent learning, both repeated ANOVA for 5 bins]. There was no significant difference in shock sensitivity [F(1,39) = 0.005, p = 0.95] (Table 2). There were no significant differences between males and females in eNRG1 effects on the above indices, either [gender  $\times$  eNRG1; F(1,37) = 1.20, p =0.28 for conditioning: gender  $\times$  eNRG1; F(1,37) = 0.41, p = 0.53 for context-dependent learning: gender  $\times$ eNRG1: F(1,37) = 1.87, p = 0.18 for tone-dependent learning]. Similar behavioral alterations were observed when we administered the lower dose of eNRG1 (0.3  $\mu/g$ ) to mouse pups (Supplemental Fig. **S1**). This dose  $(0.3 \mu/g)$  was approximately 40 pmol per gram body weight and equivalent to the molar dose of T1-NRG1 that had been given to mouse pups in our previous study [15].

To investigate the impact of neonatal eNRG1treatment on social behaviors in adulthood, we used a resident-intruder behavioral assay. In this assay, a group-housed male mouse (intruder) was placed in another home cage where a resident male mouse had

Table 1.	Summary	/ of	physical	develo	pment.
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Dose	Veh	icle	eNF	RG1		
Tooth Eruption (PND)						
0.1 μg/g	11.6	± 0.2	10.4 ±	10.4 ± 0.2***		
0.3 μg/g	11.6	± 0.2	10.2 ±	0.2***		
1.0 μg/g	10.9	± 0.2	9.4 ±	0.2***		
Eyelid Opening (PND)						
0.1 μg/g	13.9	± 0.1	11.5 ± 0.2***			
0.3 μg/g	13.7	± 0.2	10.8 ± 0.1***			
1.0 μg/g	13.1 ± 0.2		10.5 ± 0.2***			
Body Growth; P11						
0.1 μg/g	5.72 ± 0.13		5.52 ± 0.07			
0.3 μg/g	6.18 ± 0.20		5.62 ± 0.14*			
1.0 μg/g	6.05 ± 0.17		5.08 ± 0.22**			
Body Growth; P56	Male Female		Male	Female		
0.1 μg/g	24.2 ± 0.6	20.2 ± 0.3	23.8 ± 0.2	19.3 ± 0.3		
0.3 μg/g	24.2 ± 0.6	20.3 ± 0.4	21.4 ± 0.3**	18.4 ± 0.5*		
1.0 μg/g	19.4 ± 0.3 16.6 ± 0.3		17.6 ± 0.4**	14.3 ± 0.6**		

eNRG1 or vehicle was administered (s.c.) daily to neonatal mice during PND2-10. During and after treatment, we monitored eyelid opening, tooth eruption and body weight of all mice. Data are expressed as mean  $\pm$  SEM (n = 10 per group). *P*-values, compared between eNRG1-treated and vehicle-treated groups by unpaired two-tailed *t*-test. \* p < 0.05, \*\* p < 0.01, \*\*\* p < 0.001.

been housed alone until the test day (Table 2). eNRG1treated mice exhibited decreased sniffing duration compared with vehicle-treated controls (p < 0.05, unpaired *t*-test), whereas no significant difference between groups was found with sniffing counts or fighting counts (Table 2). The lower frequency of aggressive behaviors of the present mice can be ascribed to the mouse strain used in the present study (i.e., C57BL/6N) [27,28].

#### Effects of Neonatal eNRG1-Treatment on MK-801-Driven-Locomotor Activity

Sensitivity to psychostimulants, such as NMDA receptor antagonists, have often been evaluated in animal models to assess their relevancy to a schizophrenia model. To test whether neonatal eNRG1-treatment (0.1, 0.3, and 1.0 µg/g) alters acute responsiveness to an NMDA receptor antagonist, MK-801, in adulthood, mice were first acclimated to a novel environment to minimize the substantial difference of exploratory activity and/or motivation between groups. After one hour acclimation, mice were challenged with a 0.3 µg/g dose of MK-801 (Fig. 3). The given dose of MK-801 was the minimum dose that can induce hyperlocomotion in mice [29]. MK-801 markedly increased the locomotor activity of mice for approximately 2 h in both the eNRG1-treated and control groups [drug: F(1,36) = 29.7, p < 0.001]. However, the magnitude of MK-801-induced locomotor activity was significantly higher in the eNRG1-treated group than in the control group [eNRG1-treatment: F(1,36) = 5.2, p < 0.05; eNRG1 × drug: F(1,36) = 6.0, p < 0.05] (Fig. **3A**). The sensitivity appeared to depend on the dose of eNRG1 [eNRG1 dose: F(1,36) = 3.2, p < 0.05] (Fig. **3C**). Thus, these results indicate that neonatal exposure to eNRG1 enhances behavioral sensitivity to MK-801 in a dosedependent manner. However, there was no significant difference in MK-801-triggered stereotypic scores between eNRG1-treated and vehicle-treated control mice at any dose of eNRG1 [eNRG1 dose: F(3,36) =1.2, p = 0.34] (Fig. **3B**, **D**).

#### Effects of Neonatal eNRG1-Treatment on c-Fos-Expression in the Brain

The NMDA antagonist MK-801 increases both dopamine and glutamate efflux primarily in the forebrain regions [30,31] and induces the gene expression of c-Fos through MK-801-evoked glutamatergic neurotransmission and subsequent MAP kinase/AP-1 activation [22]. Employing c-Fos expression as a marker of neuronal activity, we examined the effects of neonatal eNRG1 treatment on MK-801-induced c-Fos expression and attempted to confirm the above observation; the higher sensitivity of eNRG1-treated mice to MK-801. We counted the number of c-Fos positive cells in the medial prefrontal cortex (mPFC), nucleus accumbens (NAc), piriform cortex, and





**Fig. (2).** Baseline locomotor activity and stereotypic behavior in a novel environment. (**A**) Total locomotor activity and (**B**) stereotypic behavior counts of eNRG1 (0.1, 0.3 and 1.0  $\mu$ g/g)-treated and vehicle-treated control mice for 60 min (*n* = 10 mice per group). Data is expressed as mean ± SEM. \**p* < 0.05 compared with control mice by Tukey HSD.

striatum. MK-801 challenge significantly increased the number of c-Fos-positive cells in these regions in both control and eNRG1-treated mice [drug: F(1,12) = 384.1, p < 0.001 for mPFC; F(1,12) = 139, p < 0.001for NAc; F(1,12) = 69.6, p < 0.001 for piriform cortex]. In the mPFC and NAc, however, we found significant statistical interactions between MK-801 challenge and eNRG treatment [eNRG1  $\times$  drug: F(1,12) = 8.5, p < 0.05 for mPFC, F(1,12) = 12.4, p < 0.01 for NAc]. The interactions indicate that MK-801-triggered increases in the number of c-Fos-positive cells were greater in eNRG1-treated mice than in control mice (Fig. 4A-H). In contrast, the number of c-Fos positive cells in the piriform cortex was indistinguishable in control and eNRG1-treated mice following MK-801 challenges [eNRG1-treatment: F(1,12) = 1.4, p = 0.27, eNRG1treatment × drug F(1,12) = 0.6, p = 0.45] (Fig. 4I). As

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compared between control and eNRG1-treated mice in the saline challenged-groups, however, there was a significant difference in the basal frequency of c-Fospositive cells in the mPFC (p < 0.001). In the striatum, we found no detectable increase in c-Fos expression following MK-801 challenge [drug: F(1,12) = 0.1, p = 0.79] (Fig. **4**).

	Vehicle	eNRG1
Prepulse inhibition (% i	nhibition)	
73dB	20.5 ± 3.2	-2.5 ± 3.9***
76dB	38.5 ± 3.5	4.7 ± 4.8***
79dB	47.2 ± 3.3	3.7 ± 5.3***
82dB	57.6 ± 3.2	8.7 ± 4.9***
Learning (% freezing)		
Shock sensitivity	61.3 ± 3.4	60.0 ± 4.7
Context-dependent	66.5 ± 1.5	65.7 ± 2.1
Tone-dependent	66.0 ± 1.3	41.5 ± 3.2***
Resident-intruder test		
Sniffing Duration (sec)	178.3 ± 8.9	145.8 ± 8.1*
Sniffing Counts (times)	9.7 ± 0.8	8.1 ± 0.7
Aggression (frequency)	2.28 ± 0.14	3.61 ± 0.23

Table 2. Summary of behavioral traits.

Neonatal mice were treated with eNRG1 (1.0  $\mu$ g/g) or vehicle (control) as described in the Materials and Methods. In adulthood, PPI levels (%) were measured with 73, 76, 79 and 82 dB prepulse stimuli (n = 25-26 mice per group) and a main pulse of 120 dB. eNRG1-treated and vehicle-treated mice were also subjected to context-tone-foot shock pairs to evaluate their fear learning. Mean freezing rates (%) were measured for 150 sec (30 sec/ bin) during conditioning or during the same context exposure and during tone exposure in the test paradigm (n = 20-21 mice per group). In the resident-intruder assay, we measured the total time duration of sniffing behaviors and frequency of sniffing and fighting behaviors of the resident males over a 10-min period (n = 18 mice per group). Data are expressed as mean ± SEM. \*p < 0.05, \*\*\* p < 0.001 by Tukey HSD or Student's t-test.

#### NMDA Receptor-Like Immunoreactivity in eNRG1-Treated Mice

Alterations in brain expression of NMDA receptors are often implicated in the behavioral sensitivity to the NMDA receptor blockers MK-801, phencyclidine, and ketamine [24,32]. We aimed to elucidate the molecular phenotypes underlying the hypersensitivity of eNRG1 (1.0 µg/g eNRG1)-treated mice to MK-801. Using Western blot techniques, we estimated protein levels for NMDA receptor subunits (GluN1, GluN2A-2D) and AMPA receptor subunits (GluA1-A4) in the frontal cortex, hippocampus, and striatum plus NAc (Fig. 5A). In addition to these receptors, we also examined molecular markers for GABAergic and dopaminergic neurons. Among the receptors examined, there were significant decreases in hippocampal levels of GluN2Clike immunoreactivity and frontal cortex levels of GluN2D-like immunoreactivity in eNRG1-treated mice compared with those of control mice (Fig. 5B). In addition, there was a decrease in GAD67-like



**Fig. (3).** Hyper-locomotor activity and stereotypic behavior induced by MK-801. (**A**, **C**) Horizontal locomotor activity and (**B**, **D**) stereotypic behavior of eNRG1 (0.1, 0.3 and 1.0  $\mu$ g/g)-treated and vehicle-treated control mice were monitored before and after MK-801 (0.3  $\mu$ g/g, i.p.) or saline challenge every 10 min. Prior to MK-801 challenge, mice were acclimated to a test chamber to minimize their exploratory movement. Total locomotor activity and stereotypic counts of eNRG1-treated and vehicle-treated control mice were calculated and presented (n = 10 mice per group). Data is expressed as mean ± SEM. Arrows mark the timing of MK-801 challenge. \*p < 0.05, \*\*p < 0.01 and \*\*\*p < 0.001 compared with vehicle-treated control mice challenged by MK-801 and  $^{\#}p < 0.05$  compared between marked groups by Tukey HSD. Note; There was a significant main effect of eNRG1 treatment on total locomotor activity during the acclimination period of the four groups of mice; F(1, 36) = 19.3, ###p < 0.001 for eNRG1, F(1,36) = 0.46, p = 0.503 for MK-801, F(1,36) = 1.46, p = 0.234 for eNRG1 x MK-801.

immunoreactivity in eNRG1-treated mice. As we performed multiple comparisons here, we attempted to compensate the statistics with a Bonferroni's method. However, the differences in GluN2C-like and GluN2Dlike immunoreactivites still remained to be significant. There were no significant differences in other receptors and other GABAergic/dopaminergic markers between groups after compensation.

#### Distinct Sensitivity of eNRG1-Treated and T1-NRG1-Treated Mice to MK-801 But Not to Methamphetamine

Recently, we reported that neonatal exposure to full length T1-NRG1 results in behavioral impairments and hypersensitivity to methamphetamine in mice [15]. To compare the *in vivo* effects of eNRG1 and T1-NRG1,



**Fig. (4).** Neonatal exposure to eNRG1 enhances c-Fos expression following MK-801-challenge. In adulthood, vehicle-treated mice (**A**, **C**, **E**, **G**) and eNRG1-treated mice (**B**, **D**, **F**, **H**) were subjected to c-Fos immunohistochemistry 2 h after saline (**A**, **B**, **E**, **F**) or MK-801 (**C**, **D**, **G**, **H**) challenge. Representative pictures of mPFC (prelimbic cortex; **A** - **D**) and NAc (**E** - **H**) are shown. Scale bar, 100  $\mu$ m. (**I**) The number of c-Fos-positive cells in the microscopic field (725 × 965  $\mu$ m) was counted bilaterally using five to seven sections of mPFC (+1.70 ~ +1.98 mm from Bregma), NAc, and striatum (+1.18 ~ +1.54 mm from Bregma), piriform cortex (+1.18 ~ +1.98 mm from Bregma), averaged for each mouse, and subjected to statistical analysis (*n* = 4 mice per group). Values are expressed as mean ± SEM. \**p* < 0.05, \*\*\**p* < 0.001 compared between marked groups. \*### *p* < 0.001 compared with saline-challenged groups by Scheffe's test.

we measured the sensitivity of T1-NRG1-treated and eNRG1-treated mice to the NMDA receptor antagonist and methamphetamine (Figs. **6**, **7**). We prepared both types of mouse models by daily administering the same molar dose of T1-NRG1 (1.0  $\mu$ g/g body weight) and eNRG1 (0.3  $\mu$ g/g body weight) to mouse pups, allowed

them to reach 2 months of age, and challenged with MK-801 or saline. This dose was approximately 40 pmol per gram body weight and equivalent to the molar dose of T1-NRG1 that had been given to mouse pups in our previous study [15].



#### В

	Frontal Cortex (% of control)	Hippocampus (% of control)	Striatum + Nucleus Accumbens (% of control)
GluN1 # GluN2A	93± 4 88 ± 11	$\begin{array}{c} 105\pm15\\ 111\pm12 \end{array}$	$\begin{array}{c} 99 \pm 5 \\ 99 \pm 8 \end{array}$
GluN2B #	100 ± 6	110 ± 17	98 ± 13
GluN2C GluN2D #	103 ± 17 58 ± 7**	50 ± 7** 124 ± 16	79 ± 11 96 + 10
GluA1 #	121 ± 13	96 ± 7	104 ± 6
GluA2/3 GluA4 #	$107 \pm 3$ 99 ± 2	$80 \pm 11$ $92 \pm 7$	$105 \pm 14$ $109 \pm 12$
GAD65	92 ± 3	$116\pm3$	94 ± 3
GAD67 Parvalbumin	84 ± 3* 104 ± 8	$99 \pm 2$ 96 ± 6	$\begin{array}{c} 90\pm8\\ 96\pm7\end{array}$
TH	92±3	80 ± 5	90 ± 7
β-actin (n=5-6) (n=9-10) #	$\begin{array}{c} 101\pm 6\\ 104\pm 3\end{array}$	$\begin{array}{c} 98\pm10\\ 99\pm5\end{array}$	$\begin{array}{c} 97\pm9\\ 98\pm4\end{array}$

**Fig. (5).** Influences of neonatal eNRG1 treatment on the immunoreactivity of glutamate receptors, GABAergic and dopaminergic markers. Protein extracts were prepared from the frontal cortex, hippocampus, and striatum puls NAc of vehicle-treated and eNRG1 (1.0  $\mu$ g/g)-treated mice (n = 4-5 mice per group) at the adult stage (PND70-80) and subjected to immunoblotting with the antibodies directed against NMDA receptors (GluN1, GluN2A, GluN2B, GluN2C, GluN2D), AMPA receptors (GluA1, GluA2/3 and GluA4), GABAergic markers (glutamic acid decarboxylase; GAD65/67 and parvalbumin), a dopaminergic marker (TH; tyrosine hydroxylase), and a loading control ( $\beta$ -actin). (**A**) Typical signals of two samples in each brain region are displayed. (**B**) The intensity of the immunoreactivity appearing at the authentic size(s) was measured by densitometoric analysis and subjected to an unpaired two-tailed *t*-test. \*p < 0.05, \*\*p < 0.01 without Bonferroni's compensation. #) The number of samples was increased to 9 or 10 when the values of SEM from 4-5 mice had been larger than 20%; GluN1, GluN2B, GluA1, GluA4, and  $\beta$ -actin. Relative levels of the markers in eNRG1-treated mice are presented (% of control; mean ± SEM; n = 4-5 or n = 9-10 for #).

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**Fig. (6).** Difference of eNRG1-treated and T1-NRG1-treated mice in MK-801-driven locomotor activity and stereotypic behaviors. eNRG1 (0.3 µg/g)-treated, T1-NRG1 (1.0 µg/g)-treated and vehicle-treated mice (control) as neonates were challenged with MK-801 (0.3 µg/g; black boxes) or saline (white boxes) and their horizontal locomotor activity (**A**) and stereotypic behavior (**B**) were compared. Total horizontal activity and stereotypic score of mice were measured for the 120 min period after challenge and presented with the scales of cm/120 min and counts/120 min, respectively (n = 10-23 mice per group). Data is expressed as mean ± SEM. \*\*p < 0.01, compared between control and eNRG1-treated mice challenged with MK-801 and <sup>#</sup>p < 0.05 compared between eNRG1-treated mice challenged with MK-801 by Tukey HSD.

An initial 2-way ANOVA on horizontal locomotor activity, using the subject factors of NRG1 isoform and MK-801, revealed a main effect of MK-801 [F(1,74) = 23.57, p < 0.001] and a significant interaction between NRG1 isoform and MK-801 [F(2,74) = 5.17, p < 0.01]. We then stratified on MK-801 treatment (MK-801-challenged and saline-challenged groups; Fig. **6A**). In the saline-challenged control groups, there was no







Fig. (7). Similar enhancement of eNRG1-treated and T1-NRG1-treated mice in methamphetamine-driven locomotor activity and stereotypic behaviors. eNRG1 (0.3 µg/g)-treated, T1-NRG1 (1.0 µg/g)-treated and vehicle-treated mice (control) as neonates were challenged with methamphetamine (MAP; 1.0 µg/g black boxes) or saline (white boxes) and their horizontal locomotor activity (A) and stereotypic behavior (B) were compared. Data is expressed as mean ± SEM. \*p < 0.05, \*\*p < 0.01 between control and eNRG1-treated or T1-NRG1 treated mice challenged with MAP by Tukey HSD.

difference in basal locomotor activity among T1-NRG1treated, eNRG1-treated and control mice [NRG1 isoform : F(2,37) = 0.70, p = 0.50]. A one-way ANOVA for the MK-801-challenged group revealed a significant effect of isoform [F(2,37) = 4.80, p < 0.05]. Post-hoc analyses suggest that MK-801-driven horizontal locomotor activity of T1-NRG1-treated mice was indistinguishable from that of control mice receiving MK-801 whereas eNRG1-treated mice displayed a larger locomotor response to MK-801 than control mice. A two-way ANOVA for stereotypic movement revealed no significant effect of NRG1 isoform or no interaction between NRG1 isoform and MK-801 [NRG1 isoform: F(2,74) = 0.15, p = 0.86; NRG1 isoform × MK-801: F(2,74) = 0.99, p = 0.37] (Fig. **6B**). The results from the horizontal activity test revealed a significant difference in behavioral sensitivity to the NMDA receptor antagonist between T1-NRG1-treated and eNRG1-treated mice.

We also compared the magnitude of methamphetamine effects on locomotor activity. An initial 2-way ANOVA on horizontal movement, with between subject factors of NRG1 isoform and methamphetamine, revealed a main effect of methamphetamine [F(1,92) =60.2, p < 0.001 and an interaction between NRG1 isoform and methamphetamine [F(2,92) = 5.19, p <0.01]. We then stratified on methamphetamine treatment (methamphetamine-challenged and salinechallenged groups; Fig. 7A). In the saline-challenged groups, there were no significant differences among eNRG1-treated, T1-NRG1-treated, and control groups [F(2,46) = 0.60, p = 0.56]. A one-way ANOVA for the methamphetamine-challenged groups revealed a significant effect of NRG1 isoform [F(2,46) = 5.68, p <0.01]. Post-hoc analyses detected a significant difference between eNRG1-treated and control groups and between T1-NRG1-treated and control groups, but not between eNRG1-treated and T1-NRG1-treated groups. These results suggest that neonatal treatments with eNRG1 and T1-NRG1 similarly enhance methamphetamine-induced horizontal locomotion. We analyzed the effects of NRG1 isoform on stereotypic movements in a similar manner (Fig. 7B). There was a main effect of methamphetamine [F(1,92) = 101, p <0.001] and an interaction between NRG1 isoform and methamphetamine [F(2,92) = 4.74, p < 0.05]. Thus, we stratified on methamphetamine treatment. In the salinechallenged groups, there were no significant differences among groups [F(2,46) = 0.0064, p = 0.99]. significant In the methamphetamine-challenged groups, however, there was a significant effect of NRG1 isoform [F(2,46)] = 5.19, p < 0.01]. Post-hoc tests detected a significant difference between eNRG1-treated and control groups and between T1-NRG1-treated and control groups, but not between eNRG1-treated and T1-NRG1-treated groups. These analyses suggest that neonatal treatments with eNRG1 and T1-NRG1 similarly enhance methamphetamine-triggered stereotypic movements.

#### Auditory Perception of eNRG1-Treated Mice

NRG1 signaling is involved in the survival of cochlear sensory neurons; thus, we tested sound startle responses of the mice treated with eNRG1 (0.3 and 1.0  $\mu$ g/g) to increasing noises and also measured the auditory stimulus thresholds in different tone frequencies. In the sound startle response test, the lower dose of eNRG1-treated mice exhibited a marked increase in startle responses to 110-dB and 120-dB tones, compared with control mice [eNRG1  $\times$  pulse:

F(6,114) = 30.6, p < 0.001 (Fig. 8A). Conversely, the eNRG1-treated mice exhibited marked increases in the auditory stimulus thresholds at all stimulus frequencies at the ABR test [eNRG1 × stimulus frequencies: *F*(5,90) = 11.2, *p* < 0.001] (Fig. **8B**). The mice treated with the higher dose of eNRG1 (1.0  $\mu$ g/g) similarly exhibited the increases in startle responses and the auditory stimulus thresholds [eNRG1  $\times$  pulse: *F*(6,114) = 21.4, p < 0.001 for startle responses; eNRG1  $\times$ stimulus frequencies: F(5,90) = 13.4, p < 0.001 for auditory stimulus thresholds] (Fig. 8A, B). The results from the ABR test suggest deterioration in hearing of eNRG1-trearted mice. We speculate that, in the sound startle response test, the saturated levels of tone stimuli appear to produce higher startle responses in burst-noise-naïve eNRG1-treated mice with deterioration in hearing.

#### DISCUSSION

In our preceding study, mice pups were treated with T1-NRG1 and their behavioral consequences were analyzed with respect to the relevancy to an animal model for schizophrenia [15]. The T1-NRG1-treated animals exhibit various behavioral abnormalities in PPI and social traits but their gross learning performance and hearing ability are normal [15]. In addition to the isoform of T1-NRG1, here mouse pups were challenged with eNRG1, the core domain peptide that is produced by the neuropsin-dependent processing of T1-NRG1 [7]. In agreement with the preceding results from T1-NRG1-treated mice, transient exposure of eNRG1 during the neonatal stage similarly altered adult behavioral traits in PPI and resident-intruder test. Even though the mice were treated with the same molar dose of NRG1, eNRG1-treated mice exhibited additional behavioral deficits in tone-dependent fear learning, sound-startle responses and sensitivity to the NMDA receptor antagonist MK-801.

We also found that eNRG1-treated mice have a hearing disability. This finding is consistent with the previous report that ErbB4 signals regulate the development of hair follicles in the inner ear and that the manipulation of NRG1 signals indeed impair hearing in mice [33,34]. In this context, we can suggest that the deficits in prepulse inhibition, sound-startle responses, and tone-dependent learning of eNRG1-treated mice are, in part, due to the deterioration in hearing.

The decrease of 1.0  $\mu$ g/g eNRG1-treated mice in exploratory movement is also noteworthy. In contrast to the basal locomotor reduction, these mice rather exhibited the higher locomotor responses following MK-801 challenge. Although the lower basal movement might reflect the elevation of their anxiety levels, we do not rule out the possibility that these behavioral features of eNRG1-treated mice might be indirectly influenced by their deterioration in hearing as well.

In the present study, we additionally prepared the animal treated with the full soluble type 1 isoform of T1-NRG1 at the same molar dose of eNRG1 and



**Fig. (8).** Auditory responses of eNRG1-treated mice as adults. (**A**) Relative amplitudes of startle responses of eNRG1 (left; 0.3  $\mu$ g/g and right; 1.0  $\mu$ g/g)-treated and vehicle-treated mice (control) were monitored with 90 – 120 dB tones (n = 10 mice per group). Data is expressed as mean ± SEM. \*\*\* p < 0.001 compared between groups. ## p < 0.01 and ###p < 0.001 compared with startle levels at 90 dB by Tukey HSD. (**B**) ABR thresholds of eNRG1 (left; 0.3  $\mu$ g/g and right; 1.0  $\mu$ g/g)-treated and vehicle-treated mice were determined with specific auditory stimuli (4, 8, 10, 16, 20, and 32 kHz) by varying the sound pressure levels (n = 9-11 mice per group). Data is expressed as mean ± SEM. \*\*p < 0.01 and \*\*\* p < 0.001 compared between groups by Scheffe's test.

compared the sensitivities of eNRG1-treated and T1-NRG1-treated mice to two psychotomimetics, MK-801 and methamphetamine. The MK-801-driven locomotor enhancement was larger in mice treated with eNRG1 as neonates, compared with T1-NRG1-treated mice, even though the given molar concentration of T1-NRG1 was nearly equal to the dose of eNRG1. The hypersensitivity of the eNRG1-treated mice to MK-801 was also apparent in the magnitude of c-Fos induction in the brain. Following MK-801 challenge, eNRG1treated mice contained a higher number of c-Fospositive cells in the mPFC as well as in the NAc than control mice receiving MK-801. Both brain regions are implicated as target regions of MK-801 [35]. In contrast to the responses to MK-801, there were no significant difference in methamphetamine-triggered locomotor enhancement between T1-NRG1-treated and eNRG1treated mice. The behavioral difference of T1-NRG1eNRG1-treated mice treated and might reflect qualitative differences in in vivo activities of eNRG1 and T1-NRG1. Alternatively, the quantitative difference in the efficacy of blood-brain barrier permeability or in metabolic rates in the brain might underly the behavioral difference between these NRG1 variants. However, the magnitude and peak time of ErbB4 phosphoryration appeared to be similar between

eNRG1 and T1-NRG1 (i.e. both ~3-fold increases three hours after injection of their 1.0  $\mu$ g/g dose) [15].

Several reports indicate the activity difference among NRG1 splicing variants. Major splicing variants of NRG1 (type 1; NDF, type 2; GGF, and type 3; SMDF) exhibit distinct interactions to individual ErbB1-4 receptor subtypes, presumably leading to distinct biological effects in the brain [35,36]. NRG1 isoforms exhibit qualitative differences due to their specific structures such as an immunoglobulin-like domain. This domain, which is found in T1-NRG1 but not eNRG1, carries a heparin binding activity and regulates tissue distribution and receptor interactions of the ligand [7,37,38].

eNRG1 is produced from T1-NRG1 as well as potentially from the type2 isoform of NRG1 through the neuropsin-dependent proteolytic processing [7]. The protease neuropsin recognizes and cleaves the immunoglobulin-like domain of the NRG1 precursors and liberate their carboxyl terminal domain; eNRG1. Tamura et al. [7] demonstrate the biological implication of the neuropsin-dependent NRG1 processing in hippocampal synapse plasticity. As the molar dose of eNRG1 was adjusted to that of T1-NRG1 in some of the present experiments, their dose difference cannot illustrate the behavioral differences between T1-NRG1treated and eNRG1-treated mice. Nonetheless, the present results demonstrate that the biological actions of individual NRG1 variants significantly differ in vivo and result in distinct behavioral consequences. Corroborating our findings, mice deficient in type 3 NRG1 exhibit distinct behavioral deficits from type 1 NRG1-knockout mice [8,9,11]. However, it remains to be explored whether the present behavioral differences of T1-NRG1-treated and eNRG1-treated mice are ascribed to the unique activity of the immunoglobulinlike domain or that of the neuropsin-cleaving product eNRG1 [7,38].

To elucidate the molecular underpinnings of the hypersensitivity to the NMDA receptor given antagonist, we assessed the neurochemical influences of eNRG1 in adulthood, focusing on NMDA-type and AMPA-type glutamate receptors. There were reductions in NMDA receptor-like immunoreactivities (i.e. GluN2C and GluN2D) in the frontal cortex and hippocampus of eNRG1-treated mice, which can be involved in the hypersensitivity to MK-801. The specificity of the anti-GluN2D antibody, but not that for the anti-GluN2C antibody, has been verified with the use of knockout mice [39,40] Although the molecular nature of the present GluN2C-like immunoreactivity required further validation, the above arguments are in agreement with the observation that NMDA receptorhypomorphic mutant mice show hypersensitivity to an NMDA receptor blocker [24,41]. Recently, Belforte et al. (2010) also provide supportive evidence that the psychomimetric activity of MK-801 is ascribed to a decrease in the NMDA receptor sensitivity of parvalbumin-positive GABA neurons during postnatal development [42]. As parvalbumin-positive GABA neurons are enriched with ErbB4 receptors for NRG1,

the expression of NMDA receptors in this cell population might be affected by excess eNRG1 in the present model [43-46]. However, we do not rule out the possibility that the eNRG1-driven hypersensitivity to MK-801 may involve glutamatergic, dopaminergic, or cholinergic brain circuits as suggested previously [47].

NRG1 is one of the most promising candidate genes for schizophrenia. Most of the single nucleotide polymorphisms (SNPs) associating with schizophrenia are located in the genomic promoter region and introns of the 5'-side of the NRG1 gene [4,48,49]. Although the functional effects of these polymorphisms on gene transcription or alternative splicing of NRG1 isoforms are largely speculative, postmortem studies indicate that schizophrenic alterations of NRG1 mRNA levels are isoform-specific [3,50,51]. In agreement, the individual SNPs in human NRG1 are differentially implicated in psychotic symptoms, spatial working memory capacity, and white matter density and integrity [52,53]. In addition to schizophrenia, the genetic and neuropathologic association with NRG1 is implicated in several other psychiatric disease major depression, and bipolar disorder as well although several controversies still remain [54,55]. The differential expression of NRG1 processing enzymes is reported in schizophrenia and bipolar disorder [56]. Thus differential processing of NRG1 protein may contribute to the disease specificity as well.

The present results indicate that the composition of NRG1 isoforms might influence behavioral or cognitive traits in humans as well. Future studies should investigate the biological or pathological actions of individual NRG1 splicing isoforms underlying the SNP-dependent cognitive and anatomical traits in normal subjects and/or patients with schizophrenia.

#### ABBREVIATIONS

ABR	=	Auditory brain-stem evoked response
ANOVA	=	Analysis of variance
EGF	=	Epidermal growth factor
eNRG1	=	An epidermal growth factor domain peptide of neuregulin-1
mPFC	=	Medial prefrontal cortex
NAc	=	Nucleus accumbens
NRG1	=	Neuregulin-1
PBS	=	Phosphate-buffered saline
PND	=	Postnatal day
PPI	=	Prepulse inhibition
SNP	=	Single nucleotide polymorphisms
T1-NRG1	=	Type1 neuregulin-1

#### CONFLICT OF INTEREST

The authors confirm that this article content has not conflicts of interest.

#### Neuregulin-1 Variants and Behavioral Difference

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#### SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's web site along with the published article.

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### RESEARCH



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# Assessment of copy number variations in the brain genome of schizophrenia patients

Miwako Sakai<sup>1,2</sup>, Yuichiro Watanabe<sup>2</sup>, Toshiyuki Someya<sup>2</sup>, Kazuaki Araki<sup>1</sup>, Masako Shibuya<sup>2</sup>, Kazuhiro Niizato<sup>3</sup>, Kenichi Oshima<sup>3</sup>, Yasuto Kunii<sup>4</sup>, Hirooki Yabe<sup>4</sup>, Junya Matsumoto<sup>4</sup>, Akira Wada<sup>4</sup>, Mizuki Hino<sup>4</sup>, Takeshi Hashimoto<sup>5</sup>, Akitoyo Hishimoto<sup>5</sup>, Noboru Kitamura<sup>5</sup>, Shuji Iritani<sup>3,6</sup>, Osamu Shirakawa<sup>5,7</sup>, Kiyoshi Maeda<sup>5,8</sup>, Akinori Miyashita<sup>9</sup>, Shin-ichi Niwa<sup>4</sup>, Hitoshi Takahashi<sup>10</sup>, Akiyoshi Kakita<sup>10</sup>, Ryozo Kuwano<sup>9</sup> and Hiroyuki Nawa<sup>1\*</sup>

#### Abstract

**Background:** Cytogenomic mutations and chromosomal abnormality are implicated in the neuropathology of several brain diseases. Cell heterogeneity of brain tissues makes their detection and validation difficult, however. In the present study, we analyzed gene dosage alterations in brain DNA of schizophrenia patients and compared those with the copy number variations (CNVs) identified in schizophrenia patients as well as with those in Asian lymphocyte DNA and attempted to obtain hints at the pathological contribution of cytogenomic instability to schizophrenia.

**Results:** Brain DNA was extracted from postmortem striatum of schizophrenia patients and control subjects (n = 48 each) and subjected to the direct two color microarray analysis that limits technical data variations. Disease-associated biases of relative DNA doses were statistically analyzed with Bonferroni's compensation on the premise of brain cell mosaicism. We found that the relative gene dosage of 85 regions significantly varied among a million of probe sites. In the candidate CNV regions, 26 regions had no overlaps with the common CNVs found in Asian populations and included the genes (i.e., ANTXRL, CHST9, DNM3, NDST3, SDK1, STRC, SKY) that are associated with schizophrenia and/or other psychiatric diseases. The majority of these candidate CNVs exhibited high statistical probabilities but their signal differences in gene dosage were less than 1.5-fold. For test evaluation, we rather selected the 10 candidate CNV regions that exhibited higher aberration scores or larger global effects and were thus confirmable by PCR. Quantitative PCR verified the loss of gene dosage at two loci (1p36.21 and 1p13.3) and confirmed the global variation of the copy number distributions at two loci (11p15.4 and 13q21.1), both indicating the utility of the present strategy. These test loci, however, exhibited the same somatic CNV patterns in the other brain region.

**Conclusions:** The present study lists the candidate regions potentially representing cytogenomic CNVs in the brain of schizophrenia patients, although the significant but modest alterations in their brain genome doses largely remain to be characterized further.

Keywords: CNV, Caudate, Genome instability, Schizophrenia, Somatic mutation

#### Background

Copy number variation (CNV) is defined as a deletion or duplication/multiplication of a genomic fragment spanning more than 1 kb when compared to a reference genome [1-3]. Approximately 37,000 sites of common CNVs have been identified in the human genome and they occupy 12 % of the entire genome [4, 5]. The genome-wide

\* Correspondence: hnawa@bri.niigata-u.ac.jp

<sup>1</sup>Department of Molecular Neurobiology, Brain Research Institute, Niigata University, 1-757, Asahimachi-dori, 951-8585 Niigata, Japan Full list of author information is available at the end of the article association studies (GWAS) on schizophrenia analyzed DNA which was isolated from peripheral lymphocytes and have identified risk CNV sites, some of which are not present in the patients' parents [6–9].

Somatic mosaicism of genome sequences and structures have recently drawn particular attention [10–12]. Nearly 30 % of developing brain cells in human are reported to harbor aberrant chromosomal compositions [13, 14]. In addition, there are significant genomic differences in somatic cells between monozygotic twins and among tissues [15–18]. Accordingly, aberrant cytogenomic variations in



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human brain are implicated in neurodegenerative and neurodevelopmental disorders such as Alzheimer's disease, amyotrophic lateral sclerosis, and Huntington's diseases [19–25]. It is an open question whether the brain-specific somatic mutation or CNV might also contribute to the etiology or neuropathology of schizophrenia [26–28].

To obtain hints at the above question, we prepared DNA from the brain tissue of 48 schizophrenia patients and 48 control subjects. Labeling brain DNA samples, we directly applied those to Agilent 1 M comparative genomic hybridization (CGH) arrays to measure relative gene doses without the use of reference genome. This direct comparison through the case–control pairing reduces technical data deviations and enhances the statistical power of detection [29, 30]. With the potential genomic mosaicism of heterogeneous brain cell mixtures, we expected that the target genome could be diluted with normal DNA from the off-target cells and thus assumed non-integer values of CNVs in this analysis [31]. Technical limitations of this approach are further discussed below.

#### Results

The striatum contain neural stem cells that proliferate throughout human life and carries somatic mutation in its mitochondrial genome [32, 33]. Therefore, we hypothesized that the striatum may be a potential candidate region that would exhibit somatic mosaicism in brain genome structures. DNA was extracted from postmortem striatum of patients with chronic schizophrenia (n = 48) and agematched controls who had no history of neuropsychiatric disorders (n = 48) (Additional file 1: Table S1). Although there were significant differences in postmortem intervals (PMIs) between groups, there was no detectable difference in DNA quality (data not shown). All other indices were indistinguishable between schizophrenia patients and control subjects. A DNA sample was randomly picked from each group, paired to a sample in the other group, and subjected to two-color competitive CGH analysis with 1 M SurePrint G3 Human CGH Microarrays.

We applied the ADM-2 algorithm to the CGH signals of individual microarray probes (nearly 1 million) and searched for the primary candidate CNV loci associated with schizophrenia. A flowchart of the present study design is shown in Additional file 1: Figure S1. We chose1381 chromosomal loci that exhibited large group differences in gain/loss calls (Selection 1). In each probe site located on the primary candidate loci, we plotted the distribution of log2 signal ratios from 48 sets of microarray analyses and tested the null hypothesis that the mean log2 signal ratios was zero, indicating that the two groups were indistinguishable (Selection 2). We calculated total probabilities and averaged log2 signal ratios for individual candidate loci and judged their statistical significance with Bonferroni's correction. The number of the candidate loci maintaining the statistical significance through Selection 2 was reduced to 85 (Details in Additional file 1: Table S2).

Positive CNV loci were found in almost all chromosomes except chromosome 17 and 21 (Fig. 1). Individual loci covered 1–746 probe sites (3–2200 kb) and exhibited average log2 ratios of –1.46 to +0.63 (i.e., odds ratio (OR) = 0.36-1.55). A majority of the average log2 ratios were between –0.59 to +0.59 (i.e., < 1.5-fold differences) and only 4 loci showed more than 1.5-fold differences in array CGH signals. A genomic region spanning from 6p22.2 to 6p21.32 contained six CNV loci and included genes for the major histocompatibility complex that is highly associated with schizophrenia in GWAS [34]. Among the 85 CNV loci in Selection 2, 59 loci were reported and 26 loci were not reported in the CNV study on leukocyte DNA samples of Asian populations (Additional file 1: Table S2) [2].

To validate the authenticity of the present procedure, we attempted to verify the above genome dosage changes of several candidate loci using quantitative polymerase chain reaction (qPCR). According to the following two criteria, we selected the test loci whose signal differences were larger between groups and could be detectable with the given accuracy of qPCR; (i) those exhibiting the large and consistent gain/loss calls across the limited sample pairs (from Selection 1) and (ii) the loci represented larger global effects shared in most of the schizophrenia samples (from Selection 2).

In the former category, the gene dosage of Hs03385437 (1p13.3), CC70L1J (1p36.21), Hs03318079 (Chr18:q22.1), Hs04794356 (4q24), Hs05080419 (9q22.2), and Hs07134106 (19p12) produced exclusive gain/loss calls in not less than four sample pairs. No discrepant calls were detected in any sample pairs. Using the same DNA pairs showing the difference in the penetrance call (Selection 1), we determined and confirmed the gene dosage of those DNA samples using qPCR. ANOVA detected significant gene dose differences at two loci (Hs03385437 and CC70L1J) between patient and control groups (Fig. 2).

In this measurement, we used *RNaseP* gene as an internal DNA dose control. Measured genome doses of the above regions appeared not to be integer levels in several control samples, potentially reflecting the cell mosaicism of the original tissues. We also extracted DNA from the prefrontal cortex of the same subjects of both groups and compared the genome doses of the above loci (Hs03385437 and CC70L1J). We calculated the copy number ratio of the patient' DNA dosage to that of the control subject' dosage and compared these ratios between the brain regions. At both loci, almost all the copy number ratios were markedly lower than 1.0 except the C26:S34 pair, supporting our primary observation that the absolute gene dosages of these loci were decreased in the schizophrenia samples. However, copy number ratios did not significantly differ





between these brain regions in any of the sample pairs (Fig. 2). At least at these two candidate loci, we failed to find evidence for a gene dosage difference between these brain regions.

In the latter category, Hs0358779 (6p22.1), Hs03265736 (7p21.3), Hs03765933 (11p15.4), and Hs03298358 (q21.1) exhibited higher log2 signal ratios and were thus subjected to the test evaluation. Gene dosage of these four loci were determined by qPCR using all the DNA samples in control and schizophrenia groups (n = 48 each). Differences in gene dosages were replicated by qPCR for Hs03765933 and Hs03298358 (Fig. 3). In contrast to the data distributions of Fig. 2, almost all the values of the gene doses were located

at the levels of integers but with several exceptions. These candidate CNVs appear to reflect the gene dosage differences of germinal origin.

#### Discussion

Several recent reports have indicated the neuropathological contribution of somatic CNV or DNA instability of the brain genome [19-28, 35-40]. In accordance with these findings, a small proportion few percent of brain cells are known to exhibit aneuploidy and carry large CNVs [13, 14, 41]. Aneuploidy is detected by fluorescence in situ hybridization (FISH) and appears to be increased by the onset of Alzheimer's disease [20, 22]. The aneuploidy of chromosome 1, 18 and X was also identified in the brain of schizophrenia patients [21, 41]. Despite its advantages, FISH cannot be employed in exploratory investigations, unless the specific genome region of the CNV of interest is identified. Since bonafide genome structures from off-target cells could dilute the abnormal genome DNA population, more sensitive technologies remain to be developed, which detect low quantities of CNV in heterogeneous cell mixtures of the brain tissue [42, 43]. In the present study, we attempted to evaluate the efficacy of the CGH microarray technique to extract somatic CNVs in the postmortem brains of schizophrenia patients [42, 43].

With given semi-quantitative nature of the microarray technique, we applied statistics to the 1 M array CGH results from 48 sample pairs. Using the high density CGH array and statistical approach, we found 85 candidate CNV loci in the present study; 59 CNV loci are overlapped with the common CNV regions and the remaining 26 loci are not reported in peripheral leukocyte-derived DNA of Asian people [2, 44]. Of note, the 26 candidate regions encode the seven genes that are associated with or implicated in schizophrenia or other psychiatric diseases; ANTXRL, CHST9, DNM3, NDST3, SDK1, STRC, and SKY (Additional file 1: Table S2). DNM3 in the candidate region of 1q24.3 is disruptively mutated in some of schizophrenia patients [45]. ANTXRL and CHST9 are located in the CNV regions associated with bipolar disorder and autism [46, 47]. NDST3 and STRC are the risk genes for schizophrenia and hearing impairment that are identified by GWAS, respectively [48, 49]. SDK1 and SKY are the genes whose expression levels are markedly altered in the brain of schizophrenia patients [50, 51]. Accordingly, the present listing of the candidate brain CNVs is informative for future cytogenomic studies on schizophrenia [21, 41].

It was difficult for us to validate most of the abovementioned 85 candidate loci with qPCR analysis with the given small signal differences between groups (i.e., less than 1.5-fold). Therefore, we selected the best 10 test loci that exhibited relatively large and/or wide effects on gene dosage. The six loci were chosen from Selection 1 as putative rare CNVs, which exhibited exclusive gain/loss calls in the



limited number of samples. From Selection 2, the four loci were chosen as provisional common variants, which showed large effects and higher probability levels in the above parametric analysis. The qPCR analysis confirmed the schizophrenia-associated gene dosage differences at nearly half of the candidate CNV loci, suggesting the validity of the present strategy.

Unfortunately we had neither stored peripheral tissues nor information about these CNVs in peripheral DNA of the same subjects. To estimate the contribution of somatic CNVs to the present CNV listing, therefore, we were compelled to compare the gene dosages between the two brain regions or to search for their absence in the databases of Asian CNVs of leukocyte origin [2.44]. In the test PCR, however, we could not detect significant differences in gene dosages between the striatum and prefrontal cortex, at least, at these test CNV loci. If somatic CNVs were produced prior to neuroectodermal differentiation, there should be no difference between these two neural tissues, suggesting that the present comparison between these brain regions was inappropriate. Therefore, a comparative analysis of DNA from germinal cells of the same subjects will warrant this definitive conclusion [45].

Among the CNV candidate regions in Fig. 1, 26 candidate regions are not reported as the common CNVs of Asian populations [2, 44]. The majority of these loci exhibited high statistical significance with the probabilities of less than  $10^{-100}$ , such as 4q35.2, 6p11.2, 7q11–12, 11p15.4–15.5, and 15q11.2. In contrast, their CGH signal differences between patients and controls were markedly smaller (OR = 0.988–1.055). As discussed above, these candidate CNV loci include the peculiar genes that are

implicated in schizophrenia [45-51]. These regions, which exhibited small signal differences, might represent more promising candidates of somatic CNV sites because the genome aberration of target cells is presumably diluted in the brain and should result in smaller ORs. However, such small differences in gene dosage should make the conventional qPCR verification more challenging with the given technical deviations [52]. To avoid target DNA dilution with cell mosaicism, single cell qPCR or FISH may be more beneficial in theory [20-22, 43]. However, it would be difficult to independently perform microdissection of hundreds of cells and perform single-cell analysis unless the target cell population is identified with molecular markers and its sensitivity of gene detection is high enough. FISH also requires properly fixed and processed brain tissues of the same subjects. With the given technical difficulties, therefore, we have been unable to verify these small variations.

#### Conclusion

The present CGH analysis lists the potential candidate regions of somatic CNVs associated with schizophrenia, although most of those exhibited the modest but highly significant alterations in brain genome doses. Future studies aim to develop more elaborate techniques for somatic genome mosaicism and to verify the schizophrenia-associated cytogenomic instability in the above CNV candidates [53–56].

#### Methods

#### **Ethical approval**

The study was approved by Niigata University Medical Ethics Committee (No. 683). The use of postmortem brain tissues was authorized by the Matsuzawa Hospital Ethics Committee, Kobe University Medical Ethics Committee, Fukushima Medical University Ethics Committee, and Niigata University Medical Ethics Committee. The families of the control and schizophrenia patients provided written informed consent to allow the use of brain tissues for pathological investigations.

#### Brain tissue

Postmortem brain tissue was collected from patients with chronic schizophrenia (30 men, 18 women; mean age,  $64.5 \pm 12.5$  years old) and from age-matched control subjects (30 men, 18 women; mean age,  $64.2 \pm 12.0$  years old), with no history of neuropsychiatric disorders (Additional file 1: Table S1). The diagnosis of schizophrenia was confirmed by examining the patient's report according to DSM-III or DSM-IV categories (American Psychiatric Association). Postmortem brains of schizophrenia patients were collected at Matsuzawa Hospital, Kobe University, Fukushima Medical University and Niigata University, while those of control subjects were collected at Niigata

University. In brief, the left cerebral hemisphere was fixed in formalin for diagnostic examination and the right hemisphere was frozen at -80 °C. Tissue samples were taken from postmortem brains that did not exhibit neurodegenerative abnormalities by conventional pathological staining (data not shown). The striatum (caudate) was identified in frozen coronal slices according to a human brain atlas. All tissues were collected and stored according to the principles of the Declaration of Helsinki, and tissue use was in compliance with the Human Tissue Act 2004.

#### **DNA** extraction

High molecular weight DNA was extracted by the guanidinium – phenol procedure (Gentra Pure Gene Tissue Kit, Qiagen, Tokyo, Japan) according to the manufacturer's protocol. Extracted DNA was quantified by spectrophotometry using a Nanodrop ND-2000° (Thermo Scientific Wilmington, DE, USA). Samples with absorbance ratios of  $A260/280 \sim 1.80$  and A260/230 > 1.90, respectively, were regarded as sufficiently pure and suitable for CGH analysis. Some DNA samples were subjected to 1.0 % agarose gel electrophoresis for quality control. Evidence of DNA degradation was not detected in randomly-picked DNA samples from patient or control groups (data not shown).

#### Comparative genomic hybridization (CGH)

Array-based CGH was performed by the manufacturer Takara Bio Dragon Genomics Center (Seta, Shiga, Japan). In brief, DNA (2 micro g) was fluorescentlabeled by random priming DNA synthesis in the presence of Cy3-dUTP (control group) or Cy5-dUTP (patient group) (Genomic DNA Enzymatic Labeling Kit; Agilent Technologies, Hachioji, Tokyo, Japan). DNA labeling efficiency was estimated by spectrophotometry (Nanodrop ND-2000°) measuring optical absorbance at 260 nm for DNA, at 550 nm for Cy5, and at 649 nm for Cy3. Cy5- and Cy3-labeled DNAs were randomly paired, mixed, and hybridized to SurePrint G3 Human CGH Microarrays (1 M) in the presence of human Cot-1 DNA (Oligo aCGH/ChIP-on-chip Hybridization Kit, Agilent Technologies). Following hybridization for 24 h, microarray slides were washed according to the manufacturer's instructions and immediately scanned on a DNA Microarray Scanner (Agilent Technologies). With the given limitation of the sample number, we took an advantage of the above direct comparison between case and control samples [57]. This approach allowed us to determine relative ratios of their gene dosages but not their absolute gene dosages. However this procedure decreased data deviations, compared with the CGH analysis utilizing two microarrays and reference genome DNA [30].

#### Quantitative polymerase chain reaction (qPCR)

To validate the results from the microarray experiments, we performed qPCR using TaqMan probes (Applied Biosystems, Foster City, CA) as described previously [32]. Gene dosages of the following genomic regions of interest were measured for the sample pair sets that exhibited the exclusive positive penetrance call with the Aberration Detection Method 2 (ADM-2) algorithm; CC70L1J (1p13.3), Hs03385437 (1p36.21), Hs04794356 (4q24), Hs05080419 (9q22.2), Hs03318079 (18q21.1), and Hs07134106 (19p12). Using all the samples, we also determined the gene dosages of the candidate CNV loci that exhibited lower probability scores by the global *t*-test analysis; Hs03587795 (6p22.1), Hs03265736 (7p21.3), Hs03765933 (11p15.4), and Hs03298358 (13q21.1). DNA sequences of TaqMan probes and PCR primers are shown in the Additional file 1. We obtained cycle threshold (CT) values for the region of interest for each sample with FAM<sup>™</sup>-labeled probes, simultaneously monitoring those for *RNaseP* gene (an internal control) with its VIC®-labeled probe (ABI PRISM 7900HT Sequence Detection System and SDS v2.3 software, both Applied Biosystems). These CT values of the target gene and RNaseP gene were obtained for all the DNA samples. Copy number of the target gene was estimated from CT values by CopyCaller v1.0 software (Applied Biosystems).

#### Statistics

The ADM-2 algorithm prompted by Genomic Workbench software (edition 5.0.14, Agilent Technologies, 2010) was used to identify individual and common aberrations for 48 microarray data sets. This algorithm identifies all aberrant intervals with consistently high or low log ratios based on the statistical score. The algorithm searches for intervals where a statistical score based on the average qualityweighted log ratio of the sample and reference channels exceed a user-specified threshold. For the primary screening (Selection 1), we applied the following filtering options to the human genome assembly hg19 (excluding sex chromosomes): sensitivity threshold = 6, fuzzy zero = On, bin size = 10, and centralization threshold = 6. We then selected the primary candidate loci of somatic CNVs which exhibited > =4 difference in gain/loss calls in the whole penetrance summary.

To calculate mean signal OR and the probability of CNVs between groups, we plotted individual log2 signal ratios at all the probe sites within the above candidate loci. The Kolmogorov – Smirnov test revealed that log2 signal ratios were judged to fit into the Gaussian distribution at more than 80 % of probe sites. Assuming their Gaussian distribution, we analyzed their statistical biases against log2 = 0 (i.e., the null hypothesis of equal signal intensities between patients and controls) by two tailed *t*-test at each probe position. Within a candidate CNV

locus containing multiple probe sites, their log2 signal ratios were averaged and probabilities were summed and then subjected to Bonferroni's correction (Selection 2). Statistical difference of qPCR results between individual sample pairs was determined with ANOVA or two tailed *t*-test, incorporating technical errors into account. Alternatively, group differences of qPCR results from individual samples were estimated by the chi-square and Mann-Whitney U tests. Statistical analyses were performed using SPSS software (IBM Japan, Tokyo, Japan).

#### Additional file

Additional file 1: Figure S1. A flowchart of the study design. Table S1. Autopsy and clinical information of the subjects used. Table S2. List of candidate CNV regions and their statistical details. Table S3. Custom Tagman PCR primers and probes used.

#### Abbreviations

ADM: Aberration detection method; ANOVA: Analysis of variance; CGH: Comparative genomic hybridization; CNV: Copy number variation; CT: Cycle threshold; FISH: Fluorescent in situ hybridization; OR: Odds ratio; PMI: Postmortem interval; qPCR: Quantitative polymerase chain reaction.

#### **Competing interests**

The authors have no conflicts of interest to declare except OS; The author received honoraria from Otsuka Pharmaceutical; Shionogi; GlaxoSmithKline; Eli Lilly; and Tanabe Mitsubishi Pharma, and donations for research from the Otsuka Pharmaceutical Corporation.

#### Authors' contributions

TS, RK, and HN designed the experiments and wrote the manuscript; M. Sakai, YW, KA, M. Shibuya, AM, and HN performed the experiments; KN, KO, YK, HY, JM, AW, MH, TH, AH NK, SI, OS, KM, SN, HT, and AK collected and examined postmortem tissues. All authors read and approved the final manuscript.

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#### Author details

<sup>1</sup>Department of Molecular Neurobiology, Brain Research Institute, Niigata University, 1-757, Asahimachi-dori, 951-8585 Niigata, Japan. <sup>2</sup>Department of Psychiatry, Graduate School of Medical and Dental Sciences, Niigata University, 1-757, Asahimachi-dori, 951-8510 Niigata, Japan. <sup>3</sup>Matsuzawa Hospital, Setagaya-ku, 156-0057 Tokyo, Japan. <sup>4</sup>Departments of Neuropsychiatry, Fukushima Medical University School of Medicine, 960-1295 Fukushima, Japan. <sup>5</sup>Division of Psychiatry and Neurology, Kobe University Graduate School of Medicine, 650-0017 Kobe, Hyogo, Japan. <sup>6</sup>Department of Mental Health, Nagoya University Graduate School of Medicine, 466-8550 Nagoya, Aichi, Japan. <sup>7</sup>Department of Neuropsychiatry, Kinki University Faculty of Medicine, 589-8511 Osaka-Sayama, Osaka, Japan. <sup>8</sup>Department of Social Rehabilitation, Kobe University School of Medicine, 654-0142 Hyogo, Japan. <sup>9</sup>Department of Molecular Genetics, Brain Research Institute, Niigata University, 951-8585 Niigata, Japan. <sup>10</sup>Pathology and Brain Disease Research Center, Brain Research Institute, Niigata University, 951-8585 Niigata, Japan.

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## ORIGINAL ARTICLE

# Determination of kainate receptor subunit ratios in mouse brain using novel chimeric protein standards

Izumi Watanabe-Iida,\*'† Kohtarou Konno,†'‡ Kaori Akashi,\*'† Manabu Abe,\*'† Rie Natsume,\*'† Masahiko Watanabe†'‡ and Kenji Sakimura\*'†

\*Department of Cellular Neurobiology, Brain Research Institute, Niigata University, Niigata, Japan †CREST, Japan Science and Technology Agency, Chiyoda-ku, Japan ‡Department of Anatomy, Hokkaido University School of Medicine, Sapporo, Japan

#### Abstract

Kainate-type glutamate receptors (KARs) are tetrameric channels assembled from GluK1-5. GluK1-3 are low-affinity subunits that form homomeric and heteromeric KARs, while GluK4 and GluK5 are high-affinity subunits that require coassembly with GluK1-3 for functional expression. Although the subunit composition is thought to be highly heterogeneous in the brain, the distribution of KAR subunits at the protein level and their relative abundance in given regions of the brain remain largely unknown. In the present study, we titrated Cterminal antibodies to each KAR subunit using chimeric GluA2-GluK fusion proteins, and measured their relative abundance in the P2 and post-synaptic density (PSD) fractions of the adult mouse hippocampus and cerebellum. Analytical western blots showed that GluK2 and GluK3 were the major KAR subunits, with additional expression of GluK5 in the hippocampus and cerebellum. In both regions, GluK4 was very low and GluK1 was below the detection threshold. The relative amount of low-affinity subunits (GluK2 plus GluK3) was several times higher than that of high-affinity subunits (GluK4 plus GluK5) in both regions. Of note, the highest ratio of high-affinity subunits to low-affinity subunits was found in the hippocampal PSD fraction (0.32), suggesting that heteromeric receptors consisting of high- and low-affinity subunits highly accumulate at hippocampal synapses. In comparison, this ratio was decreased to 0.15 in the cerebellar PSD fraction, suggesting that KARs consisting of low-affinity subunits are more prevalent in the cerebellum. Therefore, low-affinity KAR subunits are predominant in the brain, with distinct subunit combinations between the hippocampus and cerebellum.

**Keywords:** antibody, GluK2, GluK3, kainate receptor, quantification, western blot.

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A kainate type of the ionotropic glutamate receptor family (KAR) is widely expressed in the central nervous system and involved in neurotransmission (Lerma 2003, 2006; Pinheiro and Mulle 2006: Contractor et al. 2011). There are five members of KAR subunits, GluK1-GluK5, which significantly differ in spatio-temporal patterns of expressions in the brain (Wisden and Seeburg 1993). KAR subunits are grouped into low-affinity subunits GluK1-GluK3, and highaffinity subunits GluK4 and GluK5 (Bettler et al. 1992). When expressed in cell lines or Xenopus oocytes, lowaffinity subunits can form functional homomeric KAR channels (Bettler et al. 1990, 1992; Egebjerg et al. 1991), while high-affinity subunits require any of GluK1-GluK3 to form functional KAR channels (Herb et al. 1992; Sakimura et al. 1992). KAR subunits are expressed in various neurons, such as pyramidal neurons, granule cells, and inhibitory interneurons in the hippocampus (Bahn et al. 1994; Bureau *et al.* 1999) and Purkinje cells, granule cells, and inhibitory interneurons in the cerebellum (Wisden and Seeburg 1993).

KAR-mediated responses are recorded in cerebellar neurons (Renard *et al.* 1995; Savidge *et al.* 1999; Bureau *et al.* 2000), where GluK2 and GluK3 are mainly expressed

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Address correspondence and reprint requests to Kenji Sakimura, Department of Cellular Neurobiology, Brain Research Institute, Niigata University, Niigata 951-8585, Japan. E-mail: sakimura@bri.niigata-u.ac. jp

Abbreviations used: AMPAR, AMPA-type glutamate receptor; DG, dentate gyrus; DIG, digoxigenin; KAR, kainate-type glutamate receptor; KO, knockout; NBT/BCIP, nitro-blue tetrazolium and 5-bromo-4-chloro-3'-indolyphosphate; NMDAR, NMDA-type glutamate receptor; PB, phosphate buffer; PFA, paraformaldehyde; PSD, post-synaptic density; SDS, sodium dodecyl sulfate; TNT, Tris-NaCl-Tween.

(Gallyas *et al.* 2003). KAR-mediated excitatory post-synaptic currents (EPSCs) are detectable at mossy fiber-CA3 synapses in the hippocampus, but are lost in GluK4/GluK5double knockout mice (Fernandes *et al.* 2009), suggesting that low-affinity subunits alone are insufficient, and coexpression of high-affinity subunits are essential, to form functional KARs at this hippocampal synapse. Thus, the composition of low-affinity and high-affinity KAR subunits and their functional contribution appear to be differentially regulated depending on the brain regions. Despite the wealth of knowledge on expressions at the transcription level (Wisden and Seeburg 1993), the distribution of KAR subunits at the protein level and their relative abundance in given regions of the brain remain largely unknown.

In the present study, we undertook this issue by testing the specificity and standardizing the titer of C-terminal antibodies to each KAR subunit using chimeric GluA2-GluK fusion proteins. With this novel method, we studied the relative abundance of KAR subunits in the adult mouse hippocampus and cerebellum, and found that low-affinity subunits are predominantly expressed in both regions.

#### Materials and methods

#### Animal experiments

All animal experiments were carried out in accordance with the guidelines laid down by the animal welfare committees and the ethics committees of Niigata University.

#### Fixation and sections

Under deep pentobarbital anesthesia (100 mg/kg body weight, ip.), mice were fixed by transcardial perfusion with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) for chromogenic *in situ* hybridization. After excision from the skull, brains were further post-fixed for 3 days at 20°C and cryoprotected with 30% sucrose in 0.1 M phosphate buffer, and cryosections (30 µm) were prepared on a cryostat (CM1900; Leica Microsystems, Wetzlar, Germany).

#### In situ hybridization

Mouse cDNA fragments of GluK1 (nucleotides 46–850 bp; GenBank accession number, NM\_146072), GluK2 (42–788, NM\_001111268), GluK3 (24–798, NM\_001081097), GluK4 (59–889, NM\_175481), and GluK5 (11–894, NM\_008168) were subcloned into pBluescript II plasmid vector. Digoxigenin-labeled cRNA probes were transcribed *in vitro* (Yamasaki *et al.* 2010). Fragmentation of riboprobes by alkaline digestion was omitted to increase the sensitivity and specificity. The chromogenic *in situ* hybridization was carried out as reported previously (Konno *et al.* 2014).

#### KAR-knockout mice

GluK2, GluK3, GluK4, and GluK5-knockout (KO) mice were generated using ES cell line RENKA derived from C57BL/6N (Mishina and Sakimura 2007). Detailed characterization of GluK2-KO, GluK3-KO, and GluK5-KO mice was described in Figure S1. Production of GluK4-KO mice was previously reported (Akashi et al. 2009).

#### Antibody

We produced anti-GluK1 antibody in the guinea pig against Cterminal 36 amino acid (aa) residues of mouse anti-GluK1 (898– 934, NM\_146072) and anti-GluK3 antibody in the rabbit against Cterminal 17 aa residues of mouse GluK3 (903–919, NM\_001081097). We also used rabbit polyclonal antibodies to anti-GluK2 (Synaptic Systems, Goettingen, Germany), anti-GluK4 (Akashi *et al.* 2009), and anti-GluK5 (Millipore Corporation, Bedford, MA, USA), which were raised against the C-terminal 55 aa residues of rat GluK2 (844–908, NM\_019309), C-terminal 91 aa of mouse GluK4 (866–896, NM\_175481) and C-terminal 21 aa of rat GluK5 (960–979, NM\_031508).

#### RT-PCR

Total RNA was isolated from whole brains of adult and post-natal day 3 (P3) mice using TRIzol LS Reagent (Thermo Fisher Scientific Inc., Waltham, MA, USA) following the protocol of the manufacturer. Semiquantitative reverse transcription (RT)-PCR was performed using an RT-PCR kit from Clontech (Takara, Tokyo, Japan). Briefly, 1 µg of total RNA was reverse transcribed using random hexamer primers for 1 h at 42°C. The resulting cDNA was appropriately diluted, and amplified using the following primers: GluK1 (exon 10-12), sense, 5'-TCGCTTGCCTAGGAGTCAGT-3'; antisense, 5'-GGGTGAAAAACCACCATATTC-3'; glyceraldehyde-3-phosphate dehydrogenase, sense, 5'-AGGTCGGTGTGA ACGGATTTG-3'; antisense, 5'-TGTAGACCATGTAGTTGAGGT CA-3'. Amplified products were electrophoresed and visualized by ethidium bromide staining. The relative expression of GluK1 was measured after scanning the bands with a CS Analyzer ver.3.0 (ATTO, Tokyo, Japan).

#### Construction of chimeric proteins

To standardize the titer of each C-terminal antibody, we generated standard chimeric proteins consisting of mouse GluA2 subunit (1-833 aa, NM\_013540) in the N-terminal side and mouse KAR subunits (GluK1, 841-934; GluK2, 841-908; GluK3, 842-919; GluK4, 826-952; GluK5, 825-979) in the C-terminal side (Fig. 3a). cDNAs for these chimeric proteins were subcloned into the pEF-BOS vector (Mizushima and Nagata 1990). Plasmid vectors were transiently transfected into COS-7 cells and/or HEK293 cells using the Plus Reagent and Lipofectamine Reagent (Invitrogen, Carlsbad, CA, USA) grown in the Dulbecco's Modified Eagle Medium containing 10% fetal bovine serum at 37°C in 5% CO<sub>2</sub>. 24 h after transfection, cells were lysed at 4°C in the sodium dodecyl sulfate (SDS) sample buffer (2% SDS, 3.3% glycerol, 125 mM Tris-HCl, pH 7.4). The extract was centrifuged at 10 000g for 10-20 min to remove cell debris, and the supernatant was used for SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) experiments.

#### Preparation of brain samples

C57BL/6N mice were purchased from Charles River Laboratories Japan (Yokohama, Japan), and were decapitated by cervical dislocation. Whole brains from P3 and adulthood (7–12 weeks of age) were homogenized in the homogenate buffer (0.32 M sucrose,

5 mM EDTA, 5 mM HEPES-NaOH, pH 7.4, complete protease inhibitor cocktail tablet; Roche, Mannheim, Germany). After measuring the total protein concentration by the bicinchoninic acid (BCA) Protein Assay Reagent (Thermofisher Scientific Inc.), the whole brain lysates were recovered in the SDS sample buffer and used for western blot analysis. Fractionated protein samples were prepared from the hippocampus and cerebellum of adult male C57BL/6N mice and of adult male GluK2-KO, GluK3-KO, GluK4-KO, and GluK5-KO mice. Preparation of fractionated protein samples and western blot was performed as previously described (Abe et al. 2004; Fukaya et al. 2006; Yamazaki et al. 2010). Brain tissues were excised and homogenized in the homogenate buffer and centrifuged at 1000g for 10 min. The resulting supernatants were then centrifuged at 10 000g for 10 min to obtain P2 fractions. The pellets were resuspended with homogenate buffer and aliquoted for P2 fraction, and the remaining samples were further separated on a sucrose density gradient (0.32, 0.8 and 1.2 M sucrose) by ultracentrifugation at 90 000g for 2 h at 2°C to obtain synaptosomal fractions. Triton X-100 was added to the aliquot samples to a final concentration of 1% and incubated at 2°C for 30 min and centrifuged at 10 000g for 10 min at 2°C to obtain P2 fractions. To prepare PSD fraction, the synaptosomal fraction was further solubilized with 0.5% Triton X-100 and then centrifuged at 200 000g for 1 h. The resulting pellet (PSD fraction) was suspended in 1% SDS, 40 mM Tris-HCl, pH 8.0 and diluted in SDS sample buffer. Protein sample of each fraction was heated at 100°C for 5 min in the presence of 2-mercaptoethanol and 0.002% Bromophenol blue and subjected to SDS-PAGE for western blot.

#### Western blot

Protein samples were separated by 8% SDS-PAGE under reducing conditions and electrophoretically transferred to a nitrocellulose membrane (Amersham, Buckinghamshire, UK). Membranes were blocked with 5% non-fat milk with Tris-buffered saline-Tween 20 (TBS-T) (137 mM NaCl, 0.1% Tween 20, 20 mM Tris-HCl, pH 7.6) for 1 h at 20°C. After washing for 30 min with three changes of TBS-T, membranes were incubated with each primary antibody (1 µg/mL) in TBS-T for 3-4 h at 20°C. After three washes with TBS-T, the membranes were incubated with horseradish peroxidaseconjugated secondary antibody for 1 h at 20°C in TBS-T. After three washes with TBS-T, proteins were visualized with enhanced chemiluminescence (ECL) (GE Healthcare, Piscataway Township, NJ, USA and EZ capture MG; ATTO, Tokyo, Japan) using a medical X-ray film (FUJIFILM Corporation, Tokyo, Japan) and a luminescence image analyzer with an electronically cooled chargecoupled device camera (LAS-4000 mini; GE Healthcare).

#### Quantitative analysis of western blot

Signal intensities of immunoreacted bands were determined by densitometric measurement using ImageJ software (available from the US National Institutes of Health), imageQuant TL (GE Healthcare) and CS Analyzer ver.3.0 (ATTO).

#### Statistical analysis

All data are presented as mean  $\pm$  SE. Groups were compared using the two-tailed, unpaired Student's *t*-test. Statistical significance is indicated as follows: \*\*\*p < 0.001.

#### Results

#### GluK1-5 mRNA expressions in the mouse brain

Distinct regional expression patterns of mRNAs for five GluK subunits have been reported in the rat brain (Wisden and Seeburg 1993; Bahn et al. 1994). In this study, we performed in situ hybridization for GluK1-5 mRNAs in the adult mouse brain using Digoxigenin-labeled cRNA probes, with special interest in the hippocampus and cerebellum (Fig. 1). Expression levels were generally low for GluK1 mRNA, but the hybridizing signals were selective to interneurons in the hippocampus and Purkinje cells in the cerebellum (Fig. 1a,f,k and p). GluK2 mRNA was widely expressed, with intense signals in the olfactory bulb, striatum, hippocampus, and cerebellum (Fig. 1b). In the hippocampus, GluK2 mRNA was expressed in both principal neurons (i.e., pyramidal and granule cells) and interneurons, which were identified as cells forming compact pyramidal cell and granule cell layers or cells dispersed inside and outside the layers respectively. The intensity of GluK2 mRNA expression was in the order of the dentate gyrus > CA3 > CA1 (Fig. 1g). In the cerebellum, granule cells expressed GluK2 mRNA intensely (Fig. 11 and q). GluK3 mRNA was moderately expressed in the cerebral cortex, hippocampus and cerebellum (Fig. 1c). GluK3 mRNA was detected in granule cells in the dentate gyrus, interneurons in the hippocampal Ammon's horn, and interneurons in the cerebellum; cerebellar interneurons were identified as cells dispersed in the molecular and granular layers (Fig. 1h,m and r). Expression levels were generally low for GluK4 mRNA, but intense signals were noted in pyramidal cells in the hippocampal CA3, granule cells in the dentate gyrus, and Purkinje cells in the cerebellum (Fig. 1d,i, n and s). Strong signals for GluK5 mRNA were found in the whole brain, especially in pyramidal cells in the hippocampal CA1-CA3, granule cells in the dentate gyrus, and interneurons and granule cells in the cerebellum (Fig. 1e,j,o and t). These distinct regional and cellular expressions in the adult mouse brain were consistent with those in the adult rat brain (Wisden and Seeburg 1993). We then examined how the distinct regional expressions of respective GluK subunits were reflected at the protein level.

#### Specificity of GluK antibodies

We checked the specificity of GluK2-5 antibodies with western blot analysis using hippocampal extracts from wild-type and GluK-defective mutant mice (Fig. 2a). Each of GluK2-5 antibodies recognized protein bands at expected molecular weights in the hippocampus of wild-type mice: GluK2, 102 kDa; GluK3, 104 kDa; GluK4, 107 kDa; GluK5, 109 kDa. No such bands were detected in the hippocampus of mutant mice lacking the corresponding GluK subunits. These results verify the specificity of these GluK2-5 antibodies and further indicate their applicability to quantitative western blot analysis.

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Because commercially available GluK1 antibody was found to cross-react to GluK2 (Figure S2), we produced GluK1 antibody in the guinea pig. To test the specificity of guinea pig GluK1 antibody, we used lysates of COS-7 and HEK293 cells transfected with GluK1-3 cDNAs, and found selective protein band in GluK1-transfected lysates (Fig. 2b). However, no protein bands were observed in the extracts from adult wild-type cerebellum and striatum. Because transcription levels of GluK1 were transiently increased Fig. 1 Expression of kainate-type glutamate receptor (KAR) subunit mRNAs in mouse brain. In situ hybridization with specific BNA probes for KAB subunits: (a) GluK1 (b) GluK2 (c) GluK3 (d) GluK4 (e) GluK5, in C57BL/6N mouse whole brain. High power images of hippocampal and cerebellar layer structures; (f, k, p) GluK1, (g, l, q) GluK2, (h, m, r) GluK3, (i, n, s) GluK4, (j, o, t) GluK5. CA1-3, CA1-3 regions of the Ammon's horn; DG, dentate gyrus; GL, granular layer; ML, molecular layer; PCL, Purkinje cell layer. Arabic numerals indicate cerebellar lobules. Scale bars: a-e, 1 mm; f-j, 200 µm; k-o, 100 µm; p-t, 100 μm.

Fig. 2 Characterization of each anti-GluK subunit antibody. (a) Hippocampal lysate (30-40 µg) of wild type and GluK2, GluK3, GluK4, and GluK5-knockout (KO) mice were examined by western blot analyses with anti-GluK2 (Synaptic systems), anti-GluK3, anti-GluK4 (ours), and anti-GluK5 (Millipore) antibodies. Asterisks show correct bands of GluK2, GluK3, GluK4, and GluK5. (b) Characterization of guinea anti-GluK1 C36 antibody piq with recombinant GluK1-GluK3 proteins and P2 fractions brain prepared from cerebellum (Cb.) and striatum (St.), indicating no cross-reactivity to GluK2 and GluK3. (c) Whole brain lysates (50  $\mu$ g) from juvenile (P3) (WT pool 1, pool 2, pool 3) and adult mice were loaded and detected with our anti-GluK1 C36 antibody.

during the perinatal period (Bettler *et al.* 1990; Bahn *et al.* 1994; Figure S3), we examined brain lysates prepared from post-natal day 3 (P3) and adult mice. A putative GluK1 protein band just above 100 kDa was faintly detected in brains at P3, but not in adulthood (Fig. 2c). This indicates that the amount of GluK1 proteins is below the detection threshold in adult brains when using this GluK1 antibody. Thus, we measured GluK2-5 protein levels in the subsequent analyses.

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#### Titration of GluK antibodies

We titrated GluK2-5 antibodies using GluA2-KAR chimeric proteins, in which the C-terminal sequence of GluA2 (downstream to the M4 domain) was replaced with that of GluK2-5 (Fig. 3a). Because all of the GluK2-5 antibodies were raised against the C-terminal regions, the titer of GluK2-5 antibodies could be standardized by using GluA2 antibody raised against the N-terminal domain (Fig. 3b). The densitometric measurement of protein bands for different loads of chimeric proteins showed linear doseintensity plots for each of GluK2-5 and GluA2 antibodies (Fig. 3c). Since an extreme difference in the intensities between chimeric protein bands detected by GluA2 and GluK2-5 antibodies failed to stabilize their titer ratio, we used each of their signal intensities from the same range and generated standard curves (Fig. 3c). From these plots, the titer ratios of GluK2-5 antibodies were calculated to be 8.0, 2.4, 30.5, and 24.6 times that of GluA2 antibody.

**Relative amounts of GluK in hippocampus and cerebellum** Using the titer ratios, we estimated the relative amount of GluK2-5 proteins in the P2 and PSD fractions prepared from the hippocampus and cerebellum (Figs 4 and 5). The



**Fig. 3** Unique method of GluK subunits determination by western blot. (a) Scheme of a chimeric protein of GluA2K2-GluA2K5 used for quantification analysis whose N-terminal GluA2 was fused with four Cterminal GluK2-5. Each titer was corrected by the titer of anti-GluA2 Nterminal antibody, and used for quantitative analysis of the four GluK subunits. (b) Chimeric proteins prepared from COS-7 cells or HEK293 cells were used for determining the ratio of antibody titers. Each antibody binds to chimeric protein depending on each affinity titer, and this reaction can be seen as western blot band signals. (c) Western

blot for determination of each titer of GluK2-5 antibodies with chimeric GluA2K2-5 proteins detected by GluA2 antibody and GluK2-5 antibodies. Each number of loaded samples of transfected cell lysates represents protein amount ( $\mu$ g). Examples of standard curves of GluA2K2-5 signal intensities detected by anti-GluA2 antibody (black square) and anti-GluK2-5 antibodies (white square) are shown. The ratios of anti-GluK2-5 antibody titers to anti-GluA2 antibody were determined by using GluA2K2-5 intensities.



Fig. 4 Determination of GluK subunits in hippocampus. (a) Analytical western blots of GluK subunits in P2 and post-synaptic density (PSD) fractions of hippocampus. Graded dilutions of hippocampal P2 and PSD fractions and each of chimeric GluA2K were subjected to analytical western blot. In order to simplify the picture, loaded protein amounts are indicated with relative one (see Table S1). The molecular weights are growing larger, in the order of GluA2 < GluA2K2-

amounts of loaded protein samples on western blots were changed according to the titer of each antibody and subunit content in each fraction, as summarized in Table S1. We generated standard curves of chimeric proteins and endogenous proteins (GluA2, GluK2-5) in every experiments (n = 3). A representative example is shown in Figure S4, where we made four standard curves of GluA2K2 detected by GluA2 antibody, GluA2K2 detected by GluK2 antibody, GluA2 in hippocampal PSD fraction, and GluK2 in hippocampal PSD fraction in the same intensity range (< 30000 (intensity), see vertical axis in Figure S4). Using these standard curves, we titrated their titer ratio and determined the ratio of GluK2 to GluA2. The western blot images (Figs 4a and 5a) are of one of those experiments determining GluK2-GluK5. We averaged the amounts of GluK2-5 proteins relative to GluA2 protein in each experiment (GluK2-GluK5, n = 3, respectively), as shown in the graph and table (Figs 4b and 5b).

In the hippocampus, GluK2, GluK3, GluK4, and GluK5 amounted to  $9.2 \pm 0.8\%$ ,  $3.5 \pm 1.0\%$ ,  $0.77 \pm 0.30\%$ , and  $2.0 \pm 0.3\%$ , respectively, of GluA2 in the P2 fraction (n = 3 for each), and to  $7.0 \pm 0.6\%$ ,  $4.2 \pm 0.8\%$ ,  $0.47 \pm 0.09\%$ , and  $3.1 \pm 0.2\%$ , respectively, of GluA2 in the PSD fraction

 $5 < {\rm GluK2-5}.$  Arrow heads show correct bands of kainate-type glutamate receptor (KAR) subunits. (b) The ratios of KAR GluK2, GluK3, GluK4 and GluK5 to GluA2. Ratios of KAR subunits in hippocampal P2 and PSD fractions are shown in the table and the bar graphs. The ratios of GluK2 (white), GluK3 (gray), GluK4 (stripe), and GluK5 (black) are normalized by the amount of GluA2 in each brain region and fraction. Error bars represent SEM.

(n = 3 for each) (Fig. 4b). In the cerebellum, GluK2, GluK3, GluK4, and GluK5 amounted to  $22 \pm 2\%$ ,  $17 \pm 4\%$ ,  $1.9 \pm 0.4\%$ , and  $2.8 \pm 0.5\%$ , respectively, of GluA2 in the P2 fraction (n = 3 for each), and to  $9.9 \pm 2.4\%$ ,  $7.3 \pm 1.0\%$ ,  $1.1 \pm 0.1\%$ , and  $1.4 \pm 0.2\%$ , respectively, of GluA2 in the PSD fraction (n = 3 for each) (Fig. 5b). These data show that the composition of GluK2-5 subunits is different depending on the brain regions and biochemical fractions, with GluK2 being the major component in the hippocampus and cerebellum.

By setting the relative amount of GluK2 in each fraction as 1.00, we compared the ratio of four GluK subunits among the four hippocampal and cerebellar fractions (Fig. 6a). In all fractions, the relative abundance of GluK2-5 subunits was in the order of GluK2 > GluK3 > GluK5 > GluK4, with relative enrichment of GluK3 in the cerebellar fractions and of GluK5 in the hippocampal PSD fraction (Fig. 6a). To simplify the understanding of composition of GluK subunits in each fraction, we also set GluK2 in the hippocampus P2 fraction as 1.00 and compared the ratio between the P2 and PSD fractions in the hippocampus and cerebellum (Fig. 6b). When collectively calculating low-affinity subunits (GluK2 plus GluK3) as 1.00, the ratio of high-affinity subunits



**Fig. 5** Determination of GluK subunits in cerebellum. (a) Analytical western blots of GluK subunits in P2 and post-synaptic density (PSD) fractions of cerebellum. Detailed explanation is as shown in Fig. 4. (b) Ratios of kainate-type glutamate receptor subunits in cerebellum P2

and PSD fractions are shown in the table and the bar graphs. The ratios of GluK2 (white), GluK3 (gray), GluK4 (stripe), and GluK5 (black) are normalized by the amount of GluA2 in each brain region and fraction. Error bars represent SEM.

(GluK4 plus GluK5) was higher in the hippocampus (0.22 in the P2 and 0.32 in the PSD fraction) than in the cerebellum (0.12. and 0.15 respectively). By setting the relative amount in the P2 fraction as 1.00 in each region, each GluK subunit showed 3–5-fold enrichment in the PSD fraction of the hippocampus and cerebellum (Table 1).

## Discussion

In the present study, the relative amount of KAR subunits was compared by standardizing the titer of KAR subunit antibodies using chimeric GluA2-GluK fusion proteins. Based on the titrated antibodies, we examined the relative amount of each KAR subunit in biochemical fractions prepared from the adult mouse hippocampus and cerebellum.

This method enabled the assessment of the amount of KAR subunits relative to GluA2 subunit in the same biochemical fractions. Compared to standardized immunoblot analysis using endogenous standard proteins, such as actin, this method is advantageous in that it is applicable to the comparison of relative amounts of multiple subunits using different antibodies. Although its low sensitivity such as inability of GluK1 detection in the adult mouse brain is to be further improved, the reliability of the present comparison for GluK2-5 subunits was ensured by specific detection of GluK2-5 protein bands,

as demonstrates by using GluK2-5 defective mice as negative controls, and by linear dose-intensity plots using chimeric GluA2-KAR protein standards. Here, we have shown that low-affinity KAR subunits are predominantly expressed in the adult mouse brain, where the relative amount of GluK2 plus GluK3 was several times higher than that of GluK4 plus GluK5 in the hippocampus and cerebellum.

Native KARs are thought to be heteromeric receptors composed of various combinations of subunits. When expressed in HEK293 cells and Xenopus oocytes, GluK4 and GluK5 subunits associate with GluK1-3 subunits to form heteromeric receptors with pharmacological properties distinct from homomeric receptors consisting of the low-affinity subunits. Moreover, the amplitude of channel activity of GluK2/GluK5 heteromeric receptors is several times larger than that of GluK2 homomeric receptors (Sakimura et al. 1992). Immunoprecipitated analysis has revealed that GluK5 is co-immunoprecipitated with GluK2 in CA3 pyramidal cells (Wenthold et al. 1994). A single molecule imaging of fluorescence-tagged GluK2 and GluK5 subunits revealed that they assemble with a 2 : 2 stoichiometry (Reiner et al. 2012). Crystal structure analysis has shown that the N-terminal domains of GluK2 and GluK5 subunits preferentially coassemble as heterodimers rather than as homodimers (Kumar et al. 2011). In the hippocampus of GluK4/GluK5-double

(a) GluK2 GluK4 GluK5 GluK3 0.38 0.08 0.22 Hippcampus P2 1 0.07 Hippocampus PSD 1 0.60 0.44 Cerebellum P2 1 0.78 0.08 0.13 Cerebellum PSD 0.74 1 0.11 0.15 GiuK2 GluK3 Hippcampus P2 S GluK4 GluK5 Hippocampus PSD Cerebellum P2 Cerebellum PSD 0.5 1.5 2.5 0 1 2 (b) GluK2 GluK4 GluK3 GluK5 1 0.08 0.22 Hippcampus P2 0.38 Hippocampus PSD 3.57 2.13 0.24 1.57 Cerebellum P2 1.28 0.99 0.11 0.16 Cerebellum PSD 3.83 0.43 0.56 2.83 Gluk2 GluK3 Hippocampus P2 GluK4 GluK5 Hippocampus PSD Cerebellum P2 Cerebellum PSD

knockout mice, detectable KAR-mediated EPSCs are lost at mossy fiber-CA3 synapses (Fernandes *et al.* 2009). These findings indicate that one form of functional KARs in the brain is heteromeric receptors consisting of high- and lowaffinity subunits. GluK4 and GluK5 subunits in hippocampal and cerebellar fractions are likely to represent, mostly if not all, such heteromeric receptors. The highest GluK5-to-GluK2 ratio and its marked increment from the P2 fraction (0.22) to the PSD fraction (0.44) in the hippocampus further suggest that heteromeric receptors consisting of high- and low-affinity subunits highly accumulate at hippocampal synapses.

Another form of functional KARs in the brain is homomeric and heteromeric receptors consisting of only lowaffinity KAR subunits. Application of kainate and glutamate elicits current responses in HEK293 cells expressing GluK1 (Q), GluK2(Q/R), GluK1/GluK2, GluK3a, and GluK3b (Sommer *et al.* 1992; Coussen *et al.* 2005). GluK1 and GluK2 subunits co-assemble to generate recombinant receptors with novel functional properties (Cui and Mayer 1999). Two splice variants of GluK2 subunit (GluK2a and GluK2b)

Fig. 6 Composition kainate-type of glutamate receptor (KAR) subunits in hippocampus and cerebellum. (a) Relative ratios of KAR subunits in hippocampal P2. post-synaptic density (PSD) and cerebellar P2, PSD are shown in the table and the bar graph. The ratios of GluK3 (gray), GluK4 (stripe), and GluK5 (black) are normalized by the amount of GluK2 (white) in the brain regions respectively. (b) Relative amounts of KAR subunits normalized by the amount of GluK2 in hippocampus P2 fraction are shown in the table and the bar graph. Lowaffinity GluK2 and GluK3 are described in the left side of position 0, and high affinity GluK4 and GluK5 are described in the right side

also co-assemble to regulate KAR trafficking and function (Coussen et al. 2005). The ratio of low-affinity (GluK2 plus GluK3) to high-affinity (GluK4 plus GluK5) was higher in the cerebellum (8.5 in the P2 fraction and 6.7 in the PSD fraction) than in the hippocampus (4.6 and 3.1, respectively), suggesting that KARs consisting of low-affinity subunits are more prevalent in the cerebellum. There have been many studies on KAR-mediated synaptic transmission focusing on hippocampus CA3-dentate gyrus synapses, reporting loss of GluK4 and GluK5 showed no detectable KAR-EPSCs in hippocampus. In this study, it is reasonable to expect that GluK2 homomer in cerebellum granule cells would not show KAR-EPSCs. Indeed, cerebellar granule cells in wild type showed no KAR-mediated synaptic transmission, but loss of AMPAR activity increased GluK5 expression and GluK2/5containing KAR-mediated synaptic transmission (Yan et al. 2013). KAR activity is possibly regulated by an increase in GluK4 or GluK5 expression, when AMPAR activity decreased.

Expression patterns of five KAR subunits by *in situ* hybridization are quite similar between rat and mouse brains.

	GluK2	GluK3	GluK4	GluK5	
Hippocampus P2	1	1	1	1	
Hippocampus PSD	3.57	5.56	2.84	7.24	
Cerebellum P2	1	1	1	1	
Cerebellum PSD	2.99	2.83	4.00	3.46	

Table 1 Enrichment of GluK subunits in the post-synaptic density (PSD) fraction of the hippocampus and cerebellum

These values are calculated from Fig. 6b.

In both species, GluK5 mRNA is prominently expressed in various brain regions, including the hippocampus and cerebellum (Wisden and Seeburg 1993; this study). It was thus unexpected to find predominant protein contents of lowaffinity KAR subunits over high-affinity ones in the adult brain. This was particularly prominent in the cerebellum, where GluK4 and GluK5 mRNAs are highly expressed, but their protein contents were less than one-tenth of that of GluK2. This discrepancy raises the possibility that the predominant low-affinity subunit expression is constructed mainly at the post-transcriptional level. Considering that high-affinity subunits cannot form functional homomeric KARs (Bettler et al. 1990, 1992; Egebjerg et al. 1991; Herb et al. 1992; Sakimura et al. 1992), homomeric receptors consisting of high-affinity subunits alone might be unstable and prone to be degraded, even if they are constructed in neurons. In NMDARs, heteromeric subunit configuration is essential for channel function and synaptic expression. Without GluN2 subunits, GluN1 subunit is unable to accumulate on the post-synaptic membrane and its turnover rate is accelerated (Abe et al. 2004). Without GluN1 subunit, GluN2 subunits are retained in the ER for degradation (McIlhinney et al. 1996; Fukaya et al. 2003). In KAR, GluK5 also has an ER retention motif and enables recruiting the membrane when co-assembled with GluK1, GluK2, and GluK3 (Gallyas et al. 2003). Without them, GluK5 was also heavily retained in the ER and lost its current in heterologous cells (Hayes et al. 2003; Ren et al. 2003). We also found that the amount of GluK5 is less enriched in PSD fraction of cerebellum than that of hippocampus (Table 1), indicating the trafficking of GluK subunit to the post-synaptic plasma membrane may be differentially regulated in these regions. Perhaps its turnover rate is maintained depending on the brain region. Overall, this discrepancy of the amount of GluK5 at mRNA and protein level remains to be investigated.

In this study, we noticed the importance of low-affinity subunits as major KAR subunits, although their main roles have not been understood so far. In our data, GluK2-GluK5 showed positive concentration efficiency to PSD fractions from P2 fractions. The fractionation procedure was performed as previously described (Abe et al. 2004; Fukaya et al. 2006; Yamazaki et al. 2010; see method section). In

hippocampus, concentration levels of both GluK2 and GluK3 are fourfold higher in PSD fractions than in P2 fraction. The proportions of AMPAR and KAR subunits in enriched synaptic membrane fractions in rat cortex with synaptic purification technique (Phillips et al. 2001; Pinheiro et al. 2003, 2005) showed that GluK2/3 was predominantly present in PSD fractions (about 70-80%) with much lower levels of pre-synaptic and non-synaptic synaptosomal protein fractions (about 20-30%) (Feligioni et al. 2006), which are consistent with our data as mentioned above. On the other hand, GluA1 and GluA2 were not predominantly present in PSD fraction (40-60%) compared with GluK2/3. Biochemical distribution and protein solubility of other glutamate receptors showed that many NMDA-type glutamate receptors were in the insoluble membrane fractions, while AMPARs were in the insoluble membrane fractions and the soluble fractions (Fukaya et al. 2006; Inamura et al. 2006), suggesting that the distribution of KAR at ultrasynaptic fraction may be different from that of AMPAR. These data might elucidate the controversy of subcellular localization of KAR including pre- and/or post-synapse, and point to a hidden role of the post-synaptic afferent in the neuron. Future study will provide an insight into whether lowaffinity KAR subunits mediate neurotransmission signal at post-synaptic sites.

# Acknowledgments and conflict of interest disclosure

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All experiments were conducted in compliance with the ARRIVE guidelines.

## Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web-site:

Figure S1. (a) To construct a Grik2 targeting vector, a 770 bp DNA fragment carrying exon 14 of the Grik2 was amplified by PCR, and inserted to the targeting vector as described previously (Akashi et al. 2009). In this clone, a DNA fragment of pgk promoter-driven Neo-poly(A) flanked by two frt sites (Neo cassette) and loxP sequence was located at the site 311 bp upstream of the exon 14, while the other loxP sequence was placed at the site 241 bp downstream of the exon 14. It contained a 6.83 kb Grik2 at the 5' side and 3.79 kb Grik2 at the 3' side, followed by an MC1 promoterdriven diphtheria toxin (DT) gene. (b) To construct a Grik3 targeting vector, a 817 bp DNA fragment carrying exon 12 of the Grik3 was amplified by PCR, and inserted to the SacI sites of the middle entry clone (pDME-1). In this clone, a DNA fragment of Neo cassette and

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loxP sequence was located at the site 428 bp upstream of the exon 12, while the other loxP sequence was placed at the site 270 bp downstream of the exon 12. The 5.07 kb upstream and 5.24 kb downstream homologous genomic DNA fragments were retrieved from the BAC clone (RP23-131M1), and then subcloned to 5' entry clone (pD3UE-2) and 3' entry clone (pD5DE-2) respectively. For targeting vector assembly, the three entry clones were recombined to a destination vector plasmid (pDEST-DT; containing a cytomegalovirus enhancer/chicken actin (CAG) promoter-driven DT gene by using a MultiSite Gateway Three-fragment Vector construction Kit (Invitrogen) (Sasaki et al. 2004). (c) To construct a Grik5 targeting vector, a 770 bp DNA fragment carrying exon 13, 14, and 15 of the Grik5 was amplified by PCR, and inserted to the targeting vector as described previously (Akashi et al. 2009). In this clone, a DNA fragment of Neo cassette and loxP sequence was located at the site 411 bp upstream of the exon 13, while the other *loxP* sequence was placed at the site 96 bp downstream of the exon 15. It contained a 6.53 kb Grik5 at the 5' side and 3.13 kb Grik5 at the 3' side, followed by an MC1 promoter-driven DT gene. Each targeting vector introduced into C57BL/6N ES cell line RENKA and recombinant clones were identified by Southern blot. To produce germline chimeras, recombinant ES clones were microinjected into eight cell stage embryos of CD1 mouse strain. To generate Grik2, Grik3 and Grik5-null mice, each flowed mouse was crossed with the telencephalin-Cre mice (Nakamura et al. 2001).

**Figure S2.** Specificity of GluK1 antibody (Upstate) was checked with western blot analysis of COS-7 cell lysates transfected with each cDNA of GluK1-GluK3. Cross reactivity of GluK1 antibodies between GluK1 and GluK2 was tested using the brain extracts, hippocampal P2 fractions, derived from wild type and GluK2-KO mice. A detected GluK1 band of the GluK1 antibody in the wild type disappeared in GluK2-KO mice.

**Figure S3.** (a) RT-PCR was performed using primers spanning exon 10–12 with whole brain of adult wild type (C57BL/6N), GluK3flox/flox mice and post-natal day 3 (P3) GluK3flox/flox mice. Amplification of glyceraldehyde-3-phosphate dehydrogenase gene was used as a positive control for the RT reaction (bottom panel). P3 GluK3flox/flox mice were used instead of wild type mice. (b) GluK1 transcript was decreased in adult mice, compared to P3 mice (top panel). The expression level of adult mice GluK1 transcript was normalized by that of P3 mice. Error bars represent SEM. Student's *t*-test was used for statistical analysis.

**Figure S4.** (a) Determination of GluK2 ratio to GluA2 examined by chimeric protein and hippocampus PSD sample is shown as an example of the determination method. Amounts of loaded COS-7 cells lysate expressing GluA2K2 and hippocampal PSD sample are shown as a relative value. (b) Standard curves of GluA2K2 (left) and hippocampus PSD sample (right) were detected by GluA2 antibody (black square and circle) and GluK2 antibody (white square and circle). The protein ratio of GluK2 to GluA2 in hippocampus PSD was quantified by correcting titer ratio of GluK2 antibody to GluA2 antibody. (c) The titer of GluK2 antibody to GluA2 antibody was obtained from calculation using the slopes of GluA2K2 standard curves (b, left). The relative ratio of GluK2 protein to GluA2 and GluA2 standard curves (b, right) and GluK2 titer ratio mentioned above.

Table S1. The amount (mg) of loaded protein samples of hippocampus and cerebellum.

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# Anterograde C1ql1 Signaling Is Required in Order to Determine and Maintain a Single-Winner Climbing Fiber in the Mouse Cerebellum

Wataru Kakegawa,<sup>1,2</sup> Nikolaos Mitakidis,<sup>3</sup> Eriko Miura,<sup>1,2</sup> Manabu Abe,<sup>2,4</sup> Keiko Matsuda,<sup>1,2</sup> Yukari H. Takeo,<sup>1,2</sup>

Kazuhisa Kohda,<sup>1,2</sup> Junko Motohashi,<sup>1,2</sup> Akiyo Takahashi,<sup>1,2</sup> Soichi Nagao,<sup>5</sup> Shin-ichi Muramatsu,<sup>6</sup>

Masahiko Watanabe,<sup>2,7</sup> Kenji Sakimura,<sup>2,4</sup> A. Radu Aricescu,<sup>3</sup> and Michisuke Yuzaki<sup>1,2,\*</sup>

<sup>1</sup>Department of Physiology, Keio University School of Medicine, Tokyo 160-8582, Japan

<sup>3</sup>Division of Structural Biology, Wellcome Trust Centre for Human Genetics, University of Oxford, Oxford OX3 7BN, UK

<sup>4</sup>Department of Cellular Neurobiology, Brain Research Institute, Niigata University, Niigata 951-8585, Japan

<sup>5</sup>Laboratory for Motor Learning Control, RIKEN Brain Science Institute, Saitama 351-0198, Japan

<sup>6</sup>Division of Neurology, Jichi Medical University, Tochigi 329-0498, Japan

<sup>7</sup>Department of Anatomy, Graduate School of Medicine, Hokkaido University, Sapporo 060-8638, Japan

\*Correspondence: myuzaki@a5.keio.jp

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#### SUMMARY

Neuronal networks are dynamically modified by selective synapse pruning during development and adulthood. However, how certain connections win the competition with others and are subsequently maintained is not fully understood. Here, we show that C1ql1, a member of the C1q family of proteins, is provided by climbing fibers (CFs) and serves as a crucial anterograde signal to determine and maintain the single-winner CF in the mouse cerebellum throughout development and adulthood. C1ql1 specifically binds to the brain-specific angiogenesis inhibitor 3 (Bai3), which is a member of the celladhesion G-protein-coupled receptor family and expressed on postsynaptic Purkinje cells. C1ql1-Bai3 signaling is required for motor learning but not for gross motor performance or coordination. Because related family members of C1ql1 and Bai3 are expressed in various brain regions, the mechanism described here likely applies to synapse formation, maintenance, and function in multiple neuronal circuits essential for important brain functions.

#### INTRODUCTION

In mammals, precise neuronal network formation is generally achieved by selective synapse pruning: a few inputs are strengthened by increasing the number of their synaptic contacts, while weak inputs are physically removed. As a model of activity-dependent synapse refinement in the central nervous system (CNS), postnatal pruning of supernumerary synapses between climbing fibers (CFs, axons of inferior olivary [IO] neurons) and Purkinje cells in the cerebellum has been extensively studied (Cesa and Strata, 2009; Kano and Hashimoto, 2009; Lohof et al.,

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1996; Watanabe and Kano, 2011). Multiple CFs initially innervate a single Purkinje cell soma, but a single CF becomes dominant in a postsynaptic, voltage-dependent Ca2+ channel activitydependent manner during postnatal days (P) 3-7 in mice (Kano and Hashimoto, 2009; Watanabe and Kano, 2011). The "single-winner" CF translocates to the Purkinje cell dendrites around P9, and the rest of the CFs remaining on the soma are eventually eliminated by P21 (Hashimoto et al., 2009). CF pruning is reportedly regulated by activities in Purkinje cells mediated by parallel-fiber (PF, an axon of a granule cell) inputs through metabotropic glutamate receptor 1 (mGluR1), as well as inhibitory inputs through y-aminobutyric acid (GABA) receptors (Kano and Hashimoto, 2009; Watanabe and Kano, 2011). Although a recent live-imaging study indicated that translocation of CF synapses to dendrites may provide a competitive advantage to the winner CF (Carrillo et al., 2013), how a selected CF is strengthened remains unclear. Furthermore, although brain-derived neurotrophic factor and insulin-like growth factor are suggested to be involved in strengthening CF synapses, the site of synaptic expression and action remains largely elusive (Watanabe and Kano, 2011). In addition, while CF synapses further undergo activity-dependent expansion or retraction throughout adulthood (Cesa and Strata, 2009), how the winner CF is maintained is unknown.

C1q, which is a member of the innate immune system, recognizes various targets via its C-terminal globular domain (gC1q). Recently, C1q released from neurons has been shown to regulate postnatal elimination of inactive synapses between retinal ganglion cells and the lateral geniculate nucleus in vivo (Stevens et al., 2007). The group of proteins containing the gC1q domain is referred to as the C1q family. Like adiponectin, which is released from adipose tissues and regulates glucose and lipid metabolism in muscle and liver, most C1q family members are secreted and involved in various signaling pathways (Kishore et al., 2004; Yuzaki, 2008). In the cerebellum, Cbln1, a cerebellin family of C1q homologous, is released from granule cells and plays a crucial role in the formation and maintenance of PF-Purkinje cell synapses by binding to its postsynaptic receptor, the delta2 glutamate

<sup>&</sup>lt;sup>2</sup>Core Research for Evolutional Science and Technology (CREST), Japan Science and Technology Agency (JST), Tokyo 102-0075, Japan

receptor (GluD2) (Matsuda et al., 2010). C1q-like family members (C1ql1-C1ql4) are also expressed in the CNS (lijima et al., 2010; Shimono et al., 2010) and are implicated in synapse formation or elimination in cultured hippocampal neurons in vitro (Bolliger et al., 2011). It is interesting that C1ql1 mRNA is selectively and highly expressed in IO neurons (lijima et al., 2010) during development and throughout adulthood. Here, we examined the hypothesis that C1ql1 provided by CFs may regulate CF synapse formation, elimination, and maintenance in vivo. We found that C1ql1 plays a crucial role in strengthening and maintaining single-winner CFs by binding to brain-specific angiogenesis inhibitor 3 (Bai3), a cell-adhesion G protein-coupled receptor (GPCR) that is constitutively expressed in Purkinje cells. Furthermore, our in vivo results demonstrate that C1gl1-Bai3 signaling is essential for CFs to mediate normal motor learning but not gross motor coordination.

#### RESULTS

#### C1ql1 Is Required to Win CF Competition

We first investigated the localization of endogenous C1ql1 proteins in the wild-type (WT) mouse cerebellum. Immunohistochemical (IHC) analysis revealed punctate C1ql1 immunoreactivity in the molecular layer of the cerebellum along the proximal dendrites of Purkinje cells (Figure 1A). The C1ql1-immunopositive puncta were mostly located at the edge of CF terminals (74%  $\pm$  4%, n = 1,195 puncta from 10 slices), which were immunopositive for vesicular glutamate transporter 2 (vGluT2). Similarly, postembedding immunogold electron microscopy (EM) analysis revealed high levels of C1ql1 immunoreactivity at presynaptic sites and CF synaptic clefts (Figure 1B). These results suggest that, like Cbln1 at PF synapses, the C1ql1 protein provided by CFs localizes at CF synapses.

To investigate the physiological functions of C1ql1, we generated mice in which the C1gl1 gene was disrupted (Figures S1A and S1B available online). IHC and immunogold EM analyses revealed essentially no C1ql1 immunoreactivity (Figures 1A, 1B, and S1C) in C1ql1-null cerebellum. The cerebella of C1ql1-null mice exhibited normal foliation and laminated cortical structures (Figure S1D). IHC analysis of adult cerebellar slices (Figure 1C) revealed that the number of vGluT2-positive puncta was significantly reduced in C1ql1-null cerebella (76  $\pm$ 2, n = 10 slices) compared to WT (243  $\pm$  6, n = 10 slices, p <0.001; Figure 1D). Although CF terminals translocated to dendrites and penetrated  $85\% \pm 1\%$  (n = 10 slices) of the molecular layer thickness in adult WT cerebella, the depth reached by most distal CF terminals was significantly smaller in C1gl1-null cerebella (68%  $\pm$  1%, n = 10 slices, p < 0.001; Figure 1E). The reductions in CF terminal number and height were not observed in C1ql1-null cerebella at P9 but became increasingly evident during development (Figures 1D and 1E). In contrast, although PF and inhibitory inputs to Purkinje cells are reported to affect CF synapse elimination processes (Kano and Hashimoto, 2009; Watanabe and Kano, 2011), no changes were observed in the number or distribution of vesicular glutamate transporter 1 (vGluT1, a marker for PF terminals; Figure S1E) or vesicular GABA transporter (vGAT, a marker for inhibitory fiber terminals; Figure S1F). These results indicate that CF

refinement processes at stages later than P9 were specifically impaired in *C1ql1*-null cerebella.

Next, we evaluated CF synapse function by assessing CFevoked excitatory postsynaptic currents (CF-EPSCs) in Purkinje cells with whole-cell patch-clamp recordings. The number of functional CF synapses on single Purkinje cells was estimated by varying the stimulus intensity because a single CF input has a single threshold for excitation. At P4-P6, both WT and C1ql1-null Purkinje cells were innervated by a similar number (from three to nine) of CFs (p = 0.15; Figures 1F and 1G). Among these CF inputs, a single CF-EPSC was already dominant in both WT (disparity index = SD/mean of CF-EPSC amplitudes; 0.67 ± 0.05, n = 24) and Clql1-null (0.65  $\pm$  0.05, n = 26) Purkinje cells (p = 0.70). In contrast, although single CF-EPSCs were elicited in 89% of WT Purkinje cells by P22-P28, only 57% of the Purkinje cells attained a one-to-one relationship with CFs in C1ql1-null mice (p < 0.01; Figure 1G). Furthermore, while the amplitude of the largest CF-EPSCs steadily increased during development in WT mice, it remained constant after P7-P9 in C1ql1-null mice (Figure 1H). Finally, voltage-dependent Ca2+ channel currents, which reportedly regulate the initial functional differentiation of multiple CF inputs during P3-P7 (Hashimoto et al., 2011), were similar between WT and Clql1-null cerebella (Figure S2). These results suggest that the winning CFs, which were selected during the initial functional differentiation stage by P7, were not further strengthened in the absence of C1ql1.

To examine causal relationships between C1ql1 expression and determination of the winner CFs, we expressed C1ql1 together with enhanced GFP in WT IO neurons at P0–P1 with lentivirus vectors (Figure 2A). GFP expression was detected in essentially all CF terminals surrounding the observed Purkinje cell soma by P5 (Figure 2B). At P7–P8, Purkinje cells were innervated by multiple CFs overexpressing C1ql1, with a dominant CF producing the largest EPSC in a manner similar to that of GFPonly-expressing Purkinje cells (Figure 2C). It is interesting that the amplitudes of the strongest CF-EPSC and also of the weaker CF-EPSCs were increased by C1ql1 overexpression in CFs (Figure 2D). Thus, C1ql1, at least when overexpressed in IO neurons, could enhance synaptic connectivity between CF and Purkinje cells, regardless of the original synaptic strength.

Unexpectedly, at P14–P15, 79% of Purkinje cells had already attained a one-to-one relationship when C1ql1 was overexpressed in CFs, compared to only 57% of control Purkinje cells innervated by single CFs (p < 0.05; Figures 2E and 2F). The amplitude of the dominant CF-EPSCs was significantly larger in Purkinje cells innervated by CFs overexpressing C1ql1 at all developmental stages (Figure 2G). Thus, although synaptic connections at less dominant CFs were enhanced by C1ql1 overexpression, their elimination was also facilitated. These results indicate that C1ql1 has dual functions: strengthening existing CF synapses and inducing the elimination of inactive synapses (see Discussion).

## Purkinje-Cell-Specific *Bai3*-Null Mice Phenocopy *C1ql1*-Null Mice

Recently, affinity chromatography identified C1ql proteins as ligands for Bai3, a member of the cell-adhesion GPCR family (Bolliger et al., 2011). Although Bai3 was reported to regulate

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#### Figure 1. C1ql1 Is Localized at CF Synapses during Their Formation and Maturation

(A) IHC images of endogenous C1ql1 in adult WT (left) and C1ql1-null (right) mouse cerebella. Areas surrounded by white squares are magnified in the lower panels. Scale bars in upper and lower panels, 20 µm and 3 µm, respectively.

(B) Postembedding immunogold EM images of endogenous C1ql1 in adult WT mouse cerebellum. C1ql1 and vGluT2 (a marker for CF terminals) were immunolabeled with 10 nm and 20 nm gold particles, respectively. sp, Purkinje cell spine. Red arrows indicate gold particles labeling C1ql1 around CF synapses. Scale bar, 200 nm. Histogram shows the vertical distribution of C1ql1-positive particles around CF synapses. Distances were measured from the midline (dotted line) of the synaptic cleft to the center of immunogold particles. On the x axis, negative and positive numbers represent presynaptic or postsynaptic side, respectively, from the midline.

(C) IHC images of vGluT2 in adult WT (left) and C1q/1-null (right) mouse cerebella. Dotted lines and asterisks represent pial surface and Purkinje cell soma, respectively. Scale bar, 20 µm.

(D) Developmental changes in the density of vGluT2 puncta.

(E) Developmental changes in the localization of vGluT2 puncta. The relative height is represented as a percentage, where 100% is at the top of Purkinje cell distal dendrites and 0% is at the Purkinje cell soma.

(F) CF-EPSC traces evoked by the paired-pulse stimulation (50 ms interstimulus interval) in various intensities in P5 (top) and adult (bottom) WT (left) and C1q/1null (right) mice.

(G and H) Developmental changes in the percentage of the number of CFs innervating single Purkinje cells (G) and the strongest CF-EPSC amplitudes (H).

\*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001 by two-way factorial ANOVA followed by Bonferroni post hoc test in (D), (E), and (H). \*p < 0.05, \*\*p < 0.01 by Mann-Whitney U test in (G). Data are represented as mean  $\pm$  SEM.

See also Figures S1 and S2.

synapse formation or elimination in cultured hippocampal neurons in vitro (Bolliger et al., 2011) and affect dendrite morphogenesis (Lanoue et al., 2013), its in vivo localization and synaptic functions have not been established. We first examined endogenous Bai3 in the cerebellum. IHC analysis revealed that, like C1ql1, Bai3 immunoreactivity was mostly located adjacent to vGluT2-positive CF terminals in WT mice ( $69\% \pm 5\%$ , n = 1,003 puncta from 10 slices; Figure 3A). Superresolution microscopic analysis also revealed that, although C1ql1 and Bai3 immunoreactivities were mostly colocalized, Bai3 was located in more peripheral regions along vGluT2 (Figure 3B). Similarly,

postembedding immunogold EM analysis revealed that Bai3 immunoreactivity was highly abundant at postsynaptic sites and in CF synaptic clefts (Figure 3C). These results support the view that, like GluD2 for Cbln1 at PF synapses, Bai3 may serve as a postsynaptic receptor for C1ql1 at CF synapses.

To examine whether C1ql1 serves as an endogenous ligand for Bai3, we generated mice in which the *Bai3* gene was specifically disrupted in Purkinje cells (PC-*Bai3*-null mice; Figures S3A and S3B). IHC and immunogold EM analyses detected essentially no Bai3 immunoreactivity in PC-*Bai3*-null cerebellum (Figures 3A, 3C, and S3C), indicating that, indeed, Bai3 was



#### Figure 2. C1ql1 Promotes CF Synapse Refinement during Development

(A) Cartoon showing the lentivirus injection into IOs in WT mouse pup (P0-P1) for the expression of C1ql1.

(B) IHC images of vGluT2 (red in dashed circles) expression along GFP-positive CFs surrounding Purkinje cell soma at P5. Purkinje cells were immunostained for calbindin (white). Scale bar, 5 μm.

(C) CF-EPSCs evoked by various stimulus intensities from P7 WT cerebellar slices, in which CFs expressed GFP only (CTL, top) or GFP plus C1ql1 (C1ql1 OE, bottom).

(D) Histograms showing total CF-EPSC amplitude (Total) and the mean amplitudes of CF-EPSCs evoked by the strongest, second strongest (2nd), or third strongest (3rd) CF activation.

(E) CF-EPSCs recorded from P14 (left) and P21 (right) cerebellar slices.

(F and G) Developmental changes in the percentage of the number of CFs innervating single Purkinje cells (F) and the strongest CF-EPSC amplitudes (normalized by the amplitude in CTL, G).

\*p < 0.05, \*\*p < 0.01 by Mann-Whitney U test. Data are represented as mean ± SEM.

postsynaptically located at CF synapses. Although PC-*Bai3*-null mice had normal cerebellar structure (Figure S3D) and immuno-reactivities for vGluT1 and vGAT in cerebellar slices (Figures S3E and S3F), the numbers of vGluT2-positive puncta were significantly reduced in adult PC-*Bai3*-null cerebella (47 ± 5, n = 10 slices) compared to control littermate (185 ± 8, n = 10 slices; p < 0.001; Figures 3D and 3E). In addition, the depth of the molecular layer thickness reached by the most distal CF terminals was significantly less in PC-*Bai3*-null (74% ± 1%, n = 10 slices) than in control (84% ± 1%, n = 10 slices) cerebella

(p < 0.001; Figure 3F). Whole-cell patch-clamp recordings from adult PC-*Bai3*-null cerebellar slices revealed that only 68% of Purkinje cells attained a one-to-one relationship with CFs (p < 0.001 versus control; Figures 3G and 3H). Furthermore, the amplitudes of the strongest CF-EPSCs were significantly smaller in PC-*Bai3*-null Purkinje cells (p < 0.01 versus control; Figure 3I). Together, these results indicate that PC-*Bai3*-null mice phenocopied *C1ql1*-null mice.

To further examine whether the function of Bai3 was mediated by C1ql1 binding, we next designed a Bai3 mutant that could not

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(legend on next page)

bind C1ql1. Surface plasmon resonance (SPR) assays revealed that the extracellular domain of Bai3 (eBai3<sup>WT</sup>) bound to C1ql1 with a dissociation constant ( $K_D$ ) of 2  $\mu$ M; this interaction was significantly reduced by deleting the N-terminal CUB (complement C1r/C1s, Uegf, Bmp1) domain (eBai3<sup> $\Delta$ CUB</sup>; K<sub>D</sub> > 100  $\mu$ M; Figures 3J, S4A, and S4B). Conversely, the short N-terminal region of Bai3 containing the CUB domain (nBai3<sup>CUB</sup>) was sufficient for C1ql1 binding ( $K_D = 0.3 \mu$ M; Figures 3J and S4C). Similarly, C1ql1 proteins strongly bound to heterologous cells expressing full-length Bai3 (Bai3<sup>WT</sup>) but not to cells expressing Bai1, Bai2, or Bai3 lacking the CUB domain (Bai3<sup>ΔCUB</sup>) (Figures S4F and S4G). We introduced cDNAs encoding GFP only, GFP plus Bai3<sup>WT</sup>, or GFP plus Bai3<sup> $\Delta$ CUB</sup> into PC-*Bai3*-null Purkinje cells by in utero electroporation (IUE) at embryonic day (E) 11.5 (Figure 3K). Whole-cell patch-clamp recordings revealed that, although expression of Bai3<sup>WT</sup> in PC-Bai3-null Purkinje cells restored a one-to-one relationship with CFs, expression of Bai3 $^{{\scriptscriptstyle \Delta}{\rm CUB}}$  or GFP alone was insufficient (p < 0.05; Figures 3L and 3M). Furthermore, the amplitudes of the strongest CF-EPSCs were significantly increased in PC-Bai3-null Purkinje cells expressing Bai3<sup>WT</sup> compared to cells expressing Bai3<sup>ΔCUB</sup> or GFP alone (p < 0.001; Figures 3L and 3N). Because Bai2, a closely related cell-adhesion GPCR that shares similar domain structures (Stephenson et al., 2014), is reportedly expressed on Purkinje cells along with Bai3 (Selimi et al., 2009), we generated mice in which the Bai2 gene was disrupted specifically in Purkinje cells (PC-Bai2-null mice; Figures S5A and S5B). In contrast to PC-Bai3-null mice, PC-Bai2-null cerebellum showed no changes in the amount or distribution of vGluT2 immunoreactivity (Figures S5C-S5E). Together, these results indicate that the function of Bai3 at CF synapses is mediated by the CUB domain, which C1ql1 directly binds.

# C1ql1–Bai3 Triggers Pruning of Surplus CFs in Adult Mice

Although synaptic pruning could occur throughout adulthood (Cesa and Strata, 2009; Holtmaat and Svoboda, 2009), it is un-

clear whether the molecules involved in developmental pruning exert their functions in the adult brain. To address this question, we next expressed C1ql1 (C1ql1<sup>WT</sup>) in adult C1ql1-null IO neurons with adenoassociated virus (AAV) vectors (Figure 4A). Whole-cell patch-clamp recordings showed that, although 38% of Purkinje cells remained innervated by multiple CFs in the adult C1ql1-null cerebellum, 84% of Purkinje cells attained a one-to-one relationship with CFs by expressing C1ql1<sup>WT</sup> (p <0.05; Figures 4B and 4C). Furthermore, the amplitudes of the strongest CF-EPSCs were significantly increased by C1ql1WT expression in adult C1ql1-null mice (p < 0.001; Figure 4D). Although it is difficult to rule out the possibility that CFs in C1ql1-null mice are in a state of permanent immaturity, these results indicate that C1gl1 can strengthen CF synapses and promote CF pruning to determine a single-winner CF, even at the adult stage in C1ql1-null mice.

To test whether the function of C1ql1 in adult synaptic pruning was mediated by Bai3 as observed during development, we next designed a mutant C1ql1 that did not bind Bai3. Because the C-terminal globular C1q domain of C1ql1 (gC1ql1; Figure 4E) was sufficient for binding to Bai3 (Figure S4D), we solved the crystal structure of gC1ql1 (Figure 4F; Table 1). Based on this structure, we designed a "glycan wedge" mutant by introducing two N-linked glycosylation sites in each subunit of the trimeric gC1ql1 domain (C1ql1<sup>GW</sup>; Figures 4E and 4F); the attachment of a bulky N-glycan moiety to Asn residues has been shown to prevent specific protein-protein interactions (Luo et al., 2003; Rondard et al., 2008). Indeed, SPR assays demonstrated that C1ql1<sup>GW</sup> did not retain any binding to Bai3 (Figures 4E and S4E). Similarly, C1ql1<sup>GW</sup> proteins did not bind to cells expressing full-length Bai3 (Figures S4F and S4G). C1ql1<sup>WT</sup> and C1ql1<sup>GW</sup> were similarly expressed in vGluT2-positive CF terminals (Figure S6A), indicating that binding of Bai3 was likely to be nonessential for localizing C1ql1 at CF terminals. Whole-cell patch-clamp recordings showed that, unlike C1ql1<sup>WT</sup>, expression of C1ql1<sup>GW</sup> in CFs did not restore the normal process of CF synapse elimination in C1ql1-null mice (Figures 4B and 4C).

#### Figure 3. C1ql1 Is an Endogenous Ligand for Bai3 to Win the CF Competition

(H and I) Histograms showing the percentage of the number of CFs innervating single Purkinje cells (H) and the strongest CF-EPSC amplitudes (I).

(K) Cartoon showing IUE to PC-Bai3-null embryo at E11.5. cDNA solution was loaded into the fourth cerebral ventricle (4V) to perform IUE.

(L) CF-EPSCs recorded from adult PC-Bai3-null Purkinje cells expressing Bai<sup>WT</sup> (center), Bai3<sup>ΔCUB</sup> (right), and empty vector (CTL, left).

(M and N) Histograms showing the percentage of the number of CFs innervating single Purkinje cells (M) and the strongest CF-EPSC amplitudes (N) in each condition.

\*\*p < 0.01, \*\*\*p < 0.001 by Mann-Whitney U test in (E), (F), (H), and (I). \*p < 0.05, \*\*\*p < 0.001 by Kruskal-Wallis test followed by Steel post hoc test in (M) and (N). ns, no significance. Data are represented as mean ± SEM.

See also Figures S3–S6.

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<sup>(</sup>A and B) Confocal (A) and superresolution structured illumination (SIM; B) microscopic images of endogenous Bai3 (red in A and green in B), C1ql1 (red in B), and vGluT2 (green in A and blue in B) immunoreactivities in the molecular layer of adult WT and C1ql1-null cerebella. Purkinje cells were immunostained for calbindin (blue in A). Scale bars, 20  $\mu$ m and 3  $\mu$ m in the upper and lower left panels, respectively, in (A) and 200 nm in (B).

<sup>(</sup>C) Postembedding immunogold EM images of endogenous Bai3 in adult WT mouse cerebellum. Bai3 and vGluT2 (a marker for CF terminals) were immunolabeled with 10 nm and 20 nm gold particles, respectively. sp, Purkinje cell spine. Red arrows indicate gold particles labeling Bai3 around CF synapses. Scale bar, 200 nm. Histogram shows the vertical distribution of Bai3-positive particles around CF synapses.

<sup>(</sup>D) IHC images of vGluT2 in adult PC-*Bai3*-null (right) and its control littermate (CTL, left) mouse cerebella. Dotted lines and asterisks represent pial surface and Purkinje cell soma, respectively. Scale bar, 20 µm.

<sup>(</sup>E and F) Quantification of the density (E) and the height (F) of vGluT2 puncta in the molecular layer of each mouse.

<sup>(</sup>G) CF-EPSCs evoked by various stimulus intensities in adult PC-Bai3-null (right) and CTL (left) mice.

<sup>(</sup>J) Diagrams of a full-length Bai3 (Bai3<sup>WT</sup>, top left) and a Bai3 lacking CUB domain (Bai3<sup>ΔCUB</sup>, top right) and the equilibrium SPR data (bottom) of the C1ql1 binding to the extracellular region of Bai3<sup>WT</sup> (eBai3<sup>WT</sup>), N-terminal region containing CUB domain (nBai<sup>CUB</sup>), and extracellular region of Bai3<sup>ΔCUB</sup> (eBai3<sup>ΔCUB</sup>). TSR, thrombospondin repeats; HB, hormone-binding domain; GPS, GPCR proteolysis sequence.



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#### Figure 4. C1ql1-Bai3 Signaling Triggers Surplus CF Synapse Elimination in Adult Mutant Mice

(A) Cartoon showing the AAV injection into adult *C1ql1*-null mouse IO for the expression of C1ql1. Experiments were performed at 14–21 days postinfection (dpi).

(B) CF-EPSCs recorded from adult *C1ql1*-null Purkinje cells innervated by CFs expressing WT C1ql1 (C1ql1<sup>WT</sup>, center), C1ql1<sup>GW</sup> (right), and empty vector (CTL, left).

(C and D) Histograms showing the percentage of the number of CFs innervating single Purkinje cells (C) and the strongest CF-EPSC amplitudes (D) in each condition.

(E) Diagrams (left) for C1ql1<sup>WT</sup>, C1ql1<sup>GW</sup>, and their globular domains (gC1ql1<sup>WT</sup> and gC1ql1<sup>GW</sup>, respectively), and the equilibrium SPR data of each C1ql1 binding to eBai3 (right). RU, resonance unit.

(F) Cartoon representation of the gC1ql1<sup>WT</sup> (top) and gC1ql1<sup>GW</sup> (bottom) trimeric structures. Individual monomers are separately colored. Glycosylation sites introduced by mutagenesis (original residues were Q211 and S244) are indicated by spheres in C1ql1<sup>WT</sup>. The gC1ql1<sup>GW</sup> trimeric structure is shown with modeled Man9GlcNAc2 glycans (atoms in sphere representation).

(G) Cartoon showing the lentivirus injection into adult PC-*Bai*3-null mouse cerebellum for the expression of Bai3. Experiments were performed at 14–21 dpi.

(H) CF-EPSCs recorded from adult PC-*Bai3*-null Purkinje cells expressing WT Bai3 (Bai $^{WT}$ , center), Bai $^{\Delta CUB}$  (right), and empty vector (CTL, left).

(I and J) Histograms showing the percentage of the number of CFs innervating single Purkinje cells (I) and the strongest CF-EPSC amplitudes (J) in each condition.

\*p < 0.05, \*\*\*p < 0.001 by Kruskal-Wallis test followed by Steel post hoc test. ns, no significance. Data are represented as mean  $\pm$  SEM.

See also Figures S4 and S6 and Table 1.

Furthermore, the amplitudes of the strongest CF-EPSCs were not increased by C1ql1<sup>GW</sup> expression in adult *C1ql1*-null mice (Figures 4B and 4D). These results indicate that the function of C1ql1 in determining a single-winner CF in the adult brain is mediated by an interface used for Bai3 binding.

Finally, to examine whether expression of Bai3 in adult PC-Bai3-null Purkinje cells could also restore CF phenotypes, we used lentiviral vectors to express Bai3<sup>WT</sup> or Bai3<sup>ΔCUB</sup> in Purkinje cells (Figure 4G). Bai $3^{WT}$  and Bai $3^{\Delta CUB}$  were similarly expressed in dendritic spines of Purkinje cells (Figure S6B), indicating that binding of C1ql1 was likely to be nonessential for localizing Bai3 at postsynaptic sites. Whole-cell patch-clamp recordings showed that Bai3<sup>WT</sup>, but not Bai3<sup>ΔCUB</sup> or empty vector, restored the normal process of CF synapse elimination in PC-Bai3-null mice (p < 0.05; Figures 4H and 4I). Similarly, the amplitudes of the strongest CF-EPSCs were increased by Bai3<sup>WT</sup> expression but not Bai3<sup>ΔCUB</sup> or empty vector in adult PC-Bai3-null mice (p < 0.001; Figures 4H and 4J). These results indicate that C1ql1 provided by CFs and Bai3 in Purkinje cells likely act together to trigger signaling pathways necessary for determining single-winner CFs, even in the adult cerebellum.

## The C1ql1-Bai3 Interaction Is Required to Maintain CF Synapses

C1ql1 and Bai3 continue to be expressed in the adult brain (lijima et al., 2010; Lanoue et al., 2013). To examine the functions of C1ql1-Bai3 signaling in adults, we knocked down C1ql1 in the IO neurons of adult WT mice by infecting them with an AAV that contained an engineered microRNA targeting C1ql1 plus the *GFP* gene (miR-*C1ql1*) (Figure 5A). Expression of miR-

# Table 1. Crystallographic Data Collection and Structure Refinement Statistics

Statistics	gC1ql1
Data Collection	
Space group	H3
Cell dimensions: a, b, c (Å)	68.08, 68.08, 65.91
Cell angles: α, β, γ (°)	90.00, 90.00, 120.00
Resolution (Å)	43.94–1.44 (1.48–1.44)
Total reflections	155,787 (11,142)
Unique reflections	20,630 (1,532)
Completeness (%)	100.0 (99.9)
R <sub>merge</sub>	0.067 (0.875)
R <sub>pim</sub>	0.028 (0.378)
l/σl	16.9 (2.1)
Redundancy	7.6 (7.3)
Wilson B factor (Å <sup>2</sup> )	11.55
Refinement Statistics	
Resolution range (Å)	34.04–1.44 (1.51–1.44)
Number of reflections	20,628 (2,956)
R <sub>work</sub>	0.140 (0.205)
R <sub>free</sub> <sup>a</sup>	0.169 (0.255)
Number of atoms	1,121
Protein	1,029
Ligands (Cd, Ni, Mg, Cl)	7 (2, 2, 1, 2)
Water molecules	85
RMSD bonds (Å)	0.006
RMSD angles (°)	1.096
Average B factors (Å <sup>2</sup> )	
Protein main/side chains	14.43/18.92
Cd/Ni/Mg/Cl	13.16/10.00/28.39/45.39
Water	31.01

This structure was determined from one crystal. Numbers in parentheses refer to the highest resolution shell. Ramachandran statistics and Molprobity scores are included in the Supplemental Experimental Procedures. RMSD, root-mean-square deviation.

 $^{\rm a}{\rm R}_{\rm free}$  was calculated as per  ${\rm R}_{\rm work}$  for a 5% subset of reflections that was not used in the crystallographic refinement.

C1ql1 significantly reduced C1ql1 immunoreactivities in GFPexpressing IO neurons (17% ± 6% of IO neurons expressing scrambled microRNA [miR-SCR], p < 0.05; Figure 5B). IHC analysis of cerebellar slices revealed that the number of vGluT2-positive puncta was significantly reduced in mice expressing miR-C1ql1 (110  $\pm$  9, n = 10 slices; Figures 5C and 5D) compared to mice expressing miR-SCR (180  $\pm$  6, n = 10 slices; p < 0.001; Figures 5C and 5D). In addition, the depth of the molecular layer thickness reached by the most distal CF terminals was reduced in mice expressing miR-C1ql1 ( $80\% \pm 1\%$ , n = 10 slices; Figures 5C and 5E) compared to mice expressing miR-SCR ( $84\% \pm 1\%$ , n = 10 slices, p < 0.05; Figures 5C and 5E). In contrast, whole-cell patch-clamp recordings from adult cerebellar slices revealed that similar percentages of Purkinje cells were innervated by single CFs in mice expressing miR-C1gl1 (83%; Figures 5F and 5G) and miR-SCR (89%, p = 0.51; Figures 5F and 5G). Nevertheless,

the amplitudes of the strongest CF-EPSCs were significantly smaller in mice expressing miR-C1g/1 (p < 0.001 versus miR-SCR; Figures 5F and 5H). The reduced amplitudes of CF-EPSCs due to miR-C1ql1 expression were restored by coexpression of the miR-*C1ql1*-resistant form of C1ql1<sup>WT</sup> (rC1ql1<sup>WT</sup>; p < 0.001 versus miR-C1ql1) but not the resistant form of C1ql1<sup>GW</sup> (rC1ql1<sup>GW</sup>; p = 0.64 versus miR-C1ql1; Figures 5F and 5H). We also knocked down Bai3 by infecting adult WT Purkinje cells with a lentivirus containing an engineered microRNA targeting Bai3 plus the GFP gene (miR-Bai3; Figure S7). We obtained essentially the same results as those with miR-C1ql1. It is important to note that the reduced CF-EPSC amplitudes observed with miR-Bai3 were restored by coexpression of the miR-Bai3resistant form of Bai3<sup>WT</sup> but not Bai3<sup>ΔCUB</sup> (Figure S7). Together, these results indicate that, even after a single final winner CF was selected, C1ql1-Bai3 signaling was necessary to maintain the winner CF synapses on Purkinje cell dendrites.

#### C1ql1-Bai3 Signaling in Motor Learning

To understand the consequences of abnormal CF synapses in vivo, we examined the behaviors of *C1ql1*-null and PC-*Bai3*null mice. Unexpectedly, these mice showed no ataxia and could walk along a straight line with regular steps (Movie S1). They showed normal motor coordination on the rotor-rod test (Figures S8A and S8B). Nystagmus, which is characteristic of *GluD2*-null humans (Hills et al., 2013) and mice (Yoshida et al., 2004), was absent in the *C1ql1*-null and PC-*Bai3*-null mice (Figures S8C– S8E). These results indicate that innervation of Purkinje cells by multiple CFs does not necessarily manifest as gross motor discoordination or nystagmus.

To further clarify the role of C1ql1-Bai3 signaling in the cerebellum, we next examined adaptation of the horizontal optokinetic response (hOKR), a motor learning paradigm shown to be dependent on the cerebellum (Ito, 2006). Continuous oscillation of a screen around a stationary animal increases the hOKR gain in WT mice (Figures 6A and 6B). Although the hOKR was similarly observed in all genotypes before the training (Figure 6B; Movie S2), the gain did not increase in *C1ql1*-null and PC-*Bai3*-null mice (Figures 6C and 6D; Movie S2), indicating that motor learning was severely impaired in these mice.

According to the Marr-Albus-Ito theory, the activities of CFs encode retinal slip errors that serve as a teacher signal to induce long-term depression (LTD) of synaptic transmission at concomitantly activated PF synapses, leading to hOKR adaptation (Ito, 2013). To test this hypothesis, we examined whether LTD could be induced in cerebellar slices prepared from C1gl1-null and PC-Bai3-null mice by simultaneously activating PFs and CFs for 2 min at 1 Hz in the current-clamp mode (Figure 6E). This protocol robustly induced LTD of PF-evoked EPSCs (PF-EPSCs) in WT, but not C1ql1-null (p < 0.001; Figures 6F and 6G) or PC-Bai3-null (p < 0.001 versus control; Figures 6F and 6H), Purkinje cells. Activation of CF input triggers dendritic spikes and a burst of somatic spikes known as complex spikes in WT Purkinje cells (Eccles et al., 1966). The occurrence of the complex spike was significantly reduced in Purkinje cells from C1ql1-null (53 ± 9 in 120 trials, n = 22 versus WT [118 ± 2 in 120 trials, n = 22]; p < 0.001 by Mann-Whitney U test) and PC-Bai3-null mice (64 ± 8 in 120 trials, n = 24 versus

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#### Figure 5. C1ql1-Bai3 Interaction Is Essential for the Maintenance of CF Synapse in Adults (A) Cartoon showing the AAV injection into adult WT mouse IO for the introduction of miR-C1gl1 or miR-

SCR. Experiments were performed at 14–21 dpi. (B) IHC images (left) and the quantitative data (right) showing C1ql1 is knocked down by miR-C1ql1 (right) but not miR-SCR (left) in GFP-expressing IO neurons. Scale bar, 100 μm.

(C) IHC images of vGluT2 in adult WT mouse cerebella with CFs expressing GFP plus miR-SCR (left) or miR-C1q/1 (right). vGluT2 immunoreactivities are binarized in the bottom. Dotted lines and asterisks represent pial surface of the molecular layer and Purkinje cell soma, respectively. Scale bar, 20 μm.

(D and E) Quantification of the density (D) and the height (E) of vGluT2 puncta on the molecular layer of each condition.

(F) CF-EPSCs evoked by various stimulus intensities in adult WT cerebellar slices with CFs introducing miR-SCR, miR-C1q/1, miR-C1q/1 plus a resistant form of C1q/1 ( $rC1q/1^{WT}$ ) or miR-C1q/1 plus a  $rC1q/1^{GW}$ .

(G and H) Histograms showing the percentage of the number of CFs innervating single Purkinje cells (G) and the strongest CF-EPSC amplitudes (H) in each condition.

 $^*p < 0.05, ^{***}p < 0.001$  by Mann-Whitney U test in (B), (D), and (E).  $^*p < 0.05, ^{***}p < 0.001$  by Kruskal-Wallis test followed by Scheffé post hoc test in (H). ns, no significance. Data are represented as mean  $\pm$  SEM. See also Figure S7.





#### Figure 6. Abnormal CF Synapses Impair Synaptic Plasticity and Motor Learning in Adult Mutant Mice

(A) Cartoon showing hOKR system. The mouse was mounted on a table surrounded by a checked-pattern screen with its head fixed. Eye movements during sinusoidal oscillation of the screen (15°) were monitored.

(B–D) Representative hOKR waveforms before (pre) and 60 min after (post) training (B) and the data of hOKR acquisition for 60 min in WT versus C1q/1-null (C) mice and PC-Bai3-null versus its control littermate Bai3<sup>flox</sup> (CTL, D).

(E) An orientation of stimulus and recording electrodes (left) and the stimulus protocol (CJ-stim, right) to induce LTD at PF synapses.

(F–H) PF-EPSCs just before (black) and 30 min after (red) CJ-stim (F) and the data of LTD recordings in WT versus C1ql1-null mice (G) and PC-Bai3-null versus CTL mice (H).

(I–K) Complex spike waveforms recorded from each mouse (I) and the quantification of the amplitude of afterhyperpolarization (AHP, J) and the pause length of spontaneous spikes (K) after the complex spike.

\*\*\*p < 0.001 by two-way repeated-measures ANOVA in (C) and (D). \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001 by Mann-Whitney U test in (G), (H), (J), and (K). Data are represented as mean ± SEM.

See also Figure S8 and Movies S1 and S2.

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#### Figure 7. A Model for C1ql1-Bai3 Signaling in Synaptic Competition at CF Synapses

(A and E) The disparity is formed among multiple CFs innervating a single Purkinje cell soma by P7 in both WT (A) and C1q/1-null or PC-Bai3-null (E) cerebellum. (B) Although the C1ql1-Bai3 signaling promotes maturation of all CFs, the winning CF may trigger a signaling pathway to eliminate less dominant synapses. The winning CF may occupy the postsynaptic region close to proximal dendrites and prevent translocation of somatic synapses formed by less dominant CFs to proximal dendrites.

(C) CFs remaining on the Purkinje cell soma are eventually eliminated.

(D) Knockdown of C1ql1 or Bai3 revealed that the C1ql1-Bai3 signaling is required to maintain the winner CF in adult.

(F) In the absence of C1ql1-Bai3 signaling, the dominant CF cannot be strengthened enough to prevent the translocation of synapses formed by less dominant CFs.

(G) Weak CF synapses translocated to proximal dendrites escape from the elimination process at soma.

(H) Expression of C1ql1 or Bai3 in adult C1ql1-null or PC-Bai3-null Purkinje cells determines the single-winner CF by promoting the maturation of the dominant CF and eliminating less dominant CFs.

control [120 in 120 trials, n = 30]; p < 0.001 by Mann-Whitney U test). Furthermore, dendritic spikes reportedly convey additional signals by triggering a pause in firing following the complex spike in WT Purkinje cells (Davie et al., 2008). Such CF-evoked afterhyperpolarization (Figures 6I and 6J) and suppression of the spontaneous spikes (Figures 6I and 6K) were significantly reduced in Purkinje cells from *C1ql1*-null (both ps < 0.05 versus WT) and PC-*Bai3*-null mice (p < 0.001 and p < 0.01 versus control, respectively). These findings indicate that C1ql1-Bai3 signaling is required for normal CF functions, such as complex spikes and CF-evoked afterhyperpolarization, which are necessary for LTD and motor learning in the adult cerebellum.

## DISCUSSION

In the present study, we demonstrated that C1ql1 from CFs plays crucial roles in determining a single-winner CF during

development by binding to its postsynaptic receptor Bai3 in Purkinje cells. CF synapse competition during development is divided into at least three stages: selection of functionally dominant CFs by P7 (Figure 7A), further strengthening and translocation of the dominant CF to Purkinje cell dendrites (Figure 7B), and elimination of weak CFs remaining on the soma while maintaining a single-winner CF (Figure 7C). We propose that C1gl1-Bai3 signaling is required for the second and third stages; in the absence of C1ql1-Bai3 signaling, the dominant CF cannot be strengthened enough to prevent the translocation of synapses formed by less dominant CFs (Figure 7F), which escape from the later elimination process (Figure 7G). These phenotypes could be rescued by expression of C1ql1 and Bai3 in adult C1ql1-null and PC-Bai3-null mice, respectively (Figure 7H). Conversely, maintenance of the winner CF requires C1ql1-Bai3 signaling throughout adulthood (Figure 7D). Furthermore, C1ql1 and Bai3 were essential for CFs to mediate cerebellar synaptic plasticity necessary for motor learning. Therefore,

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C1ql1-Bai3 signaling represents a new mechanism that regulates the selection and maintenance of functional winner inputs by an anterograde signal supplied by the input neuron itself.

# Dual C1ql1-Bai3 Signaling in CF Synapse Formation and Elimination

The P/Q-type Ca<sup>2+</sup> channel is the major Ca<sup>2+</sup> channel in Purkinje cells and reportedly regulates developmental CF pruning (Kano and Hashimoto, 2009; Watanabe and Kano, 2011). Ca<sup>2+</sup> channel activities are thought to mediate the selective enhancement of a single strong CF input by inducing long-term potentiation (LTP) (Bosman et al., 2008) and activating Sema3A signaling in Purkinje cells (Uesaka et al., 2014). Ca2+ channel activities also reportedly induce synapse elimination by activating the Arc/Arg3.1 pathway in Purkinje cells (Mikuni et al., 2013). GABAergic inputs also likely regulate CF elimination by controlling Ca<sup>2+</sup> channel activities in Purkinje cells (Nakayama et al., 2012; Watanabe and Kano, 2011). Indeed, mice lacking functional P/Q-type Ca<sup>2+</sup> channels do not exhibit disparity among multiple CF inputs by P7, and Purkinje cells in adult mice remain innervated by multiple CFs (Hashimoto et al., 2011; Miyazaki et al., 2004). In contrast, disparity is normally formed among CF inputs in C1ql1-null mice by P7, which is consistent with the normal  $Ca^{2+}$  channel activities in these mice (Figure S2). The CF-EPSC amplitudes in Purkinje cells lacking P/Q-type Ca<sup>2+</sup> channels (Hashimoto et al., 2011) or GABAergic inputs (Nakayama et al., 2012) increased at later developmental stages to a level similar to that in WT mice. By contrast, the amplitude of the largest CF-EPSCs remained constant after P7-P9 in C1ql1null mice (Figure 1H). These findings indicate that C1ql1 does not simply mediate signaling downstream of Ca<sup>2+</sup> channels.

Another major pathway that reportedly mediates CF elimination in the later developmental stage (approximately the P12 stage in mice) is mGluR1 and its downstream signaling in Purkinje cells, which involves molecules such as Gag, phospholipase C $\beta$ 4, protein kinase C $\gamma$  (PKC $\gamma$ ) (Kano and Hashimoto, 2009; Uesaka et al., 2014; Watanabe and Kano, 2011), and Sema7A (Uesaka et al., 2014). In mice lacking one of these mGluR1 signaling pathway components, Purkinje cells remain innervated by multiple CFs, even in adulthood. Because mGluR1 is activated mainly by PF inputs, innervation of Purkinje cells by multiple CFs in GluD2-null or Cbln1-null mice is thought to be caused by impaired PF synapse formation (Watanabe and Kano, 2011). However, unlike in C1ql1-null or PC-Bai3-null mice, the numbers and heights of the CF terminals are unaffected in mGluR1 knockout mice (Hashimoto et al., 2001). Similarly, CF-EPSC amplitudes were also unaffected in Sema7A knocked-down Purkinje cells (Uesaka et al., 2014), indicating that signaling pathways downstream of mGluR1 are distinct from C1ql1-Bai3.

If this is the case, then what is the function of C1ql1-Bai3 signaling? C1ql1 enhanced the amplitudes of the strongest CF-EPSC and the weaker CF-EPSCs when it was overexpressed in all CFs at P0–P1 (Figure 2D). In addition, when C1ql1 or Bai3 was knocked down in adult WT mice, the CF-EPSC amplitudes and the number of CF synapses decreased (Figures 5 and S7). Similarly, the number of CF terminals was reduced in *C1ql1*-null and PC-*Bai3*-null cerebella (Figures 1

and 3). These results indicate that the primary function of C1ql1-Bai3 signaling is to promote CF synapse maturation regardless of their synaptic strength. Synapse maturation could be caused by enhanced formation or/and reduced removal of synapses. Because the height of the CF synapses were significantly reduced in the absence of C1ql1-Bai3 signaling (Figures 1, 3, 5, and S7), CFs are likely more dependent on C1ql1-Bai3 signaling to form and maintain synapses at distal regions of Purkinje cell dendritic shafts.

C1ql1-Bai3 signaling exerts an apparently opposite function on loser CFs: while overexpression of C1ql1 at P0-P1 strengthened winner CFs, it promoted early elimination of loser CFs at P14-P15 (Figure 2). Similarly, reintroduction of C1ql1 or Bai3 in mice deficient in the corresponding molecule induced the elimination of weak CF inputs in the adult cerebellum (Figure 4). C1ql1-Bai3 signaling may simply promote maturation of the dominant CF to the level where it could trigger signaling pathways, such as Ca2+ spikes and Arc/Arg3.1, to eliminate weaker CF inputs, whereas the winner CFs with strong C1ql1-Bai3 signaling may be resistant to the elimination process. The autoproteolysis domain of Bai3 may also serve as a bistable switch to distinguish the winner from losing synapses (Arac et al., 2012). Alternatively, C1ql1-Bai3 signaling may trigger a pathway, which is independent of maturation of a winner CF, to eliminate weak CF inputs. For example, Bai1, a relative of Bai3, reportedly interacts with Elmo1/Dock180 (Park et al., 2007) or Par3/Tiam1 (Duman et al., 2013) to control the activity of the small GTPase Rac, a powerful regulator of actin cytoskeletal organization. Indeed, Bai3 was shown to interact with Elmo1 in vitro (Lanoue et al., 2013). Further studies are clearly warranted to clarify how C1ql1-Bai3 signaling is regulated and functions at CF synapses.

The Purkinje cell dendrites consist of two domains, a proximal one innervated by single CFs and a distal one on which many PF synapses are formed. These two domains are dynamically established and maintained by competition between PFs and CFs (Cesa and Strata, 2009; Watanabe and Kano, 2011). For example, when PF synapses are reduced in Cbln1-null (Hirai et al., 2005) or GluD2-null (Hashimoto et al., 2001) mice, CFs form synapses on distal dendrites of Purkinje cells. Conversely, when CF synaptic activity was pharmacologically inhibited or IO neurons were lesioned, CF synapses were lost and PFs formed synapses on proximal Purkinje cell dendrites (Cesa and Strata, 2009; Watanabe and Kano, 2011). Similarly, PFs invaded CF territories in P/Q-type Ca2+ channel-deficient Purkinje cells (Miyazaki et al., 2004). Notably, although CF synaptic inputs were reduced, PF synapses did not form on proximal dendrites of C1ql1-null or PC-Bai3-null Purkinje cells (Figures S1E and S3E). These findings indicate that, although changes in C1ql1-Bai3 signaling may be involved in the dynamic regulation of CF territories, its deficiency was not sufficient to allow PFs to form synapses on the proximal domain of Purkinje cell dendrites. PF synapse formation requires CbIn1 released from PFs to bind to GluD2 (Matsuda et al., 2010). Because GluD2 clustering is destabilized by Ca2+ channel activities (Hirai, 2001), reduction of Ca<sup>2+</sup> channel activities may be a prerequisite for PFs to form synapses on the proximal dendritic domain of Purkinje cells.

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#### **C1q Family Proteins and Synaptic Functions**

Based on the behavioral phenotypes of ataxic mutant mice lacking mGluR1, PKCy, and GluD2, monoinnervation of Purkinje cells by CFs was thought to be required for motor coordination (Chen and Tonegawa, 1997). In contrast, although C1ql1-null and PC-Bai3-null Purkinje cells remain innervated by multiple CF inputs, these mice have normal gaits without significant impairment in beam or rotor-rod tests (Figure S8; Movie S1). However, motor learning, as measured by hOKR, was severely impaired in these mice (Figure 6). Recently, mGluR1<sub>β</sub>-rescue mice, in which the splice variant of mGluR1 is expressed in mGluR1-null Purkinje cells, were shown to display normal motor coordination, even though their Purkinje cells were innervated by multiple CFs. These mice showed impaired LTD and eyeblink conditioning, another form of cerebellum-dependent motor learning (Ohtani et al., 2014). Similarly, a single injection of recombinant Cbln1 restored LTD and eyeblink conditioning in adult CbIn1-null mice without significantly affecting the CF innervation pattern or LTP at PF-Purkinje cell synapses (Emi et al., 2013). Furthermore, GluD2<sup>△CT7</sup>-rescue mice, in which GluD2 lacking seven C-terminal amino acids was expressed in GluD2-null Purkinje cells, displayed impaired LTD and eyeblink conditioning despite morphologically normal PF and CF synapses (Kakegawa et al., 2008). These findings indicate that LTD, rather than monoinnervation of Purkinje cells by CFs, is likely linked to motor learning in the cerebellum and indicate that C1ql1-Bai3 signaling is required for normal CF functions, such as complex spikes and CF-evoked afterhyperpolarization, which are necessary for LTD.

Family members of C1ql1 (C1ql2-C1ql4) are expressed in various brain regions (lijima et al., 2010). Bai3 and its related members Bai1 and Bai2 are also widely expressed (Kee et al., 2004) and have been implicated in psychiatric disorders, such as schizophrenia and bipolar disorders (Lanoue et al., 2013). Thus, signaling mechanisms similar to the one mediated by C1ql1 and Bai3 in the cerebellum likely regulate synaptic competition and maintenance in various neuronal circuits essential for brain functions. In the hippocampus, C1ql2 and C1ql3 are highly expressed in adult dentate gyrus granule cells, and their mossy fiber afferents make dynamic branched axon-type synapses like CFs. Notably, mossy fiber synapses reportedly display learning-induced structural plasticity related to the precision of learning in adult mice (Ruediger et al., 2011). Therefore, future studies should assess whether and how C1ql proteins mediate activity-dependent structural plasticity essential for memory and learning.

#### **EXPERIMENTAL PROCEDURES**

All procedures related to animal care and treatment were performed in accordance with the guidelines set down by the Animal Resource Committee of Keio University. *C1ql1*-null, PC-*Bai2*-null, and PC-*Bai3*-null mice were generated and maintained on C57BL/6N backgrounds. As controls, WT mice were used for *C1ql1*-null mice and *Bai2*<sup>flox</sup>, and *Bai3*<sup>flox</sup> mice were used for PC-*Bai2*-null and PC-*Bai3*-null mice. For the behavioral analyses, littermates were used as controls. Experimental details of mouse generation as well as electrophysiological, anatomical, biochemical, structural, and behavioral analyses are described in the Supplemental Experimental Procedures. Data are

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represented as mean  $\pm$  SEM. Statistical analyses were performed using the Excel statistics 2012 add-in software (Social Survey Research Information), and significant differences were defined as \*p < 0.05, \*\*p < 0.01, and \*\*\*p < 0.001.

#### **ACCESSION NUMBERS**

The coordinates and structure factors for the reported crystal structure are deposited in the Protein Data Bank (PDB) under PDB ID 4d7y.

#### SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, eight figures, and two movies and can be found with this article online at http://dx.doi.org/10.1016/j.neuron.2014.12.020.

#### **AUTHOR CONTRIBUTIONS**

W.K. designed the project, carried out the electrophysiological and behavioral works, analyzed the data, and wrote the paper. N.M. and A.R.A. performed structural and SPR analyses and designed mutant constructs. E.M. performed the anatomical experiments and the IUE approach. M.A. and K.S. designed and generated mutant mice. K.M. performed the cell binding assays. Y.H.T. designed and prepared the microRNA constructs. K.K., A.T., J.M., and S.M. designed and prepared the recombinant viruses. S.N. supervised the hOKR experiment. M.W. generated essential antibodies. M.Y. designed and supervised the project and wrote the paper.

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# ARTICLE

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# A CDC42EP4/septin-based perisynaptic glial scaffold facilitates glutamate clearance

Natsumi Ageta-Ishihara<sup>1</sup>, Maya Yamazaki<sup>2</sup>, Kohtarou Konno<sup>3</sup>, Hisako Nakayama<sup>4</sup>, Manabu Abe<sup>2</sup>, Kenji Hashimoto<sup>5</sup>, Tomoki Nishioka<sup>6</sup>, Kozo Kaibuchi<sup>6</sup>, Satoko Hattori<sup>7</sup>, Tsuyoshi Miyakawa<sup>7,8</sup>, Kohichi Tanaka<sup>9</sup>, Fathul Huda<sup>10</sup>, Hirokazu Hirai<sup>10</sup>, Kouichi Hashimoto<sup>4</sup>, Masahiko Watanabe<sup>3</sup>, Kenji Sakimura<sup>2</sup> & Makoto Kinoshita<sup>1</sup>

The small GTPase-effector proteins CDC42EP1-5/BORG1-5 interact reciprocally with CDC42 or the septin cytoskeleton. Here we show that, in the cerebellum, CDC42EP4 is exclusively expressed in Bergmann glia and localizes beneath specific membrane domains enwrapping dendritic spines of Purkinje cells. CDC42EP4 forms complexes with septin hetero-oligomers, which interact with a subset of glutamate transporter GLAST/EAAT1. In *Cdc42ep4*<sup>-/-</sup> mice, GLAST is dissociated from septins and is delocalized away from the parallel fibre-Purkinje cell synapses. The excitatory postsynaptic current exhibits a protracted decay time constant, reduced sensitivity to a competitive inhibitor of the AMPA-type glutamate receptors ( $\gamma$ DGG) and excessive baseline inward current in response to a subthreshold dose of a nonselective inhibitor of the glutamate transporters/EAAT1-5 (DL-TBOA). Insufficient glutamate-buffering/clearance capacity in these mice manifests as motor coordination/learning defects, which are aggravated with subthreshold DL-TBOA. We propose that the CDC42EP4/septin-based glial scaffold facilitates perisynaptic localization of GLAST and optimizes the efficiency of glutamate-buffering and clearance.

<sup>&</sup>lt;sup>1</sup> Division of Biological Sciences, Department of Molecular Biology, Nagoya University Graduate School of Science, Nagoya 464-8602, Japan. <sup>2</sup> Department of Cellular Neurobiology, Brain Research Institute, Niigata University, Niigata 951-8585, Japan. <sup>3</sup> Department of Anatomy, Hokkaido University Graduate School of Medicine, Sapporo 060-8638, Japan. <sup>4</sup> Department of Neurophysiology, Graduate School of Biomedical and Health Sciences, Hiroshima University, Hiroshima 734-8551, Japan. <sup>5</sup> Division of Clinical Neuroscience, Chiba University Center for Forensic Mental Health, Chiba 260-8670, Japan. <sup>6</sup> Department of Cell Pharmacology, Nagoya University Graduate School of Medicine, Showa, Nagoya 466-8560, Japan. <sup>7</sup> Division of Systems Medical Science, Institute for Comprehensive Medical Science, Fujita Health University, Toyoake 470-1192, Japan. <sup>8</sup> Center for Genetic Analysis of Behavior, National Institute for Physiological Sciences, Okazaki 444-8585, Japan. <sup>9</sup> Laboratory of Molecular Neuroscience, Medical Research Institute, Tokyo Medical and Dental University, Tokyo 113-8510, Japan. <sup>10</sup> Department of Neurophysiology and Neural Repair, Gunma University Graduate School of Medicine, Maebashi, Gunma 371-8511, Japan. Correspondence and requests for materials should be addressed to M.K. (email: kinoshita.makoto@c.mbox.nagoya-u.ac.jp).

lutamate transporters (Excitatory Amino Acid Transporter 1-5/EAAT1-5) are membrane-bound solute carrier proteins that terminate glutamatergic neurotransmission and maintain the glutamate homeostasis by the symport of extracellular glutamate and Na<sup>+</sup>/H<sup>+</sup> into the glial and neuronal cytoplasm<sup>1</sup>. The extracellular glutamate concentration surges to  $160-250\,\mu\text{M}$  near perisynaptic domains of Bergmann glia in the cerebellum, which is rapidly buffered and cleared by binding to and reuptake through EAATs<sup>2,3</sup>. When the glutamate-buffering/clearance capacity falls short of glutamate-release activities, glutamate pervades the extracellular space. Excessive external glutamate entails protracted activation of glutamate receptors in the nearest synaptic and extrasynaptic membranes, and in the neighbouring synapses, and of glial responses. In the cerebellar molecular layer, GLAST/EAAT1/SLC1A3 is highly concentrated along perisynaptic Bergmann glial membranes<sup>4</sup> and plays a major role in the clearance of glutamate released from the parallel fibres (PFs, from granule cells in the cerebellar granule cell layer) and climbing fibres (CFs, from neurons in the inferior olivary nuclei) to Purkinje cells (PCs), while GLT-1/EAAT2, the major glial transporter in the forebrain, and neuronal EAAT4 play relatively minor roles<sup>5</sup>. Genetic loss of GLAST results in anomalous CF-PC innervations and defective motor coordination/learning<sup>6</sup>, attesting its central role in Bergmann glia-mediated glutamate homeostasis in the cerebellum.

Despite the developmental and physiological significance of GLAST, and the linkage to human episodic ataxia and schizophrenia<sup>7–9</sup>, little is known about the post-translational regulation. Physical interactions with beta-III spectrin/GTRAP41 and ARHGEF11/GTRAP48 were reported to facilitate the anchorage and activity of EAAT4 (ref. 10). However, physiological significance of these findings from heterologous cells, and whether these proteins modulate the localization and/or activity of GLAST, remains unknown. Previous studies demonstrated a physical interaction between GLAST and septins (a family of polymerizing GTPases that constitute the membrane skeleton) *in vitro* and in heterologous cells, and their partial co-localization in Bergmann glia<sup>11,12</sup>. However, hypothetical septin dependence of the perisynaptic targeting and activity of GLAST has never been directly tested *in vivo*, partly because of the redundancy among the septin family (see Discussion).

Another line of biochemical and cell biological studies showed that a family of CRIB-domain proteins CDC42EPs/BORGs binds to septin hetero-oligomers or CDC42 (a signalling small GTPase that controls cytoskeleton and cell morphogenesis) in a mutually exclusive manner. The major determinant of the binding preference of CDC42EPs is the status of CDC42-bound nucleotide; GTP-CDC42 > septins > GDP-CDC42 (refs 13–15). However, again, *in vivo* relevance of the hypothetical CDC42EP-septin pathway remains unclear.

Given the above background, genetic deletion of the dominant CDC42EP species in Bergmann glia is a rational approach to address the two open issues: physiological roles of the CDC42-CDC42EP-septin pathway and of the septin–GLAST interaction in the brain. We find robust co-expression and co-localization of CDC42EP4 and septins in Bergmann glia, generate CDC42EP4-null mice and conduct biochemical, fine morphological, electrophysiological, pharmacological and behavioural analyses. The unique and systematic approach reveals the requirement of CDC42EP4 in Bergmann glia for the septin-mediated perisynaptic localization of GLAST, and for the efficient buffering and clearance of glutamate from around synapses towards PCs.

# Results

Selective expression of CDC42EP4 in Bergmann glia. We conducted immunoblot (IB) for the expression profiling of the CDC42EP4 protein in the whole brain, two brain subregions (cerebellum and hippocampus) and seven non-neural tissues from adult C57BL/6N mice (Fig. 1a). The major band of ~39 kDa, which fits the calculated molecular mass of 37,980, was the most abundant in the cerebellum (Fig. 1b and Supplementary Fig. 15) as predicted from public gene expression databases including National Center for Biotechnology Information.

Fluorescence *in situ* hybridization (FISH) for *Cdc42ep4* mRNA highlighted the PC layer in the cerebellum (Fig. 1c, left), which is consistent with the data in the Allen Mouse Brain Atlas #71723875 (Allen Institute for Brain Science). Double-label FISH showed complementary distribution of mRNAs for CDC42EP4 and calbindin (PC marker) and overlap between mRNAs for CDC42EP4 and GLAST (Bergmann glia marker; Fig. 1c, right), demonstrating highly Bergmann glia-selective expression of the *Cdc42ep4* gene.

**Clustering of CDC42EP4 in perisynaptic glial processes.** Consistently, double-label immunofluorescence (IF) for CDC42EP4 and calbindin, respectively, highlighted Bergmann glia and PCs in a mutually exclusive manner (Fig. 1d). At a higher magnification with a lower gain level, CDC42EP4 immunoreactivity appeared as 'hotspots', which were interspersed along PC dendrites and tightly apposed to dendritic spines (Fig. 1d, right). CDC42EP4 was far more concentrated in the processes of Bergmann glia than in the cell bodies (Fig. 1e).

To analyse the subcellular distribution of CDC42EP4 at higher resolutions, we conducted silver-enhanced immunoelectron microscopy. Bergmann glial processes thoroughly ensheathed dendritic spines of PCs, except for synaptic contact sites with axon terminals. Gold particles for CDC42EP4 were commonly found as submembranous clusters in Bergmann glial processes that surrounded dendritic spines, particularly around the spine neck (Fig. 1f). Quantitative analysis demonstrated a gradient of CDC42EP4 from the base (neck) to the apex (head) of spines (Fig. 1g). These data indicate that CDC42EP4 in Bergmann glia is localized beneath specific membrane domains that are facing dendritic spines of PCs. The characteristic distribution of CDC42EP4 is reminiscent of those of septin subunits, SEPT4/ H5 (ref. 16), SEPT7/hCDC10 (ref. 16) and SEPT2/Nedd5 (ref. 11), whose physiological role has been unknown.

Generation of *Cdc42ep4*-floxed and -null mice. To explore physiological roles of *Cdc42ep4* using a reverse genetic approach, we generated a line of C57BL/6N mice harbouring a floxed allele, which was subsequently converted to a null allele by crossing with another line that ubiquitously expresses Cre recombinase (Fig. 2a-c). Analyses of *Cdc42ep4*<sup>-/-</sup> offspring demonstrated the total absence of CDC42EP4 in IB and IF (Fig. 2d,e and Supplementary Fig. 15). For the following analyses, we consistently compared *Cdc42ep4*<sup>-/-</sup> (knockout; KO) and *Cdc42ep4*<sup>fl/fl</sup> (wild type; WT) male littermates generated from *Cdc42ep4*<sup>fl/fl</sup> (heterozygous) parents. There was no difference by genotype in the gross appearance, body weight and fertility (Supplementary Fig. 1).

**Normal architecture of the** *Cdc42ep4<sup>-/-</sup>* **cerebellum**. We conducted IF with specific markers for the four major neuronal and glial components (PFs, CFs, PCs and Bergmann glia), which did not show obvious anomaly in the cerebellar cortex of KO mice (Figs 3a and 5b). Transmission electron microscopy (TEM) images showed no recognizable ultrastructural defects in the



Figure 1 | Bergmann glia-selective expression and unique perisynaptic localization of CDC42EP4. (a) The CDC42EP/BORG family and gene expression pattern in the mouse. Each CDC42EP contains a set of basic-CRIB-BD domains. Anti-CDC42EP4 antibody was raised against a region between the CRIB and BD3 (septin-binding) domains. The numbers denote amino-acid residues. CDC42EP1/2/3/4/5, respectively, corresponds to BORG5/1/2/4/3. (b) Lysates from adult mouse tissues (50 μg total protein per lane) were immunoblotted for CDC42EP4 and were reprobed for β-actin as a loading control. The major ~39-kDa band was most abundant in the cerebellum. (c) FISH for Cdc42ep4 mRNA in the adult mouse cerebellum. (Left) Labels for Cdc42ep4 (green) highlighted the PC layer in a parasagittal section. (Top) Double-label FISH for mRNAs for Cdc42ep4 and a PC marker calbindin (red). Cdc42ep4 mRNA was excluded from PCs (\*). (Bottom) Double-label FISH for Cdc42ep4 and Glast (red) mRNAs, and TOTO-3 stain for DNA (blue). The two mRNA signals overlapped in all (n = 115) Bergmann glial cells identified in a representative section. Scale bars, 1 mm and 20  $\mu$ m. (**d**) (Left and centre) Double-label IF for CDC42EP4 (red) and calbindin (green) in the cerebellar cortex. The diffuse granular signals for CDC42EP4 distributed throughout the molecular layer and in the PC layer, which were excluded from PCs and the granule cell layer. (Right) At a higher magnification, CDC42EP4-positive puncta were interspersed and aligned along PC dendrites. Scale bars, 20 and 5 µm. (e) Double-label IF for CDC42EP4 (red) and a Bergmann glial marker Phgdh (green). The limited overlap in Bergmann glial cell bodies (arrowheads) indicated selective localization of CDC42EP4 in glial processes. Scale bar, 5 µm. (f) (Left) Immunoelectron microscopy image for CDC42EP4 in the molecular layer. Gold particles for CDC42EP4 were found as submembranous clusters in terminal processes of Bergmann glia (tinted), each surrounding a dendritic spine (Sp) of a PC. PF, parallel fibre terminal. Scale bar, 200 nm. (Right) Quantification of glia-selective localization of CDC42EP4. Data represented as mean ± s.e.m. (g) Histogram showing a gradient of CDC42EP4 signals relative to the geometry of dendritic spines of PCs; higher in regions facing spine base (arrows in f) than in regions facing the spine head.

molecular layer (Fig. 3b), including the lengths of the postsynaptic density (PSD) in PF–PC synapses (Fig. 3c). Four major proteins at glutamatergic synapses, GluA1, GluA2, GluA4 and PSD-95, did not show quantitative differences by genotype (Fig. 3d and Supplementary Fig. 15). These data indicate that CDC42EP4 is dispensable for the morphological development of the major neuronal and glial components, and synapse architecture in the cerebellar cortex.

**Septin oligomers as the major binding partners of CDC42EP4**. For unbiased identification of physiological binding partners of CDC42EP4 in Bergmann glia, we conducted proteomic analysis



**Figure 2 | Generation of** *Cdc42ep4<sup>-/-</sup>* **mice and morphology of the major neuronal components. (a)** The KO strategy of the *Cdc42ep4* gene. A schematic diagram showing the wild-type (*Cdc42ep4*<sup>+</sup>), floxed (*Cdc42ep4*<sup>fl</sup>) and null (*Cdc42ep4*<sup>-</sup>) alleles, and the targeting vector. Note that Cremediated loxP recombination leaves no coding exon. The restriction sites for Sac I (S), EcoR V (E) and Hinc II (H), three probes (grey bars) used for Southern blot analysis and three PCR primer sites are indicated. Signs; coding region (grey box), untranslated region (open box), loxP (black triangle), frt (open half-circle), neomycin resistance cassette (Neo), diphtheria toxin A-chain cassette (DT). (b) Southern blot analysis. Genomic DNAs purified from WT and the chimera (*Cdc42ep4*<sup>+/+</sup>;*Cdc42ep4*<sup>fl/+</sup>) mice were digested with the restriction enzymes and hybridized with the probes as indicated. The band patterns, as seen in preceding Southern blot analysis of ES cell clones, reconfirmed successful homologous recombination of the clone. See Methods for details. (c) PCR genotyping. Two sets of primers discriminated genomic DNAs from *Cdc42ep4*<sup>+/+</sup>, *Cdc42ep4*<sup>fl/fl</sup> (WT), *Cdc42ep4*<sup>fl/-</sup> and *Cdc42ep4<sup>-/-</sup>* (KO) mice. (d) Expression and extractability of CDC42EP4 in the adult mouse cerebellum. The pellet/supernatant assay showed that CDC42EP4 is partitioned mostly to the detergent-extractable, supernatant (s) fraction but not to the inextractable, pellet (p) fraction. CDC42EP4 was absent from KO tissues. α-Tubulin was used as a loading control. (e) IF for CDC42EP4 on parasagittal brain sections of adult male littermate WT and KO mice. The molecular layer of the WT cerebellum (Cb) was intensely labelled for CDC42EP4, which was absent from the KO brain. The faint, diffuse labelling of the entire brain is attributed to astrocytes. These results are consistent with the immunoblot data (Figs 1b and 2d) and warrant the specificity (high signal-to-noise ratio) of the antibody. Scale bar, 1mm. UTR, untranslated repeat.

of cerebellar lysates. Immunoaffinity chromatography for CDC42EP4 followed by mass spectrometry identified CDC42EP4, nine septin subunits (SEPT2/3/4/5/6/7/8/10/11) and a few other cytoskeletal proteins including Myosin-10 (nonmuscle myosin heavy chain IIB) and  $\alpha$ -II/ $\beta$ -II spectrins (Table 1). The minimal background noises in the three negative control experiments (for example, anti-CDC42EP4 IgG captured no peptides from KO samples) reconfirmed the absence of CDC42EP4 in the KO cerebellum and the antibody specificity. Intriguingly, however, CDC42 and Tc10/RhoQ (another small GTPase that can bind to CDC42EPs *in vitro*<sup>13</sup>) were not detected (Table 1 and Supplementary Fig. 13). Thus, the major physiological binding partners of CDC42EP4 in the adult mouse cerebellum are not small GTPases, but hetero-oligomers of septins.

**Physiological network of CDC42EP4, septins and GLAST**. Previous studies with recombinant proteins separately demonstrated direct interactions between CDC42EP5/BORG3 and septin hetero-oligomers (for example, SEPT6/7 and SEPT2/6/7, but not individual subunits)<sup>14,15</sup>, and between SEPT2 and GLAST<sup>11</sup>. However, higher-order molecular network composed of CDC42EPs, septin hetero-oligomers and GLAST has never been tested. We addressed this with their co-immunoprecipitation (co-IP) from cerebellar lysate (Fig. 4a and Supplementary Figs 13–15), and double-label IF that showed their partial overlap in Bergmann glia (Fig. 4e). These data, along with the previous studies and our proteomic and IF findings, indicate physiological molecular network that contains CDC42EP4, septin hetero-oligomers and a subset of GLAST.

Loss of CDC42EP4 diminishes septin–GLAST interaction. To validate the significance of CDC42EP4 in the molecular network, we compared the status with or without CDC42EP4. Pellet/ supernatant assay of cerebellar lysates showed that the amount and solubility of SEPT7, SEPT4 and GLAST were unaffected by the loss of CDC42EP4 (Fig. 4b–d and Supplementary Fig. 15). Their distribution patterns assessed using IF showed no recognizable difference by genotype (Fig. 5b). Intriguingly, however, a co-IP/IB assay revealed a significant reduction ( $\Delta$ 50%) of GLAST that was pulled down with SEPT4 (a Bergmann glia-selective septin subunit) from the *Cdc42ep4*<sup>-/-</sup> cerebellum, when the interactions with two other major septin subunits in Bergmann



**Figure 3 | Morphological analysis of the neuronal and glial components in**  $Cdc42ep4^{fl/fl}$  and  $Cdc42ep4^{-1-}$  cerebellar cortices. (a) Double-label IF of WT and KO cerebellar cortices for a Purkinje cell marker Car8 (red) and a parallel fibre (that is, granule cell) marker VGluT1 (top, green) or a climbing fibre marker VGluT2 (bottom, green). No obvious morphological anomaly, including aberrant CF-PC innervation, was found in the major neuronal components of KO-derived samples. Scale bar, 20 µm. (b) Transmission electron microscopy images of WT and KO molecular layers. No obvious ultrastructural difference was found between the genotypes. PF, parallel fibre terminal or bouton. PC, dendritic spine of Purkinje cell. Bergmann glial processes are tinted. Scale bar, 200 nm. (c) Cumulative histogram of PSD length of the PF-PC synapses, showing no significant difference between the genotypes (n = 92 synapses from two littermates for each genotype, NS, P > 0.05 by Kolmogorov-Smirnov test). (d) Quantitative immunoblot of WT and KO cerebellar PSD fractions for GluA1, GluA2 and GluA4 (the major subunits of the AMPARs), each normalized with PSD-95. There was no significant quantitative difference by genotype (n = 3, NS, P > 0.05 by t-test).

glia (SEPT2 and SEPT7) were unaffected (Fig. 5a and Supplementary Fig. 15). The significant dissociation of GLAST from septin hetero-oligomers by the loss of CDC42EP4 indicates a role for CDC42EP4 as a stabilizer and/or an adapter for the association between GLAST and septin hetero-oligomers.

**GLAST delocalizes away from perisynapse without CDC42EP4**. Previous fluorescence recovery after photobleaching assay showed that septin depletion via RNA interference augmented diffusional mobility of green fluorescent protein-tagged GLAST on the plasma membrane, while septin filament stabilization gave the opposite effect, indicating a role for septins as submembranous scaffold and/or diffusion barrier for GLAST<sup>12</sup>. To assess whether the dissociation from septins by the loss of CDC42EP4 could alter the distribution of GLAST, we conducted quantitative mapping of GLAST in Bergmann glia by postembedding immunoelectron microscopy technique (Fig. 5c-e). As expected, gold particles for GLAST were distributed along Bergmann glia membrane enwrapping pre- and postsynaptic elements of PF-PC synapses, whereas neuronal membranes were

Identified proteins (69)	Peptide number			Sequence coverage (%)				
	KO IgG	KO ep4	WT lgG	WT ep4	KO IgG	KO ep4	WT IgG	WT ep4
CDC42EP4				20				63
SEPT7			1	21			2.1	45
SEPT4			1	19			1.9	43
SEPT11				14				34
SEPT5				12				42
SEPT2				11				47
α-II spectrin			8	18			4.7	9.1
SEPT3				7				22
Myosin-10	2		1	10	1.3		0.81	6.7
SEPT8				6				24
SEPT10				5				16
SEPT6				5				26
β-II spectrin			1	4			0.8	2.8

List of proteins purified from WT cerebellar lysate with CDC42EP4-immunoaffinity column and identified LC-MS/MS analysis (rightmost columns). The numbers denote the count of peptides assigned to each protein (left) and the sequence coverage (right). The blank columns are zero. The specificity of the counts is corroborated by few false-positive counts in the controls (three left columns, that is, KO lysate captured with nonimmune IgG or anti-CDC42EP4 antibody, and WT lysate captured with nonimmune IgG). Another co-IP method used for the immunoblot analyses detected GLAST (peptide number = 2, sequence coverage = 6.45), but not CDC42. Co-IP, Co-immunoprecipitation; KO, knockout; WT, wild type.

scarcely labelled. The distribution and density of immunogold particles were comparable between WT and KO mice (Fig. 5d). Intriguingly, however, the distance from perisynaptic GLAST signals to the nearest PSD edge (arrowheads, Fig. 5c) was significantly longer in KO mice than that in WT mice (Fig. 5e). Together, the biochemical and fine morphometric data consistently indicate that CDC42EP4 in Bergmann glia is required for GLAST to interact with septins and to localize along perisynaptic membrane domains enwrapping PF–PC synapses.

 $Cdc42ep4^{-/-}$  mice exhibit insufficient glutamate clearance. To explore whether the anomalies of GLAST (that is, dissociation from septins and delocalization from synapses) in Cdc42ep4mice affect glutamatergic neurotransmissions, we assessed electrophysiological properties of the major glutamatergic synapses in cerebellar slices. We first examined the CF-PC synapse by stimulating CFs in the granule cell layer and recording the evoked excitatory postsynaptic currents (EPSCs) with a whole-cell voltage clamp configuration (Fig. 6a). Most PCs in 7-week-old WT (n = 16/18, 89%) and KO (n = 17/21, 81%) mice elicited a single large EPSC in an all-or-none manner as stimulus intensity gradually increased (Fig. 6a, left). The number of steps, which reflects the number of CFs innervating a given PC<sup>17,18</sup>, was comparable between WT and KO mice (Fig. 6a, right), indicating that CDC42EP4 is dispensable for the postnatal establishment of CF-PC monoinnervation. The major parameters of CF-EPSC kinetics (that is, amplitude, 10-90% rise time and decay time constant) were also comparable (Supplementary Table 1). These data indicate that properties of the CF-mediated synaptic transmission are normal in young adult KO mice.

Given the major role for GLAST in glutamate clearance from extracellular space around PF–PC synapses<sup>5</sup>, the delocalization of GLAST away from PSD (Fig. 5e) might affect PF–EPSC kinetics in KO mice. To test this, we measured the decay time constant of PF-EPSC, which is known to protract as glutamate reuptake delays<sup>19–22</sup>. The PF-EPSCs elicited by the stimulation in the molecular layer of KO mice showed normal paired-pulse facilitation (Fig. 6b and Supplementary Table 1), indicating that the presynaptic functions were largely unaffected. As previous studies showed that desensitization of the AMPA receptors (AMPARs) masks the effects of GLAST insufficiency on PF–PC synapses<sup>20</sup>, we also employed cyclothiazide (CTZ) to block

desensitization of AMPARs. While bath application of CTZ prolonged the decay time constant of PF-EPSCs in both genotypes, it was significantly longer in KO than in WT (P = 0.010, Fig. 6c). These data suggest a recognizable delay in the glutamate clearance from PF-PC synapses in KO mice.

To corroborate the above findings, we compared glutamate transients in PF-PC synapses using a low-affinity competitive inhibitor of the AMPARs,  $\gamma$ -D-glutamylglycine ( $\gamma$ DGG).  $\gamma$ DGG is a rapidly unbinding competitive antagonist that is readily displaced from AMPARs by released glutamate<sup>23</sup>. Therefore, the degree of inhibition of the EPSC roughly reflects the relative size and kinetics of the glutamate transient in the synaptic cleft. Bath application of 1 mM  $\gamma$ DGG inhibited the PF-EPSC amplitude to ~ 50% of the basal level in WT, while only to ~ 80% in KO, suggesting that the size of glutamate transient at PF-PC synapses was significantly higher in KO than in WT (WT versus KO, 51.5 ± 2.2/79.1 ± 4.2%, n=7, P < 0.001, Fig. 6d).

The above findings, in conjunction with the molecular-level anomalies, suggest that PF-PC synapses in KO mice have hotspots around active zones with subnormal glutamate-buffering capacity. Since the density of AMPARs is the highest in postsynaptic active zones, we hypothesized that KO mice are sensitive to subthreshold doses of glutamate clearance inhibitors. To test this, we applied 50 µM DL-TBOA, which nonselectively inhibits glutamate transporters (EAAT1-5) but only partially as the half-maximal inhibitory concentration for EAAT1/GLAST is 70  $\mu$ M. The treatment with the low-dose DL-TBOA in addition to CTZ elicited a significantly larger baseline inward current in KO PCs (WT versus KO,  $310 \pm 26/864 \pm 185$  pA, n = 11/12, P < 0.001, Fig. 6e, trace 3). The aberrant baseline inward current of KO mice was abolished by additional application of an AMPAR antagonist, NBQX (from  $895 \pm 260$  to  $367 \pm 84$  pA, n = 7, P = 0.016, Fig. 6e, trace 4). Thus, the large baseline inward current in KO PCs is generated by AMPARs exposed to excessive external glutamate. A previous study showed that 200 µM DL-TBOA augmented AMPAR-mediated inward current of PCs in WT mice<sup>19</sup>, while KO PCs were sensitive enough to react to 50 µM DL-TBOA. These results further support the compromised glutamatebuffering/clearance in KO mice, which may be due to the delocalization of GLAST from PF-PC synapse active zones (Fig. 5e), and/or their dissociation from the perisynaptic clusters of septins and CDC42EP4 (Fig. 5a).



**Figure 4 | Biochemical analysis of binding partners of CDC42EP4 in**  $Cdc42ep4^{fl/fl}$  **and**  $Cdc42ep4^{-/-}$  **cerebella. (a)** Co-IP/IB assay of CDC42EP4 with representative septin subunits and GLAST from WT and KO cerebellar lysates. (Input) IB for SEPT4, SEPT7, SEPT2 and GLAST, respectively, detected a quadruplet of 54, 52, 48 and 44 kDa, a doublet of 51 and 48 kDa, a single 42 kDa band and a broad 55 kDa band in the cerebellar lysate. (IP) Anti-CDC42EP4 antibody pulled down SEPT4, SEPT7, SEPT2 and GLAST only from WT cerebellar lysate. The graphs show densitometric quantification of the yield (n = 3, \*\*\*P < 0.001, \*\*P < 0.05, NS, P > 0.05 by one-way ANOVA with *post hoc* Tukey test). (Note: the extraction condition including the lysis buffer composition was optimized to detect GLAST, which was distinct from the one used mainly for the proteomic analysis (Table 1). See Methods.) (**b-d**) Pellet/supernatant assay results on the quantity and extractability of SEPT7, SEPT4 and GLAST in WT and KO cerebella. There was no significant difference in their amount and partitioning by genotype (n = 3, NS, P > 0.05 by t-test). The same membranes were reprobed for  $\alpha$ -tubulin as a loading control, which was used for normalization. (**e**) Double-label IF for GLAST (green) and CDC42EP4 (red) in WT cerebellar cortex showing their partial co-localization in Bergmann glial processes. Scale bars, 20 and 5  $\mu$ m.

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**Figure 5 | Loss of CDC42EP4 dissociates the GLAST-septin interaction and delocalizes GLAST away from synapses.** (a) Quantitative co-IP/IB assay between SEPT4 and SEPT7, SEPT2 or GLAST in WT and KO cerebellar lysates. While the interaction among the septin subunits did not differ, the relative amount of GLAST pulled down with SEPT4 from KO lysate was significantly less than that from WT (n = 3, \*P < 0.05, NS, P > 0.05 by *t*-test), indicating that septin-GLAST interaction depends on CDC42EP4. See Fig. 4a-d for the comparable amount and solubility of these proteins in WT and KO cerebella. (b) Double-label IF for GLAST (green) and a Purkinje cell marker Car8 (red) in WT and KO cerebellar cortices. Genetic loss of CDC42EP4 caused no recognizable difference in the distribution of GLAST up to the resolution. Scale bars, 20 and 1 µm. (c) Immunoelectron microscopy images for GLAST in WT and KO molecular layers. PF, parallel fibre terminal or bouton. PC, dendritic spine of Purkinje cell. Bergmann glial processes are tinted. The pattern of GLAST distribution appears comparable to that of a previous study<sup>4</sup>. Scale bar, 200 nm. (d) Quantitative analysis of immunoelectron microscopy data. Bergmann glia selectivity and labelling density of GLAST were comparable between WT and KO mice: Bergmann glia; 22.4 ± 1.3/21.1 ± 1.2 particles per µm (n = 51/467 particles from two littermate pairs, NS, P > 0.05 by Mann-Whitney *U*-test). Postsynaptic membrane; 0.50 ± 0.21/0.68 ± 0.24 particles per µm (n = 57/8 particles, P = 0.68 by Mann-Whitney *U*-test; *cf*. Figs 4d and 5a-c). (e) Cumulative histogram of the distance of GLAST measured from the nearest PSD edge (for example, arrowheads in c). A significant right shift of the curve for KO mice demonstrates delocalization of GLAST away from PSDs of PF-PC synapses (median, WT = 0.27 µm, KO = 0.31 µm from the nearest edge of PSD; n = 22/19 synapses from two littermates for each genotype, \*\*\*P < 0.001 by Kolmogorov-Smirnov test).



**Figure 6 |** *Cdc42ep4*<sup>-/-</sup> mice exhibit insufficient glutamate-buffering/clearance capacity. (a; Left) Sample traces of CF-EPSCs. Two to three traces were superimposed. Scale bars, 10 ms and 500 pA. (Right) Summary histogram showing the number of CF-EPSC steps (n = 21/18, P = 0.513 by Mann-Whitney *U*-test). Holding potential was -10 mV in **a** and -70 mV in **b**-e. (b; Left) PF-EPSCs in response to paired stimuli at 50 ms intervals. Scale bars, 10 ms and 100 pA. (Right) Summary graph of the paired-pulse ratio (n = 22/19, P = 0.824 by Mann-Whitney *U*-test). (**c**; Left) Sample traces of PF-EPSCs with (grey) or without (black) 100  $\mu$ M CTZ in a WT and a KO mice. Scale bars, 25 ms and 50 pA. (Right) The decay time constant of PF-EPSCs. Although CTZ prolonged the decay time constant both in WT and KO (n = 11/17, WT, from  $9.3 \pm 0.8$  to  $17.9 \pm 1.8$  ms, \*\*\*P < 0.001; KO, from 11.6  $\pm 0.8$  to 22.6  $\pm 1.3$  ms; \*\*\*P < 0.001), the effect was significantly larger in KO than in WT (\*\*P = 0.006 by two-way ANOVA with *post hoc* Tukey test), which resulted in more protracted postsynaptic response in KO PCs (\*P = 0.010). (**d**) (Left) PF-EPSCs before (black), in the presence of 1 mM  $\gamma$ DGG (grey), and after washout (dashed). Scale bars, 10 ms and 200 pA. (Right) Summary histogram showing the effects of  $\gamma$ DGG. KO PF-EPSCs were significantly more insensitive to  $\gamma$ DGG than WT ones (n = 77, 7\*\*P < 0.001 by Mann-Whitney *U*-test). (**e**; Left) Sample traces of PF-EPSCs in a WT and a KO mice in control ACSF (1), in the presence of CTZ (2), CTZ plus 50  $\mu$ M TBOA (3), CTZ, TBOA plus 10  $\mu$ M NBQX (4). Grey and dashed lines, respectively, indicate the zero offset level and baseline holding current level in the control ACSF. Scale bars, 200 ms and 100 pA. (Right) The summary graph showing the holding current. The effects of CTZ plus TBOA was significantly larger on KO than on WT (n = 11/12, \*\*\*P < 0.001 by two-way repeated measures ANOVA with *post hoc* Tukey test). (Two right plots) Additional appl

In addition, the content of glutamate, glutamine and related amino acids and monoamines in cerebellar tissues from WT and KO mice was comparable, as assessed using high-performance liquid chromatography (Table 2). Thus, the total amount of the glutamate/glutamine shuttle components, and other major neurotransmitters and metabolites, are unaffected by the loss of CDC42EP4.

**Impaired motor coordination/learning in**  $Cdc42ep4^{-/-}$  mice. As a means of unbiased screening for neural dysfunctions, a cohort of WT and KO littermates were subjected to a systematic battery of behavioural tests. In 14 behavioural test paradigms including the rotating rod (rota-rod) test, WT and KO mice performed almost comparably (Table 3 and Supplementary Figs 1–12). Given the robust compensatory potential of the motor control circuitry, and the molecular and electrophysiological endophenotype of KO mice, we assessed their motor coordination and motor learning with the balance beam test, which is superior to the rota-rod test in detection sensitivity<sup>24</sup>. While the moving speed of 12-week-old KO mice on the initial trial (the intercept) was normal, the slopes of their learning curves were significantly flatter than those of WT mice

transmitters and metabolites in the and $Cdc42ep4^{-/-}$ littermate mice.	cerebella of <i>Cdc42ep4<sup>fl/fl</sup></i>
Neurotransmitters and metabolites (pe	r mg tissue)
WT	КО

	•••	ĸo
Glu (nmol)	8.07 ± 0.521	7.97 ± 0.253
Gln (nmol)	$5.54 \pm 0.459$	5.25 ± 0.128
Gly (nmol)	$0.66 \pm 0.065$	$0.81 \pm 0.13$
GABA (nmol)	$1.43 \pm 0.102$	1.48 ± 0.133
L-Ser (nmol)	$0.40 \pm 0.028$	$0.40 \pm 0.028$
D-Ser (nmol)	0.003 ± 0.0001	$0.003 \pm 0.0000$
5-HT (ng)	$0.16 \pm 0.021$	$0.16 \pm 0.013$
5-HIAA (ng)	$0.09 \pm 0.01$	$0.11 \pm 0.007$
NE (ng)	$0.35 \pm 0.037$	0.39 ± 0.013
MHPG (ng)	$0.12 \pm 0.062$	$0.07 \pm 0.0006$
HVA (ng)	$0.005 \pm 0.003$	$0.008 \pm 0.002$
DA (ng)	$0.004 \pm 0.001$	$0.005 \pm 0.003$
DOPAC (ng)	$0.006 \pm 0.002$	$0.008 \pm 0.004$
The amount of glutamate, g tissues $(n = 3, 3)$ . There w	utamine and other substances extra as no significant difference. KO. kn	icted from WT and KO cerebellar ockout: WT, wild type.

(n = 13/13, P = 0.0003 and 0.0004, respectively, for the beam diameters of 28 and 11 mm; Fig. 7a). In a follow-up of the same cohort at 24 weeks of age, the learning curve of KO mice was consistently lower than that of WT mice (n = 12/12, P = 0.018 and 0.014; Fig. 7a).

To test whether the persistent underperformance is caused by the insufficient glutamate-buffering/clearance capacity in Bergmann glia, and not by the loss of extracerebellar astrocytic CDC42EP4 (Fig. 2e), we attempted the partial inhibition of EAATs with DL-TBOA. Direct subpial injection of 100  $\mu$ M DL-TBOA with CTZ above lobule VI of the cerebellar cortex did not affect the motor performance of WT mice, whereas the pharmacological decompensation elicited significant and transient motor incoordination in KO mice, as was obvious now in the rota-rod test (n = 5/4, P = 0.0011, Fig. 7b). The remarkable hypersensitivity to the subthreshold DL-TBOA recapitulated *in vivo* has corroborated the insufficient glutamate-buffering/ clearance capacity in KO mice.

#### Discussion

Previous studies indicated that CDC42EPs bind to small GTPases (CDC42, RhoQ/Tc10) and septins in a mutually exclusive manner<sup>13,14</sup>, and that interaction of CDC42EP1/BORG5 with CDC42 and atypical protein kinase C is required for cell motility in early mouse embryo<sup>25</sup>. A recent study revealed defective angiogenesis in  $Cdc42ep1^{-/-}$  mice, which is due partly to septin/ actomyosin dysregulation that affects migration of vascular endothelial cells<sup>26</sup>. In contrast, CDC42EP4 in Bergmann glia is mainly in complex with septins and GLAST, and is dispensable for developmental cell migration and post-developmental morphological integrity. The distinct binding partners and physiological roles of CDC42EPs may reflect their distinct primary structure and/or expression pattern. Since Cdc42ep1 mRNA is also expressed in Bergmann glia (Allen Mouse Brain Atlas #68342384),  $Cdc42ep1^{-7-}$  mice and  $Cdc42ep1^{-7-}$ ; Cdc42ep4<sup>-/-</sup> double-mutant mice would exhibit intriguing cerebellar phenotypes.

Genetic loss of a vital septin subunit (that is, SEPT7, SEPT9 or SEPT11) causes embryonic lethality<sup>27–29</sup>, whereas obvious brain anomaly or motor incoordination has never been reported in mice that lack one or two other septin subunits (that is, SEPT3, SEPT4, SEPT5, SEPT6, SEPT3 and SEPT5, or SEPT4 and SEPT6),

despite their abundance in the cerebellum and other brain regions<sup>30-34</sup>. In contrast, *GLAST<sup>-/-</sup>* mice exhibit protracted glutamate transient, multiple CF–PC innervations and failure in the rota-rod test<sup>6,21</sup>.

The present study demonstrated that Cdc42ep4-/- mice develop CF-PC innervations with largely normal wiring pattern and electrophysiological properties (Figs 3a and 6a), and pass the rotating rod (rota-rod) test (Table 3). However, pharmacological decompensation with inhibitors of AMPAR, AMPAR desensitization or EAATs consistently indicated their insufficient glutamate-buffering/clearance capacity in electrophysiological analyses (Fig. 6c-e), and caused failure in the rota-rod test (Fig. 7b). Thus, the severity of cerebellar phenotypes is ranked  $GLAST^{-/-} > Cdc42ep4^{-/-} >$  septin-null mice, which would justify an interpretation of the phenotype of  $Cdc42ep4^{-/-}$  mice as a mild insufficiency of GLAST function, rather than as a partial loss of septin function. Since  $Sept4^{-/-}$  mice and Bergmann gliaselective  $Sept7^{-/-}$  mice exhibit subnormal motor coordination only in infancy (Kinoshita, Ageta-Ishihara et al., unpublished), the pharmacological decompensation technique may help differentiate these and other septin mutant mice from WT littermates.

Glutamate released from PF boutons/terminals not only depolarizes PCs via AMPARs; however, the spillover fraction evokes  $Ca^{2+}$  responses in the perisynaptic compartments ('microdomains') of lamellar Bergmann glial processes mainly via mGluR1 (refs 35,36). A subdomain in such a compartment tightly enwraps a PF-PC synapse, where GLAST outnumbers other EAATs<sup>4,37</sup>. While EAAT4 is localized to PC spines for slow glutamate clearance<sup>20</sup>, GLAST is concentrated along perisynaptic processes of Bergmann glia and contributes to the fast and efficient limitation/termination of glutamate neurotransmission and spillover<sup>1,5,20</sup>. However, the molecular basis to target and concentrate GLAST to the proximity of synaptic gap remains unclear.

Physical and functional interactions of the cytoplasmic tail of GLAST with submembranous septins have been consistently demonstrated *in vitro*, in tissue culture cell cortex and Bergmann glial processes<sup>11,12</sup>. The interaction with submembranous septins has been hypothesized to provide membrane-bound GLAST trimers with scaffolds, limit their lateral diffusion and/or augment their transport activity, which collectively contribute to perisynaptic concentration of glutamate clearance activity. However, the hypothesis has never been tested *in vivo* partly because of the aforementioned redundancy problem in septin reverse genetics.

This study demonstrated that CDC42EP4 clusters beneath Bergmann glial membrane subdomains enwrapping dendritic spines where septins and GLAST abound<sup>4,11,16</sup>. In the absence of CDC42EP4, GLAST is dissociated from septins (Fig. 5a) and delocalized away from synapses (Fig. 5e). These findings support the above hypothesis of septins as scaffold/barrier for GLAST, and may further implicate subtle alterations in tripartite synapse geometry, for example, retraction of glial components from synaptic clefts, with or without secondary remodelling of neuronal components.

The distribution of GLAST visualized with IF and immunoelectron microscopy appears more diffuse along Bergmann glial membranes than that of CDC42EP4 (Figs 1f, 4e and 5c), which corresponds to the IP/IB data that only a subset of GLAST is pulled down with CDC42EP4 and/or septins. These data suggest other membrane-proximal proteins that contribute to perisynaptic localization of GLAST. One such candidate is NHERF1 (Na<sup>+</sup>/H<sup>+</sup>-exchange regulatory factor-1) whose PDZ domain interacts with the cytoplasmic tail of GLAST<sup>12,38</sup>. So far, however, neither GLAST-related phenotype for *Nherf1<sup>-/-</sup>* mice<sup>39</sup> nor

Tests	Mental/physical activities	Indices measured (inexhaustive)	Alteration from wild-type Cdc42ep4 <sup>-/-</sup>	Related figures*
General health and	General health	Body weight	$\rightarrow$	1
neurological screening		Rectal temperature	$\rightarrow$	
		Grip strength	$\rightarrow$	
		Hanging persistence	$\rightarrow$	
Light/dark transition test	Exploratory activity	Distance travelled in the light chamber	$\rightarrow$	2
	Light avoidance	Distance travelled in the dark chamber	$\rightarrow$	
		Latency to the first entry to the light chamber	$\rightarrow$	
		Time stayed in the light chamber	$\rightarrow$	
		Number of transitions between chambers	$\rightarrow$	
Open field test	Exploratory activity	Distance travelled	$\rightarrow$	3
	Avoidance from open space	Centre time	$\rightarrow$	
	Anxiety-like behaviour	Vertical activity	$\rightarrow$	
		Stereotypical movements	$\rightarrow$	
Elevated plus maze test	Exploratory activity	Distance travelled	$\rightarrow$	4
	Height avoidance	Entries into open arms	$\rightarrow$	
		Number of entries	$\rightarrow$	
		Time stayed on open arms	$\rightarrow$	
Acoustic startle response	Startle reflex to loudness	Amplitude of body motion	$\rightarrow$	5a
Prepulse inhibition (PPI) test	Sensorimotor gating	Decrement of startle amplitude	$\rightarrow$	5b
Porsolt forced swim test	Despair-like behaviour	Latency to immobility	$\rightarrow$	6
Home cage monitoring	Diurnal cycle of locomotor activity	Activity level (distance travelled)	$\rightarrow$	7
	Social behaviour	Mean number of particles	$\rightarrow$	
Social interaction test (one-chamber, stranger pair)	Social behaviour, anxiety-like behaviour	Distance travelled	$\rightarrow$	8
		Number of contacts	$\rightarrow$	
		Total duration of active contacts	$\rightarrow$	
		Mean contact duration	$\rightarrow$	
		Total duration of contacts	$\rightarrow$	
Social interaction test (three chambers, one to two caged strangers	Social behaviour, anxiety-like behaviour	Time spent with novel stranger	$\rightarrow$	9
		Distance travelled	$\rightarrow$	
Rota-rod test	Motor coordination/learning	Latency to fall	$\rightarrow$ ( $\downarrow$ with CTZ+TBOA)	Fig. 7b, 10
Tail suspension test	Behavioural despair	Latency to immobility	$\rightarrow$	11
Barnes maze test	Spatial memory	Time spent around target hole	$\rightarrow$	12
	Motor coordination/learning	Moving speed		Fig. 7a

# Table 3 | Systematic behavioural test results of $Cdc42ep4^{fl/fl}$ and $Cdc42ep4^{-/-}$ littermate mice.

expression and localization NHERF1 in Bergmann glia has been hig reported. Thus, the unique phenotype of  $Cdc42ep4^{-/-}$  mice provides the first *in vivo* evidence for the significance of syn perisynaptic concentration of GLAST by submembranous AM scaffold and/or diffusion barrier.

GLAST abounds near PF-PC synapses and the buffering/ clearance capacity far surpasses the level of glutamate release, which confers remarkable tolerance to EAAT inhibitors. Our electrophysiological analysis with pharmacological interventions revealed insufficient glutamate-buffering/clearance capacity in  $Cdc42ep4^{-/-}$  PF-PC synapses. The decay time constant of EPSC, which reflects the retention of extracellular glutamate, was mildly protracted on inhibition of AMPAR desensitization with CTZ (Fig. 6c). Additional inhibition of EAATs with subthreshold DL-TBOA significantly augmented AMPAR-mediated inward current only in the  $Cdc42ep4^{-/-}$  cerebellum (Fig. 6d,e). Further, a low-affinity glutamate analogue  $\gamma$ DGG was less effective in the inhibition of EPSC (Fig. 6d). These data concordantly indicate higher extracellular glutamate level in the mutant. Given the properties of EAATs and their distribution around glutamatergic synapses to  $PCs^{5,20,37}$ , and unaltered expression of the major AMPAR subunits in the  $Cdc42ep4^{-/-}$  cerebellum (Fig. 3d), the phenotype is attributed principally, if not all, to the insufficiency of GLAST function. As the total amounts of glutamate and GLAST are unaltered in the  $Cdc42ep4^{-/-}$  cerebellum, the delocalization of GLAST from perisynaptic glial processes (Fig. 5e), with or without altered transporter activity due to the dissociation from CDC42EP/septins, appears to be the major cause of the glutamate intolerance.

Overall, the unique phenotype of  $Cdc42ep4^{-/-}$  mice provided the first *in vivo* evidence for submembranous molecular network that is required for the perisynaptic concentration and efficiency of GLAST. This study illuminated the spatial regulation of GLAST by the CDC42EP4/septin-based scaffold beneath perisynaptic glial membranes, which ensures optimal buffering/clearance of extracellular glutamate from around synapses. Since CDC42EPs



Figure 7 | Motor coordination and motor learning defects in Cdc42ep4<sup>-/-</sup> mice. (a; Top) Set-up for the balance beam test. The height (0.5 m above the floor) and illumination (100 lux) motivate mice to escape by traversing along a horizontal rod  $(1 \text{ m} \times 28 \text{ or } 11 \text{ mm})$  into a dark box. (Bottom) The learning curves of a cohort of WT and KO mice measured at 3 and 6 months of age. The moving speeds are plotted for seven trials with a 28-mm rod and subsequent five trials with an 11-mm rod over 4 days. The motor coordination defects in KO mice remained uncompensated up to 6 months of age (n = 13/13 and 12/12, P = 0.0003, 0.0004, 0.018, 0.014 by two-way repeated measures ANOVA). (b) The learning curves of 6-week-old WT and KO littermate mice assessed by the rota-rod test before and after direct cerebellar cortical injection of CTZ plus DL-TBOA (100 µM each per 10 µl). The local inhibition of EAATs with the subthreshold dose of DL-TBOA elicited a significant motor coordination defects transiently (around 4 h post-injection) and only in KO mice (n = 5/4, P = 0.551, 0.0011, 0.957, 0.518 by two-way repeated measures ANOVA). The aberrant hypersensitivity of KO mice indicates their glutamate clearance deficit that is adaptively compensated.

and septins, as with GLAST, are implicated in human neurological and psychiatric disorders including schizophrenia by unbiased proteomic and genome-wide association studies<sup>8,9,40–43</sup>, whether relevant mechanisms underlie the pathophysiology in other brain regions is to be tested in future studies.

#### Methods

**Animal experiments.** Animal experiments had been approved by the institutional review committees and conducted in accordance with the regulations for the care and use of animals at Nagoya University, Niigata University, Hokkaido University, Hiroshima University, Fujita Health University and Gunma University. We consistently compared male littermates raised in the same cages unless otherwise noted.

**Antibodies**. We raised rabbit polyclonal antibodies against seven antigens and consistently used one (#2) against a recombinant polypeptide Arg<sup>58</sup>-Met<sup>100</sup> from

CDC42EP4 that gave the best signal/noise ratio in IB and IF analyses. The specificity was warranted by the absence of the signals in *Cdc42ep4*<sup>-/-</sup> tissues (Fig. 2d,e). We used antibodies for septins, SEPT2 (1:2,000), SEPT4 (1:3,000) and SEPT7 (1:4,000) as previously described<sup>33,44</sup>, GLAST<sup>45</sup>, 3-phosphoglycerate dehydrogenase (Phgdh)<sup>46</sup>, calbindin<sup>47</sup>, carbonic anhydrase 8 (Car8)<sup>48</sup>, VGluT1, 2 (ref. 49) and commercial antibodies for GluR1, 2 (Alomone Labs, AGC-004, 1:200, AGC-005, 1:150), GLAST (Frontier Institute, Rb-Af660, 1:2,000), PSD-95 (Cell Signaling, 3450, 1:1,000), CDC42 (Santa Cruz, L0809, 1:100),  $\beta$ -actin (Sigma, A5441, 1:5,000) and  $\alpha$ -Tubulin (Sigma, T9026, 1:10,000). For secondary antibodies, we used Alexa 488-, Cy3- or horseradish peroxidase-conjugated IgGs from rabbit, goat or guinea pig (Jackson ImmunoResearch, 706-545-148, 711-165-152, 111-035-003) diluted at 1:200–1,000. Histidine-tagged recombinant CDC42 (Cytoskeleton) was used to quantify endogenous CDC42.

**Biochemical fractionation and IB analysis**. Each tissue was dissected, weighed and homogenized by sonication in  $3 \text{ ml g}^{-1}$  of buffer A (10 mM Tris-HCl at pH 7.6, 0.15 M NaCl, 1% Triton X-100, protease inhibitors). The supernatant after centrifugation at 20,400g at 4 °C for 0.5 h was labelled as soluble fraction. The pellet was dissolved with sonication in 1 ml g<sup>-1</sup> buffer B (3% SDS, 5% 2-mercaptoethanol), which was termed pellet fraction. The samples were resolved by 10%PAGE (SDS-PAGE), transferred to reinforced nitrocellulose or PVDF membranes and subjected to IB analysis using a blocking buffer containing 5% skim milk in TBST (0.1 M Tris-HCl at pH 7.4, 0.15 M NaCl, 0.05% Tween 20). Chemiluminescence detection and densitometry were performed with horseradish peroxidase-conjugated secondary antibodies, ECL-Plus reagent (PerkinElmer), TrueBlot (Rockland) and an image analyser LAS-3000mini with MultiGauge software (Fuji). Uncropped version of each image is shown in Supplementary Fig. 15.

**Immunoprecipitation.** For IP/IB experiments, whole cerebella were lysed with buffer A' (0.2 M Tris-HCl at pH 7.35, 0.3 M NaCl, 0.2% SDS, 0.2% Triton X-100, 0.2% sodium deoxycholate, 20 mM 2-mercaptoethanol and protease inhibitors), centrifuged and the supernatant was mixed with protein A Sepharose CL-4B (GE Healthcare) or Affi-Prep Protein A Support (Bio-Rad) that had been pre-incubated with anti-CDC42EP4, anti-SEPT4 or nonimmune rabbit IgG (as a negative control). After washing the beads with the same buffer, bound proteins were lysed for IB. For IP/MS experiments, we used buffer C (20 mM Tris-HCl at pH 8.0, 0.15 M NaCl, 1 mM EDTA, 1% NP-40 and protease inhibitors) with more stringent wash condition<sup>50</sup>.

**Mass spectrometry and peptide mass fingerprinting.** After IP, bead-bound proteins were eluted with solution (7 M guanidine, 50 mM Tris-HCl at pH 8.0), reduced with 5 mM dithiothreitol for 0.5 h, alkylated with 10 mM iodoacetamide for 1 h in the dark, demineralized and concentrated by methanol/chloroform precipitation and digested (0.5 µg trypsin in 1.2 M urea and 50 mM Tris-HCl at pH 8.5).

Mass spectrometric analysis (LC-MS/MS) was carried out as previously described<sup>50</sup>. Briefly, we used an LTQ Orbitrap XL mass spectrometer (Thermo Scientific) connected to an HTC-PAL autosampler and a Paradigm MS4 HPLC with a C18 reversed-phase column and ADVANCE Plug and Play Nano Source (Michrom Bioresources). The reversed-phase chromatography condition was a linear gradient (0 min, 5% B; 70 min, 100% B) of solvent A (2% CH<sub>3</sub>CN with 0.1% trifluoroacetic acid (TFA)) and solvent B (98% CH<sub>3</sub>CN with 0.1% TFA) at a flow rate of 500 nl min<sup>-1</sup>. The mass spectrometer was operated in the data-dependent MS2 mode. Peak lists were generated and calibrated by using the Mascot software (Matrix Science), and validation of the MS/MS-based peptide and protein identifications was made with the Scaffold programme (ver. 3, Proteome Software). The peptide probability by Mascot was used for the Scaffold Local FDR algorithm. Peptide identification was approved by the presence of at least two peptides that exceeded the probability of 95%. Proteins that contained similar peptides and could not be differentiated by MS/MS data alone were grouped to satisfy the principle of parsimony.

**Measurement of neurotransmitters and metabolites.** We used previously described methods for the extraction and measurement of amino acids, amines and metabolites<sup>51–54</sup>. Briefly, cerebellar hemispheres dissected on ice were frozen in liquid N<sub>2</sub> and stored at - 80 °C until extraction. For measurement of amino acids, each sample was weighed, homogenized in 1.5 ml CH<sub>3</sub>OH on ice, centrifuged at 3,000g for 6 min at 4° C and 20 µl of the supernatant was dried by evaporation at 40 °C. The residue was dissolved with 20 µl water, 20 µl 0.1 M borate buffer at pH 8.0 and 60 µl 50 mM 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F; Tokyo Kasei Kogyo) in CH<sub>3</sub>CN, reacted at 60 °C for 2 min and stopped by adding 100 µl 0.1% TFA and 10% CH<sub>3</sub>CN in water. Total D-/L-serine levels were measured using a column-switching HPLC system (SCL-10A VP, Shimadzu)<sup>51</sup>. Glutamate, glutamine, glycine and GABA were measured using an HPLC system (LC-20AT, Shimadzu)<sup>52</sup>. Fluorescence emission at 530 nm was measured with an excitation at 470 nm. For monoamines and their metabolites, tissue samples were homogenized in 0.2 M HClO<sub>4</sub> containing 0.1 mM EDTA and 100 µg1<sup>-1</sup> isoproterenol (internal standard), and centrifuged at 20,000g for 15 min at 4°C. Supernatants were filtered

through 0.45-µm pore membranes (Millex-LH, Millipore). We used a reversed-phase column (SC-5ODS) and an electrochemical detector (ECD-300) in an HPLC system (EP-300, DG-300 and EPC-300, all from Eicom) with the mobile phase containing 0.1 M acetate–citric acid buffer at pH 3.5, 16% CH<sub>3</sub>OH, 5 mgl<sup>-1</sup> EDTA and 0.19 gl<sup>-1</sup> sodium octyl sulfate.

**Tissue preparation for histochemistry.** Under deep pentobarbital anaesthesia (0.1 mg per g of body weight), mice were fixed transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer (PB) at pH 7.2 for IF and immunoelectron microscopy, or with 4% paraformaldehyde/0.1% glutaraldehyde in PB for TEM. After excision from the skull, brains for immunohistochemistry were immersed in the same fixative for 2 h. In IF for CDC42EP4, fixed brains were sliced at 50  $\mu$ m with a microslicer (VT1000S, Leica) and subjected to free-floating incubation, while 4- $\mu$ m-thick paraffin sections with a sliding microtome (SM1000R, Leica) were used for Car8, GLAST, VGluT1 and VGluT2. Brains for FISH were removed under deep pentobarbital anaesthesia and immediately frozen in powdered dry ice, cut at 20  $\mu$ m with the cryostat and mounted on silane-coated glass slides.

**FISH.** Mouse cDNA fragments for *Cdc42ep4* (nucleotide 331–1,118, GenBank BC003857), calbindin (37–1,071, BC016421), GLAST (1,620–2,576, BC066154) and Phgdh (1–1,799, BC110673) were subcloned into the pBluescript II plasmid vector. Digoxigenin (DIG)- or fluorescein-labelled cRNA probes were transcribed *in vitro*<sup>55</sup>.

Sections were processed as follows: acetylation with 0.25% acetic anhydride in 0.1 M triethanolamine-HCl at pH 8.0 for 10 min and prehybridization for 1 h in buffer A (50% formamide, 50 mM Tris-HCl at pH 7.5, 0.6 M NaCl, 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin, 200 mg l<sup>-1</sup> tRNA, 1 mM EDTA and 10% dextran sulfate). Hybridization with cRNA probes was performed in buffer A at 63.5 °C for 12 h, and then sections were washed at 61 °C with 5 × standard sodium citrate (SSC) for 0.5 h, 4 × SSC containing 50% formamide for 0.7 h, 2 × SSC containing 50% formamide for 0.7 h and 0.1 × SSC for 0.5 h. Sections were incubated successively in NTE buffer (0.5 M NaCl, 0.01 M Tris-HCl at pH 7.5, 0.15 M NaCl), each for 0.3 h at room temperature.

For double-labelling FISH, sections were reacted with peroxidase-conjugated anti-fluorescein antibody (Invitrogen, A21253, 1:1,500) and incubated with the FITC-TSA plus amplification kit (PerkinElmer). After inactivation of residual peroxidase activities with 3%  $H_2O_2$ , the sections were incubated successively with DIG-labelled cRNA probe, the peroxidase-conjugated anti-DIG IgG (Roche, 11 207 733 910, 1:1,000) and the Cy3-TSA Plus reagents (PerkinElmer), and then counterstained with TOTO-3 (Invitrogen, T3604, final 0.2  $\mu$ M) in PBS.

**Immunohistochemistry.** For immunohistochemistry, 4-µm-thick paraffin sections were incubated with 10% normal donkey serum for 20 min, a mixture of primary antibodies  $(1 \text{ mgl}^{-1})$  overnight and a mixture of Alexa 488- or Cy3-labelled species-specific secondary antibodies for 2 h at a dilution of 1:200. Fluorescence imaging was conducted using scanning laser confocal microscopes IX81/FV1000 (Olympus) with a × 40 objective lens (numerical aperture (NA) 1.0) and LSM-780 (Zeiss) with × 40 (NA 1.1) and × 63 (NA 1.4) objective lenses, and wide-field microscopes (BZ-9000, Keyence or BX-60, Olympus) with × 40 and × 20 objective lenses (NA 1.3, Nikon or NA 0.7, Olympus).

**Electron microscopy**. Conventional electron microscopy, pre-embedding silverenhanced immunoelectron microscopy and postembedding immunogold electron microscopy were conducted as described previously<sup>56,57</sup>. Briefly, pre-embedding immunoelectron microscopy was conducted by incubation in blocking solution for goat gold conjugates (Aurion, Electron Microscopy Sciences) for 0.5 h, and then with CDC42EP4 antibody ( $1 \text{ mg} 1^{-1}$ ) diluted with 1% BSA and 0.004% saponin in PBS overnight. Anti-rabbit IgG linked to 1.4 nm gold particles (1:100, Nanogold labelling reagents, Nanoprobes, #2003) were incubated for 2 h, and gold particles were intensified using a silver enhancement kit (R-Gent SE-EM, Aurion). In postembedding immunogold, 80-nm-thick sections were cut with an ultramicrotome (Ultracut, Leica), incubated overnight with rabbit GLAST antibody ( $20 \text{ mg} 1^{-1}$ ) and then with colloidal gold (10 nm)-conjugated anti-rabbit or anti-guinea pig IgG (British Biocell, EM.GAR10, 1:100) for 2 h. The samples were mounted on grids and stained with 2% uranyl acetate for 20 min, and observed with a TEM (H-7100, Hitachi).

**Electrophysiology.** We prepared 250-µm-thick cerebellar slices as described previously<sup>58,59</sup>. In brief, 5–7-week-old mice deeply anaesthetized with CO<sub>2</sub> were decapitated. The cerebella were quickly cut parasagittally with a microslicer (VT1200S, Leica) in a chilled cutting solution ((in mM) 120 choline-Cl, 3 KCl, 1.25 NaHPO<sub>4</sub>, 28 NaHCO<sub>3</sub>, 8 MgCl<sub>2</sub> and 25 glucose), incubated in normal ACSF (artificial cerebrospinal fluid; 125 NaCl, 2.5 KCl, 2 CaCl<sub>2</sub>, 1 MgSO<sub>4</sub>, 1.25 NaH<sub>2</sub>PO<sub>4</sub>, 26 NaHCO<sub>3</sub> and 20 glucose, bubbled with 95% O<sub>2</sub> and 5% CO<sub>2</sub>) at 36 °C for 0.5 h and then kept at room temperature. All recordings were performed at 32–34 °C. Whole-cell recordings were made from visually identified PCs under an upright microscope (BX51WI, Olympus). Pipette solution contained (55 CsCl, 10 Cs D-gluconate, 20 TEA-Cl, 20 BAPTA, 4 MgCl<sub>2</sub>, 4 ATP, 0.4 GTP, 5 QX314-Cl and

30 HEPES at pH 7.3, adjusted with CsOH). To block inhibitory synaptic transmission, 10  $\mu$ M bicuculline was consistently supplemented to the normal ACSF. All the drugs were obtained from Tocris Bioscience. Stimulation pipettes (~5  $\mu$ m tip diameter) filled with the normal ACSF were used to apply square pulses for focal stimulation (duration, 0.1 ms; amplitude, 0–90 V). PFs were stimulated in the molecular layer. CFs were stimulated in the granular layer around the PC soma under investigation. The stimulation was given at 0.2 Hz (Fig. 6a,b) or 0.05 Hz (Fig. 6c–e). The number of CFs innervating the recorded PC was estimated by the number of discrete CF-EPSC steps in response to a gradual rise in the stimulus intensity. The stimulation pipette was systematically moved around each PC soma. Ionic currents were recorded with a patch clamp amplifier (EPC10, HEKA Elektronik). The pipette access resistance was compensated by 70–80%. Online data acquisition and offline data analysis were conducted with software, PATCHMASTER and FITMASTER (HEKA).

Generation of Cdc42ep4-floxed and null mice. The targeting strategy is illustrated in Fig. 2a. We constructed the targeting vector using the MultiSite Gateway Three-Fragment Vector Construction kit (Invitrogen) and BAC Subcloning Kit (Gene Bridges) with some modifications. Briefly, a DNA fragment carrying the loxP sequence and pgk-promoter-driven neomycin-resistance (Neo) cassette flanked by two Flp recognition target sites was inserted in reverse orientation directly downstream of the sole coding exon of Cdc42ep4 isolated from a C57BL/6 mouse genomic BAC clone RP23-366J17. The other loxP site was inserted into the intron directly upstream of the coding exon. The resulting targeting vector, which consisted of 5'- and 3'-homology arms, the floxed coding region with the Neo cassette and diphtheria toxin gene (DT) for negative selection, was linearized and electroporated into the C57BL/6N mouse embryonic stem (ES) cell line, RENKA<sup>60</sup>. Genomic DNA from neomycin/G418-resistant ES cell clones were analysed with Southern blot analysis, and positive clones with proper homologous recombination were microinjected to host embryos as described previously<sup>61</sup>. The null allele  $(Cdc42ep4^{-})$  was created by crossing heterozygous floxed  $(Cdc42ep4^{fl/+})$  mice with CAG-Cre transgenic mice<sup>62</sup>. For Southern blot analysis, genomic DNA was digested with Sac I, EcoR V and Hinc II, and hybridized with 5', 3' and neo probes, respectively. The predicted fragment sizes for the WT and floxed alleles for each probe, 13/11.3, 16.4/12.7 and N.A./16.1 (kb), respectively, were confirmed. We thereafter conducted genotyping using PCR with the following primers: (a) 5'-TGCTTCAGTACCTTCGGAC-3', (b) 5'-TTCGAGTTCACAGAGCTGGA-3' and (c) 5'-TCATAGAGAAGGTGGCAGC-3' to distinguish the WT, floxed and null alleles by 490-bp (with primers a + b), 580-bp (a + b) and 480-bp (a + c) products, respectively. After confirming germline transmission and Mendelian inheritance of the  $Cdc42ep4^{fl}$  and  $Cdc42ep4^{-}$  alleles, the lines have been deposited to RIKEN Bioresource Center (RBRC04894 and RBRC09539, respectively).

**Balance beam and rota-rod tests.** All systematic behavioural tests were conducted on male littermates (10–38 weeks old) as described previously<sup>33,63</sup>. In the balance beam test, the motor coordination was quantitatively assessed by the performance on the first trial, the moving speed and the numbers of pauses and slips along a 1-m-long rod (diameter, 28 mm). After six trials on Days 1–3, five trials with a thinner rod (diameter, 11 mm) were conducted on Days 3–4. The increment in the moving speed and decrements in the numbers of pauses and slips were quantified as indices of motor learning. In the rota-rod test, we measured the latency to fall from a rotating rod (diameter, 30 mm) that was accelerated from 4 to 40 r.p.m. over 5 min per trial. For pharmacological experiment (see below), each mouse was subjected to four sessions (four trials per session) at 24 h before injection, and 4, 6 and 24 h after injection.

**Direct cerebellar cortical injection.** We applied a method established for viral delivery<sup>64</sup> with minor modifications. Briefly, 6-week-old mice (WT, 4F + 1M; KO, 3F + 1M) anaesthetized with ketamine (100 mg kg<sup>-1</sup>) and xylazine (16 mg kg<sup>-1</sup>) were mounted in a stereotactic frame, and the occipital bone was drilled at 5 mm caudal to the bregma. A blunt-ended Hamilton syringe tip (33 G) attached to a micropump and a controller (UltramicroPump II, Micro4; World Precision Instrument) was placed beneath the pia mater above lobule VI. The mixture of CTZ and <sub>DL</sub>-threo-β-benzyloxyaspartic acid (DL-TBOA; 100 µM each in 10 µl) was injected at a rate of 333 nl min<sup>-1</sup>. After suturing the scalp, and recovery from anaesthesia on a warm pad, the mice were returned to home cages.

**Systematic behavioural analysis.** We applied our standard protocols<sup>63,65,66</sup> to a cohort of male littermates (n = 13, 13) reared in the same cages: behavioural testing was conducted between 9 a.m. and 6 p.m. except for the continuous home cage monitoring. Each apparatus was cleaned with sodium hypochlorite solution to minimize odour after use. We conducted tests in the following order: general health and neurological screening (including body weight and temperature measurements, grip strength test, righting test, whisker touch test and ear twitch reflexes, wire hang test), light/dark transition test, open field test, elevated plus maze test, one-chamber social interaction test, rota-rod test, three-chamber socialility and preference for social novelty test, prepulse inhibition test of acoustic startle response, Porsolt forced swim test, Barnes maze test, tail suspension test and long-term monitoring of locomotion and social interaction in home cage. Intervals between tests were >24 h.

**Neuromuscular strength tests.** Neuromuscular strength was assessed with the forelimb grip strength test and wire hang test. Forelimb grip strength was measured by pulling a mouse in the tail while its forepaws hung on to a wire grid attached to a spring balance. The tensile force (N) when the mouse released the grid was measured three times, and the greatest value was analysed. In the wire hang test, a wire mesh with a mouse on top was slowly inverted and the latency to fall was measured.

**Light/dark transition test.** The apparatus had a pair of differentially illuminated (390 versus 2 lux) chambers  $(21 \times 41 \times 25 \text{ cm})$  connected with a door in the middle. Each mouse was released in the dark chamber, and image data were acquired from the top with a CCD (charge-coupled device) camera for 10 min. The latency until the first entry into the light chamber, the time spent in each chamber, the number of transitions and the total distance travelled were automatically measured using ImageLD software (see Image analysis).

**Open field test.** Voluntary locomotor activity was measured in an open field test. Each mouse was placed in the centre of the open field apparatus ( $40 \times 40 \times 30$  cm; Accuscan Instruments) illuminated at 100 lux. The following indices were monitored for 120 min: total distance travelled, time spent in the centre area of  $20 \times 20$  cm, number of rearing and beam-breaks were automatically measured by counting interruptions of infrared beams.

**Elevated plus maze test.** The apparatus had two open arms  $(25 \times 5 \text{ cm}, \text{ with } 3\text{-mm-high plastic ledges) and two closed arms <math>(25 \times 5 \text{ cm}, \text{ with } 15\text{-cm-high transparent walls) interconnected via a central crossing <math>(5 \times 5 \text{ cm})$ , which was set at 55 cm height and illuminated at 100 lux. The numbers of entries into, and the time spent in the open and enclosed arms, were recorded for 10 min. Image data were acquired from the top with a CCD camera, and the number of entries into and the time spent in the open/closed arms, and total distance travelled, were measured automatically using the ImageEP software (see Image analysis).

Acoustic startle response and prepulse inhibition test. A mouse restrained in a cylinder was placed in the chamber of a startle reflex measurement system (O'Hara & Co.) with 70-dB background white noise. After 10 min, the mouse's startle response to a startle stimulus (110 or 120 dB white noise for 40 ms) was measured by a motion sensor for 140 ms. A test session was a random sequence of four trials each with a prepulse stimulus (74 or 78 dB white noise for 20 ms that preceded the startle stimulus 100 ms) and two without. Six blocks of six trials were presented in a pseudorandom order with the average intertrial interval of 15 s.

**Porsolt forced swim test.** Each mouse was released in 7.5-cm-deep water at 23  $^{\circ}$ C in an acrylic cylinder (10 cm in diameter), and the duration of the motion for evacuation was measured up to 10 min automatically using the ImageTS/PS software (see Image analysis).

Social interaction and voluntary activity in the home cage. The position of each mouse housed alone in a cage was monitored from the top continuously for a week. The distance travelled along the diurnal cycle was measured automatically using the ImageHA software (see Image analysis). Two mice of the same genotype that had been separately reared were housed together in a home cage and their two-dimensional (2D) images from the top were captured at 1 fps for a week. Their physical contact and separation were represented, respectively, as one and two particles, and their locomotor activity was quantified by the differentials of pixels between successive frames by using the ImageHA software (see Image analysis).

**One-chamber social interaction test.** The positions of two mice placed in a novel chamber ( $40 \times 40 \times 30$  cm) were monitored from the top at three frame s<sup>-1</sup>. Their horizontal distance travelled and the number of contacts were measured automatically using the ImageSI software (see Image analysis).

**Three-chamber test for sociability and social novelty.** The apparatus for Crawley's test had three chambers ( $20 \times 40 \times 22$  cm) separated by two transparent partitions each with an opening ( $5 \times 3$  cm) and a lid with an infrared CCD camera. A male mouse (9-week old, C57BL/6J, termed Stranger 1) that had no prior contact with the subject mice was enclosed in a cylinder cage (9 cm in diameter, set in the left chamber) that allowed nose contacts. Each subject mouse was released in the middle chamber and allowed to explore for 10 min, while the time spent in each chamber and within 5 cm from each cage was measured automatically using the ImageCSI software (see Image analysis). Subsequently, another unfamiliar mouse (Stranger 2) was placed in another cylinder cage (in the right chamber) and monitored likewise for another 10 min.

**Rota-rod test.** For the initial screening, each mouse was subjected to nine trials over 3 days. See Balance beam and rota-rod tests for details.

**Tail suspension test.** The movement of each mouse suspended by the tail at a height of 30 cm was recorded for 10 min and analysed by using the ImageTS/PS software (see Image analysis).

**Barnes maze test.** The apparatus consisted of a blight (800 lux), white circular platform (diameter, 1 m) with 12 holes along the perimeter, 0.75 m above the floor, and a dark escape box (containing cage bedding) set under one of the holes ('target'). Following habituation sessions on the day before, each mouse was consistently trained to learn a randomly assigned target position (1–3 trials per day  $\times$  6 days). The platform was turned 90° per day to minimize the influence from local cues. The distance travelled, time spent and number of errors from the centre to the target hole were measured from time-lapse images with a video-tracking software, ImageBM. One day (24 h) after the last training session, the first probe test was carried out without escape box to exclude local cue-dependent navigation, when mice were allowed to explore for 3 min and the time spent around each hole was measured. The probe test was followed by a training session with the target. To assess long-term spatial memory retention, the mice were again subjected to the probe test 1 month later.

**Image analysis.** The application programmes for behavioural data acquisition and analysis (ImageLD, EP, SI, CSI, TS/PS, BM, HA) were created on the ImageJ (http://rsb.info.nih.gov/ij/). ImageLD and EP are freely available from http:// www.mouse-phenotype.org/software.html.

**Statistical analysis.** Quantitative data are represented as mean  $\pm$  s.e.m. Prism 4.0 (GraphPad Software), Stat View (SAS Institute) or Sigma Plot (SYSTAT), Excel (Microsoft) were used for statistical analyses. Behavioural data were analysed by analysis of variance (ANOVA; one-way and two-way repeated measures), unless otherwise noted. For the other data, *t*-test, Mann–Whitney *U*-test, Wilcoxon signed-rank test (two rightmost plots in Fig. 6e) and two-way (Fig. 6c) or two-way repeated measures ANOVA (Fig. 6e) followed by Tukey's *post hoc* comparisons were applied. For behavioural tests, either one-way ANOVA or two-way repeated measures ANOVA was applied for statistical analyses unless otherwise noted.

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#### **Author contributions**

M.Y., M.A. and K.S. established the *Cdc42ep4*<sup>th</sup> line; N.A.-I., K.Ko. and M.W. conducted the biochemical and morphological analyses; H.N. and Ko.H. conducted electrophysiological analyses, Ke.H. supervised transmitter measurement; T.N. and K.Ka. conducted mass spectrometric analysis, K.T. provided reagents and critical suggestions for GLAST experiments; S.H. and T.M. supervised the behavioural tests; F.H. and H.H. designed and conducted drug injection surgery; and M.K. designed the study and wrote the manuscript with W.M. and Ko.H. All authors read and approved the final manuscript.

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## Spinal mechanisms underlying potentiation of hindpaw responses observed after transient hindpaw ischemia in mice

Tatsunori Watanabe<sup>1,2</sup>, Mika Sasaki<sup>2</sup>, Seiji Komagata<sup>1</sup>, Hiroaki Tsukano<sup>1</sup>, Ryuichi Hishida<sup>1</sup>, Tatsuro Kohno<sup>2</sup>, Hiroshi Baba<sup>2</sup> & Katsuei Shibuki<sup>1</sup>

Transient ischemia produces postischemic tingling sensation. Ischemia also produces nerve conduction block that may modulate spinal neural circuits. In the present study, reduced mechanical thresholds for hindpaw-withdrawal reflex were found in mice after transient hindpaw ischemia, which was produced by a high pressure applied around the hindpaw for 30 min. The reduction in the threshold was blocked by spinal application of LY354740, a specific agonist of group II metabotropic glutamate receptors. Neural activities in the spinal cord and the primary somatosensory cortex (S1) were investigated using activity-dependent changes in endogenous fluorescence derived from mitochondrial flavoproteins. Ischemic treatment induced potentiation of the ipsilateral spinal and contralateral S1 responses to hindpaw stimulation. Both types of potentiation were blocked by spinal application of LY354740. The contralateral S1 responses, abolished by lesioning the ipsilateral dorsal column, reappeared after ischemic treatment, indicating that postischemic tingling sensation reflects a sensory modality shift from tactile sensation to nociception in the spinal cord. Changes in neural responses were investigated during ischemic treatment in the contralateral spinal cord and the ipsilateral S1. Potentiation already appeared during ischemic treatment for 30 min. The present findings suggest that the postischemic potentiation shares spinal mechanisms, at least in part, with neuropathic pain.

The mechanism of postischemic tingling sensation has been attributed to enhanced excitability of peripheral nerves during recovery from ischemic conduction  $block^{1,2}$ . However, nerve conduction block is known to quickly induce plasticity in the central nervous system in experimental animals<sup>3</sup> and humans<sup>4-6</sup>. Cessation of low frequency spontaneous firing of A $\beta$  tactile afferents after partial denervation produces potentiation of neural responses in the primary somatosensory cortex (S1) elicited via the remaining nerves<sup>7</sup>. Reduction in the mechanical thresholds for paw-withdrawal reflex is observed at the same time<sup>7</sup>. This reduction in mechanical thresholds can be inhibited by spinal application of LY354740<sup>7</sup>, a specific antagonist of group II metabotropic glutamate receptors (mGluRs)<sup>8</sup>. If similar spinal mechanisms are also responsible for postischemic tingling sensation, this should be abolished by spinal application of LY354740. In the present study, we reproduced postischemic mechanical allodynia in mice, and tested roles of spinal mechanisms using this model.

Neuropathic pain is produced by nerve injury in animal models<sup>9–11</sup>, and the resulting pain is usually evaluated using behavioral tests more than 24h after the injury to allow the recovery of animals from surgical injury. Therefore, the findings obtained from behavioral tests are affected by complex cascades

<sup>1</sup>Department of Neurophysiology, Brain Research Institute, Niigata University,1-757 Asahi-machi, Chuo-ku, Niigata 951-8585, Japan. <sup>2</sup>Department of Anesthesiology, School of Medicine, Niigata University, 1-757 Asahi-machi, Chuo-ku, Niigata 951-8510, Japan. Correspondence and requests for materials should be addressed to K.S. (email: shibuki@bri.niigata-u.ac.jp)





of inflammation and gene expression during the recovery period<sup>12-14</sup>. However, postischemic mechanical allodynia is induced very quickly after transient hindpaw ischemia. Thus, if neural changes reflecting postischemic tingling sensation are found, these changes may be useful for investigating the neural mechanisms that trigger neuropathic pain. Activity-dependent flavoprotein fluorescence signals reflect aerobic energy metabolism in mitochondria<sup>15</sup>, and are useful for investigating fine neural activities and plasticity<sup>16-18</sup>. Flavoprotein signals are resistant to and spontaneously recover from photobleaching<sup>19</sup>, and quantitatively reflect neural activities<sup>20,21</sup>. Spinal activities elicited by peripheral stimulation have been visualized using flavoprotein fluorescence signals<sup>22</sup>. An initial phase of neuropathic pain after partial denervation is observed as long-lasting potentiation of flavoprotein fluorescence signals in S1<sup>7</sup>. Furthermore, a modality shift from tactile sensation to nociception can be visualized as reappearance of S1 responses to tactile stimulation in mice with ipsilateral dorsal column lesioning<sup>7</sup>. It is because the tactile sensation is mediated via the ipsilateral dorsal column to the contralateral S1<sup>23</sup>, while nociception is mediated via the contralateral spinothalamic tract<sup>24</sup>. In the present study, we confirmed that such a modality shift from tactile sensation to nociception was produced within 30 min after ischemic treatment.

#### Results

**Reduced mechanical thresholds for paw-withdrawal reflex after ischemic treatment.** Because unpleasant postischemic tingling sensation is exacerbated by external mechanical forces, we measured the thresholds for hindpaw-withdrawal reflex using von Frey filaments<sup>25</sup>. The thresholds before hindpaw ischemia were  $0.57 \pm 0.10$  g (mean  $\pm$  SEM, n = 6). Mice were transiently anesthetized with 1% isoflurane, and a pressure of 250 mmHg was applied for 30 min to a rubber cuff set around the thigh (Fig. 1a). At 30 min after the ischemic treatment was finished, the mechanical thresholds were significantly reduced compared with the corresponding data in sham-treated mice with no pressure application (P < 0.01, Fig. 1b). The thresholds reached the minimal values of  $0.07 \pm 0.02$  g (n = 6) at 2 h after the ischemic treatment (Fig. 1b), and were clearly and significantly smaller than the corresponding values in sham-treated mice (P < 0.005). Isoflurane anesthesia alone produced no reduction in the threshold in sham-treated mice (Fig. 1b).

Next, we tested whether the reduction in the thresholds was sensitive to spinal application of 10 nM LY354740 (Fig. 1c), which can block the initial phase of neuropathic pain after partial denervation<sup>7</sup>.



**Figure 2.** Potentiation of the spinal responses after hindpaw ischemia. (a) Example of ipsilateral spinal responses elicited by vibratory stimulation applied to the left hindpaw at T13 and L1 level (upper panels). The left-most panels are original fluorescence images, and others are pseudocolor images of response magnitudes in  $\Delta F/F_0$  recorded at the time before and after the stimulus onset shown on each image. Another example of ipsilateral spinal responses elicited by vibratory stimulation applied to the left hindpaw at T12 and T13 level (lower panels). (b) Example of spinal responses recorded in the same mouse before, during, and 30–120 min after ischemic treatment applied to the left thigh for 30 min. The response amplitudes were measured in the square window of  $100 \times 25$  pixels shown in the second panel. (c) Relative amplitudes of spinal responses during and after ischemic treatment. The amplitudes were normalized by those recorded before hindpaw ischemia. (d) Example of spinal responses recorded before and at 60 min after sham treatment. (e) Example of spinal responses recorded before and at 60 min after sham treatment. (e) Example of spinal responses recorded before and at 60 min after sham treatment application of 10 nM LY354740. (f) Comparison of the normalized response amplitudes in mice at 60 min after hindpaw ischemia, sham treatment, or hindpaw ischemia with spinal application of 10 nM LY354740.

When the ischemic treatment was applied to the mice that received spinal application of LY354740, the thresholds at 2h after hindpaw ischemia were  $0.63 \pm 0.12$  g (n = 6). In contrast, the thresholds were  $0.12 \pm 0.06$  g (n = 6) in mice that received spinal application of saline alone (Fig. 1c). The difference at 2h after hindpaw ischemia or sham-treatment was statistically significant (P < 0.01). We confirmed that spinal application of LY354740 alone had no apparent effect on the mechanical thresholds for paw-withdrawal reflex (Fig. 1d). Interestingly, a slight but significant reduction in the threshold was observed in the right hindpaw (Supplementary Fig. S1), suggesting that transient hindpaw ischemia might also have some effects on the contralateral spinal cord.

**Flavoprotein fluorescence responses in the spinal cord.** Because the behavioral test data suggested the presence of some spinal plasticity, we recorded ipsilateral spinal responses elicited by vibratory hindpaw stimulation using flavoprotein fluorescence imaging. Hindpaw stimulation produced an increase in fluorescence on the dorsal surface of the ipsilateral spinal cord corresponding to the dorsal horn at the T13 and L1 level (Fig. 2a). Because minimal response was found at T12, the fluorescence changes were attributed mainly to localized activities in the dorsal horn but not to ascending afferent activities mediated via the dorsal column. The responses started at 0.2 s after the onset of the stimulation,

and peaked at approximately 1% in  $\Delta$ F/F<sub>0</sub> at 0.6–0.8 s after the stimulus onset. Although these properties were similar to those recorded in cortical areas<sup>7,26</sup>, the decay was slower than that of the cortical responses, as the hemodynamic responses were not marked in the spinal cord (Supplementary Figs S2).

The spinal responses to vibratory hindpaw stimulation were abolished by a pressure of 250 mmHg applied to the thigh, indicating that ischemic conduction block was successfully produced at 250 mmHg (Fig. 2b). The responses reappeared within 30 min after hindpaw ischemia, and the magnitude of the responses estimated at 30 min after hindpaw ischemia was slightly but significantly potentiated (P < 0.03, Fig. 2b,c). At 1 h after hindpaw ischemia, the response amplitudes reached maximal values, and were potentiated to  $138 \pm 11\%$  (n = 7) compared with those before hindpaw ischemia (Fig. 2c). No clear potentiation was observed in sham-treated mice, and the difference between the two groups was statistically significant (P < 0.002, Fig. 2d,f).

Next, we applied 10 nM LY354740 on the surface of the spinal cord during imaging experiments. Application of LY354740 had no immediate effect on the spinal responses to vibratory hindpaw stimulation (Fig. 2e). However, the spinal responses were not clearly potentiated after hindpaw ischemia, and the relative amplitudes at 1 h after hindpaw ischemia were significantly smaller than those in mice with no LY354740 (P < 0.003, Fig. 2e,f). These data suggest that transient hindpaw ischemia produced spinal potentiation, and the reduction in mechanical thresholds for paw-withdrawal reflex, likely via similar spinal mechanisms sensitive to LY354740.

The spinal responses occasionally appeared in separated segments (for example, Fig. 2a), which might reflect the presence of functional units. Therefore, we tested the fine somatotopic maps in the spinal responses elicited by vibratory stimulation applied to each toe. The spinal responses to stimulation of each toe appeared in small areas that were different each other (Supplementary Fig. S2). The responsive areas were arranged almost linearly (Supplementary Figs S2). These results were compatible with a previous morphological study on spinal distribution of afferent fibers from each digit<sup>27</sup>. Therefore, the apparent segments in the spinal responses likely reflected strong stimulation of the skin convexes by the brush used for hindpaw stimulation.

Flavoprotein fluorescence responses in S1. Flavoprotein fluorescence responses elicited by hindpaw stimulation appeared in the contralateral S1 (Fig. 3a). The initial time course of S1 responses elicited by hindpaw stimulation was similar to that of spinal responses, while hemodynamic responses obscured the later phase of the responses (Supplementary Figs S3). Although neural responses in S1 are arranged according to somatotopic maps over a large scale<sup>26,28,29</sup>, the responses elicited by stimulation of each toe were not clearly separated, and no fine somatotopic map was found (Supplementary Figs S3). When a pressure of 250 mmHg was applied to the thigh, S1 responses were almost completely abolished (Fig. 3b). However, the responses reappeared and were potentiated after hindpaw ischemia (Fig. 3b,c). The potentiation was maintained up to 3h after hindpaw ischemia. The relative amplitudes at 1h after hindpaw ischemia (165  $\pm$  18%, n = 13) were significantly larger than those in sham-treated mice (99  $\pm$  4%, n = 8, P < 0.003, Fig. 3d,g). Potentiation of S1 responses after ischemic treatment was almost completely abolished by spinal application of 10 nM LY354740 (Fig. 3e,f). The relative amplitudes of S1 responses in mice with spinal application with LY354740 were  $105 \pm 6\%$  (n = 9) at 1 h after hindpaw ischemia, and were significantly smaller than those in mice with spinal application of saline alone ( $150 \pm 16\%$ , n = 8, P < 0.007, Fig. 3g). Therefore, the suppression of S1 potentiation is likely attributable to the pharmacological effects of LY354740, but not to non-specific spinal injury caused by the drug application.

**Modality shift from tactile sensation to nociception induced by hindpaw ischemia.** Potentiation in S1 after ischemic treatment may be produced by a modality shift from tactile sensation to nociception, as observed after partial denervation<sup>7</sup>. When we disrupted the ipsilateral dorsal column at the T11 level (Fig. 4a), S1 responses elicited by hindpaw stimulation were abolished (Fig. 4b). However, S1 responses reappeared at 30 and 60 min after ischemic treatment applied to the hindpaw (Fig. 4b). As no such response was observed in sham-treated mice (Fig. 4c), the reappearing S1 responses cannot be attributed to spontaneous recovery from the injury caused by dorsal column lesioning. The difference in S1 responses in the operated mice was statistically significant at 30 and 60 min after hindpaw ischemia or sham treatment (P < 0.01 for both, Fig. 4d). The reappearing S1 responses suggest that the modality shift from tactile sensation to nociception was induced within 30 min after ischemic treatment.

Potentiation in the contralateral spinal cord and the ipsilateral S1 during hindpaw ischemia. Changes in neural responses were not observable during ischemic treatment in the ipsilateral spinal cord or in the contralateral S1 because of conduction block of the peripheral nerves. However, we found that the spinal responses elicited by vibratory stimulation applied to the hindpaw contralateral to the ischemic treatment were significantly potentiated during ischemic treatment (Fig. 5a,b). The potentiation was maintained for at least 60 min after hindpaw ischemia. Cortical responses in S1 ipsilateral to the ischemic treatment were similarly potentiated during and after hindpaw ischemia (Fig. 5c,d), indicating that spinal and cortical potentiation had already been initiated during ischemic treatment. Comparison of the potentiation at 60 min after hindpaw ischemia in the spinal cord (ipsilateral:  $138 \pm 11\%$ , n = 7; contralateral:  $140 \pm 6\%$ , n = 5) and S1 (contralateral:  $165 \pm 18\%$ , n = 13; ipsilateral;  $171 \pm 14\%$ , n = 14) revealed that the neural changes in the contralateral spinal cord and the ipsilateral



**Figure 3. Potentiation of the S1 responses after ischemic treatment.** (**a**) Example of contralateral S1 responses elicited by vibratory stimulation applied to the left hindpaw. (**b**) Example of S1 responses recorded in the same mouse before, during, and 30–180 min after ischemia applied to the left thigh for 30 min. The response amplitudes were measured in the square window of  $60 \times 60$  pixels shown in the second panel. (**c**) Relative amplitudes of S1 responses during and after hindpaw ischemia. (**d**) Example of S1 responses recorded before and at 60 min after sham treatment. (**e**) Example of S1 responses recorded before and at 60 min after hindpaw ischemia in a mouse with spinal application of saline alone. (**f**) Example of S1 responses recorded before and at 60 min after hindpaw ischemia in a mouse with spinal application of saline alone. (**f**) Example of S1 responses recorded before and at 60 min after hindpaw ischemia in a mouse with spinal application of saline alone. (**f**) Example of S1 responses recorded before and at 60 min after hindpaw ischemia in a mouse with spinal application of 10 nM LY354740. (**g**) Comparison of the normalized response amplitudes in mice at 60 min after hindpaw ischemia, sham treatment, hindpaw ischemia with spinal application of saline alone, or hindpaw ischemia with spinal application of 10 nM LY354740.

S1 were comparable to those in the opposite sides. These results suggest that reduction in the mechanical thresholds of the right hindpaw-withdrawal reflex could be observed after ischemic treatment applied to the left thigh. In accordance with these results, a slight but significant reduction in the mechanical threshold (P < 0.02) was observed in the right hindpaw withdrawal reflex at 3h after ischemia applied to the left hindpaw. However, S1 responses to forepaw stimulation were not clearly affected by ischemia applied to the hindpaw (Supplementary Figs S4).

#### Discussion

**Postischemic mechanical allodynia produced by spinal mechanisms in mice.** In the present study, we found that mechanical thresholds for hindpaw-withdrawal reflex were reduced after ischemic treatment. Previous studies also reported that hyperalgesia<sup>30</sup> or mechanical allodynia<sup>31</sup> is observed after hindpaw ischemia in animal experiments. However, it is difficult to differentiate between tingling sensation (or dysesthesia) and hyperalgesia or mechanical allodynia using behavioral tests, because all of these symptoms produce reduced mechanical thresholds. The reduced mechanical thresholds after ischemic treatment in the present study can be regarded as dysesthesia, because tingling sensation rather than pain is frequently experienced by human subjects after reversible hindpaw ischemia. Usually, postischemic dysesthesia due to hyperexcitability of peripheral nerves continues for approximately 5 min after ischemic treatment<sup>1</sup>, while use of a tourniquet to prevent bleeding for more than 2 h can produce long-lasting dysesthesia<sup>32</sup>.

The reduced mechanical thresholds were accompanied by potentiation in the spinal cord and S1. These three changes after ischemic treatment were abolished by spinal application of LY354740. Spinal and S1 responses to the right hindpaw stimulation were also potentiated during ischemia applied to the left hindpaw. Although long lasting-ischemia and reperfusion produce peroxides and resulting cytokine-mediated adverse pathophysiological effects on peripheral tissues<sup>33,34</sup>, potentiation in the



**Figure 4. Sensory modality shift induced by ischemic treatment.** (a) Lesion in the left dorsal column at T11 level. (b) Example of the right S1 responses modified by the left column lesioning and ischemic treatment applied to the left thigh. (c) Example of the right S1 responses modified by the left column lessoning and sham treatment applied to the left thigh. (d) Response amplitudes immediately after dorsal column lesioning, and at 30 min and 60 min after ischemic or sham treatment.

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contralateral spinal cord or in the ipsilateral S1 was observed before reperfusion was started, and S1 responses to forepaw stimulation were not affected by ischemia applied to the hindpaw. Taken together, these data suggest that postischemic tingling sensation was produced by localized spinal mechanisms in our mouse model.

**Spinal neural circuits and mechanisms producing postischemic plasticity.** Neural imaging is useful for investigating mechanisms underlying postischemic tingling sensation. We used endogenous fluorescence signals derived from the mitochondrial flavoproteins<sup>15</sup>. Because flavoproteins are endogenous proteins of the electron transfer system in the mitochondria, it is very unlikely that flavoproteins work as a calcium chelator and have some artificial effects on the calcium dynamics, which play essential roles in induction of synaptic plasticity. We have successfully recorded various types of neural plasticity using this imaging method<sup>16,17,20,35</sup>. Flavoprotein fluorescence responses are mainly derived from synaptically-driven neuronal activity<sup>21</sup>, and no clear fluorescence response was found from the ipsilateral dorsal column that mediates tactile sensory information during hindpaw stimulation. Therefore, the spinal flavoprotein fluorescence response of lamina I–VI<sup>36</sup>. Of these, tactile inputs mediated by A $\beta$  fibers are terminated mainly in lamina III–V. Lamina II, or the substantial gelatinosa, has a critical role in neuropathic pain<sup>37,38</sup>, and receives nociceptive inputs mediated mainly by C fibers<sup>39</sup>. Group II mGluRs are located in the substantial gelatinosa<sup>40</sup>. Taken together, the spinal fluorescence responses elicited by tactile stimuli to the hindpaw are likely derived from neuronal activity in lamina III–V, and the potentiation observed after ischemic treatment likely reflects the recruitment of neuronal activity in the substantial gelatinosa.

An initial phase of neuropathic pain after partial denervation is abolished by spinal application of LY3547407, and this compound and similar group II mGluR agonists alleviate neuropathic pain<sup>8,41</sup>. Because the potentiation in the present study was also susceptible to spinal application of LY354740, these two types of potentiation may share similar mechanisms, at least in part, with neuropathic pain (Fig. 6a). Activation of postsynaptic group II mGluRs reduces presynaptic transmitter release<sup>42</sup>, produces membrane hyperpolarization by opening inward rectifier K<sup>+</sup> channels<sup>43,44</sup>, and modulates spontaneous Ca<sup>2+</sup> spikes<sup>45</sup>. Therefore, failure of group II mGluR activation during conduction block of peripheral nerve activities may increase the excitability and intracellular  $Ca^{2+}$  concentration of postsynaptic neurons. These changes induce down-regulation of the neuron-specific KCl cotransporter (KCC2) in dorsal horn neurons and a resulting reduced neuronal Cl<sup>-</sup> gradient<sup>46-48</sup>. Nociceptive spinal neurons are dynamically regulated by inhibition from various sources<sup>49,50</sup>, and modulation of the Cl<sup>-</sup> gradient can explain the increased responsiveness of spinothalamic tract neurons to innocuous mechanical stimuli in animals with neuropathic pain<sup>48,51</sup>. The spinal potentiation is induced not only in the ipsilateral site that fails to receive basal afferent firing, but also in the contralateral site or nearby site after partial nerve cutting. Because stimulation of different skin areas produces separate spinal responses (for example, Fig. 3), the potentiation in nearby spinal sites may have been induced by diffusible mediators produced in spinal neurons that failed to receive basal afferent firing (Fig. 6b). It is suggested that hindpaw ischemia produced





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some diffusible mediators that produced not only potentiation of contralateral hindpaw responses but also contralateral mechanical allodynia. Diffusible mediators may also play a role in potentiation of spinal responses ipsilateral to ischemic treatment. In accordance with this concept, neuropathic pain was reported to be facilitated by a number of diffusible mediators such as nitric oxide<sup>52</sup> and ATP/cytokines<sup>53</sup>. The mechanical allodynia observed after hindpaw ischemia seemed to be more clearly observed in mice compared with similar phenomena in humans. Furthermore, humans do not clearly exhibit tingling sensation contralateral to the ischemic side or after partial nerve cutting. This difference may be attributed to the small size of spinal circuits in mice, in which diffusible messengers such as nitric oxide can be very effective. Another important consideration is that the potentiation observed during ischemic treatment in the present study could not be induced by repetitive activity in peripheral nerves, another condition known to induce spinal potentiation<sup>54,55</sup>.

**Postischemic changes as an experimental model for investigating neuropathic pain.** Neuropathic pain is usually induced by peripheral nerve injury in animal models<sup>9-11</sup>. It is produced by a complex cascade of inflammation and gene expression, and not only neurons but also glial cells in the spinal cord have important roles in neuropathic pain<sup>12-14</sup>. We have reported that an initial phase of neuropathic pain is observed at a few hours after partial denervation<sup>7</sup>. However, postischemic tingling sensation and plasticity, which share similar spinal mechanisms with neuropathic pain, were observed at 30 min after reversible hindpaw ischemia. Furthermore, potentiation of spinal responses contralateral to the ischemic side appeared within 30 min of ischemic treatment. Regardless of the species difference, the very early onset of neural plasticity during reversible hindpaw ischemia or transient functional deafferentation in mice make them useful as an experimental model for observing and



**Figure 6. Mechanisms underlying postischemic plasticity.** (a) Sensory modality shift from tactile to tingling sensation at the spinal cord level. (b) Schematic drawings of spinal potentiation ipsilateral and contralateral to ischemic treatment. Expected spinal potentiation induced after partial nerve cutting may also be induced by similar mechanisms.

investigating the detailed cellular and molecular cascades that trigger human neuropathic pain. However, further studies on neuronal and glial activities in the spinal cord are required for establishing a new preclinical model of early neuropathic pain based on the present studies.

#### Materials and methods

The experiments in the present study were approved by the ethics committee of animal experiments in Niigata University (approved number: 233-4), and were carried out in accordance with the approved guidelines. Male C57BL/6 mice between 7 and 10 weeks old, purchased from Charles River Japan (Tokyo, Japan), were used in the present study.

**Estimation of mechanical thresholds for hindpaw-withdrawal reflex.** The mechanical thresholds for hindpaw-withdrawal reflex were measured using von Frey filaments<sup>25</sup>. The forces produced by von Frey filaments were between 0.008 and 1.4g (respective sizes: between 1.65 and 4.17). Mice were separately placed into a transparent plastic box with a mesh floor, and accustomed to the state for 30 min. The thresholds were determined from the minimal force at which hindpaw withdrawal reflex was induced more than twice in eight trials. Transient ischemia was applied to the left hindpaw of mice lightly anesthetized with 1% isoflurane. Urethane anesthesia (1.65 g/kg, i.p.) was also used for imaging experiments. A small rubber cuff was set around the left thigh, and covered with a hard plastic tube (Fig. 1a). Air pressure at 250 mmHg was applied for 30 min to the tubing connected to the cuff using a mercury manometer. The pressure was directed to the thigh, because inflation of the rubber cuff was limited by the hard plastic tube. We confirmed that this treatment was sufficient to produce transient ischemia of the hindpaw, as neural responses elicited by vibratory stimulation to the hindpaw were blocked by this manipulation (for example, Figs 2c,4b). No apparent impairment except reduced threshold of paw-withdrawal reflex was found in the left leg after the mice recovered from isoflurane anesthesia.

**Imaging experiments.** The surgical procedures were performed as described previously<sup>7</sup>. Mice were anesthetized with urethane (1.65 g/kg, i.p.), and a tracheotomy was performed for facilitating spontaneous respiration. During the experiments, body temperature was monitored using a rectal probe and maintained at 38 °C using a silicon rubber heater. These surgical operations were usually finished within 60 min. Recordings were started at 30 min after the surgical operations. Additional doses of urethane (0.1–0.2 g/kg, s.c.) were administered when necessary. When spinal responses to hindpaw stimulation were investigated, the vertebral arch was removed at the T13 and L1 level, and the dorsal surface of the spinal cord with the intact dura mater was exposed. The surface was cleaned with saline, and covered with 2% agarose to prevent spinal movement. The surface of the agarose gel was covered with a mixture of petroleum jelly and liquid paraffin to prevent drying. The spinal cord was fixed under a microscope using a clamp (STS-A; Narishige, Tokyo, Japan). Spontaneous respiration was maintained during the imaging experiment, because the movement of the spinal cord caused by respiration was minimal.

Flavoprotein fluorescence imaging was performed as described previously7. Endogenous green fluorescence ( $\lambda = 500-550$  nm) was recorded in blue light ( $\lambda = 450-490$  nm). Images ( $128 \times 168$  pixels) of the spinal cord or S1 were recorded at 9 frames/s using a cooled charge coupled device camera (ORCA-R2; Hamamatsu Photonics, Hamamatsu, Japan). The camera was attached to a binocular epifluorescence microscope (M165 FC; Leica Microsystems, Wetzlar, Germany) with a 75-W xenon light source and a  $1 \times$  objective lens. Serial images were taken in recording sessions repeated at 50s intervals. Brush vibration (amplitude: 0.2 mm; frequency: 50 Hz) was applied for 600 ms to the sole of the hindpaw using a mechanical stimulator (DPS-290; Dia Medical, Tokyo, Japan). When fine somatotopic maps were investigated in the spinal cord or S1, brush vibration was applied to each toe. Fluorescence changes elicited by the stimulation were averaged over 24 trials. Because it took approximately 20 min to obtain data from 24 trials, the recording time of the averaged data was defined as the middle point of the recording period. Spatial averaging in  $5 \times 5$  pixels and temporal averaging in three consecutive frames were used for smoothing and improving the image quality. The images were normalized, pixel by pixel, with respect to a reference image, which was obtained by averaging five images taken immediately before the stimulation. In the figures, parts of the normalized images are shown in a pseudocolor scale representing the fractional fluorescence changes ( $\Delta$ F/F<sub>0</sub>). The response amplitude was evaluated as values of  $\Delta$ F/F<sub>0</sub> in a square window of  $100 \times 25$  pixels or  $3.84 \times 0.96$  mm. The location of the window was adjusted to maximize the response amplitude in  $\Delta F/F_0$ . After the recordings, mice were euthanized with an overdose of pentobarbital (300 mg/kg, i.p.).

For investigating the S1 responses to hindpaw stimulation, the disinfected head skin was incised, and the skull over the right S1 was exposed. The surface of the skull was cleaned with sterile saline, and a small piece of metal was attached to the skull with dental acrylic resin (Super Bond; Sun Medical, Shiga, Japan) to fix the head under a microscope. The surface of the skull was covered with a mixture of petroleum jelly and liquid paraffin to keep the skull transparent. The response amplitude was evaluated as values of  $\Delta F/F_0$  in a square window of  $60 \times 60$  pixels or  $1.55 \times 1.55$  mm.

**Dorsal column lesioning.** The left dorsal column was disrupted using an ultrasonic cutter (NE87; NSK, Kanuma, Japan) at the T11 level, as described previously<sup>7</sup>. To verify the lesion, the spinal cord was isolated after the recording experiments and fixed with 10% paraformaldehyde. Serial spinal cord sections of 100- $\mu$ m thickness were cut using a microslicer (PRO-7; Dosaka, Kyoto, Japan), and the translucent images were observed.

**Spinal application of LY354740 to the spinal cord.** In behavioral experiments, LY354740 (10 nM,  $5\mu$ l), obtained from Santa Cruz Biotechnology (Santa Cruz, USA), was applied to the spinal cord with intrathecal injection before hindpaw ischemia. In imaging experiments of the spinal responses, epidural application and infiltration of 10 nM LY354740 for at least 30 min was performed before the epidural surface was covered with 2% agarose. Epidural application and infiltration of saline alone was naturally performed in other imaging experiments of the spinal responses. In imaging experiments of S1 responses, the epidural application and infiltration of 10 nM LY354740 or saline alone was performed at the T13–L1 level.

**Statistics.** Statistical significance in data was analyzed using StatView software (SAS Institute Inc., Cary, USA). Unpaired data obtained from different mice were evaluated by the Mann Whitney U-test. Paired data obtained from the same mice were evaluated by the Wilcoxon signed rank test. Only P values less than 0.05 are shown.

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#### **Author Contributions**

T.W., M.S, S.K., and K.S. designed the experiments; T.W., M.S. and H.T. conducted experiments; T.W., H.T., R.H., T.K., H.B. and K.S. analyzed the data; T.K. and H.B. provided materials; T.W., and K.S. wrote the manuscript.

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## Delineation of a frequency-organized region isolated from the mouse primary auditory cortex

## <sup>(D)</sup> Hiroaki Tsukano,<sup>1</sup> Masao Horie,<sup>2</sup> Takeshi Bo,<sup>1</sup> Arikuni Uchimura,<sup>3</sup> Ryuichi Hishida,<sup>1</sup> Masaharu Kudoh,<sup>4</sup> Kuniyuki Takahashi,<sup>5</sup> Hirohide Takebayashi,<sup>2</sup> and Katsuei Shibuki<sup>1</sup>

<sup>1</sup>Department of Neurophysiology, Brain Research Institute, Niigata University, Niigata, Japan; <sup>2</sup>Division of Neurobiology and Anatomy, Graduate School of Medicine and Dental Sciences, Niigata University, Niigata, Japan; <sup>3</sup>KOKORO-Biology Group, Laboratories for Integrated Biology, Graduate School of Frontier Biosciences, Osaka University, Osaka, Japan; <sup>4</sup>Department of Physiology, Teikyo University School of Medicine, Tokyo, Japan; and <sup>5</sup>Department of Otolaryngology, Graduate School of Medicine and Dental Sciences, Niigata University, Niigata, Japan

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Tsukano H, Horie M, Bo T, Uchimura A, Hishida R, Kudoh M, Takahashi K, Takebayashi H, Shibuki K. Delineation of a frequencyorganized region isolated from the mouse primary auditory cortex. J Neurophysiol 113: 2900-2920, 2015. First published February 18, 2015; doi:10.1152/jn.00932.2014.-The primary auditory cortex (AI) is the representative recipient of information from the ears in the mammalian cortex. However, the delineation of the AI is still controversial in a mouse. Recently, it was reported, using optical imaging, that two distinct areas of the AI, located ventrally and dorsally, are activated by high-frequency tones, whereas only one area is activated by low-frequency tones. Here, we show that the dorsal high-frequency area is an independent region that is separated from the rest of the AI. We could visualize the two distinct high-frequency areas using flavoprotein fluorescence imaging, as reported previously. SMI-32 immunolabeling revealed that the dorsal region had a different cytoarchitectural pattern from the rest of the AI. Specifically, the ratio of SMI-32-positive pyramidal neurons to nonpyramidal neurons was larger in the dorsal high-frequency area than the rest of the AI. We named this new region the dorsomedial field (DM). Retrograde tracing showed that neurons projecting to the DM were localized in the rostral part of the ventral division of the medial geniculate body with a distinct frequency organization, where few neurons projected to the AI. Furthermore, the responses of the DM to ultrasonic courtship songs presented by males were significantly greater in females than in males; in contrast, there was no sex difference in response to artificial pure tones. Our findings offer a basic outline on the processing of ultrasonic vocal information on the basis of the precisely subdivided, multiple frequency-organized auditory cortex map in mice.

auditory cortex; mapping; multiple frequency organization; courtship song; mice

THE PRIMARY SENSORY CORTEX of the mammalian brain is widely known to receive the first thalamic inputs that convey peripheral sensory information, such as hearing and vision. In the auditory cortex, the primary auditory cortex (AI) is the main recipient of information from the ears into the cortex and transfers this information to higher-order auditory cortical regions (Kaas and Hackett 2000). The AI is important in terms of being both the receiver and the relay point in hierarchical auditory processing and has been studied using various animals, including mice. The mouse is widely used in auditory physiological research because of its merits as an animal model in auditory cortical studies involving two-photon Ca<sup>2+</sup> imaging (Bandyopadhyay et al. 2010; Bathellier et al. 2012; Honma et al. 2013; Issa et al. 2014; Rothschild et al. 2010), voltage-sensitive imaging (Sawatari et al. 2011; Takahashi et al. 2006), anatomical studies (Barkat et al. 2011; Hackett et al. 2011; Hofstetter and Ehret 1992; Horie et al. 2013; Llano and Sherman 2008; Oviedo et al. 2010), genetic manipulation (Barkat et al. 2011; Rotschafer and Razak 2013; Xiong et al. 2012), and behavioral analysis (Tsukano et al. 2011). Accordingly, the attainment of precise knowledge of the AI in mice is essential. However, the delineation of the map of the mouse auditory cortex is under debate, as maps obtained by electrophysiology have been amended in recent studies using optical imaging.

The classical mouse auditory cortex map was drawn using unit recording. The anterior auditory field (AAF) and AI with clear frequency gradients are placed at the center as the core and are surrounded by the belt region, considered the higherorder region, including the secondary auditory field (AII), the ultrasonic field (UF), and the dorsoposterior field (DP) (Stiebler et al. 1997). The AAF and AI have frequencyorganized arrangements covering the frequencies up to 40 kHz; neurons with characteristic frequency over 50 kHz are localized in the UF (Fig. 1A). A subsequent study indicated that a distinct UF region does not exist, with the AAF and AI having the full range of frequency organization, processing sounds from 4 kHz up to 64 kHz (Guo et al. 2012). The term "UF" has thus been redefined as subparts within the AAF and AI that process high-frequency sounds (Fig. 1B). Recently, however, Issa et al. (2014) have reported elegantly that the AI is divided into two rostral areas that process high-frequency tones, despite only one caudal area being activated by low-frequency tones. This leads to the generation of forked, dual-frequency gradients inside of the AI (Issa et al. 2014). The larger dorsal branch of the fork-shaped frequency gradients travels toward the high-frequency area of the AI, referred to as the UF, as reported by Guo et al. (2012), whereas the smaller ventral division has an axis of the frequency organization toward the AII (Fig. 1C) (Issa et al. 2014). This new map was obtained using optical imaging. Moreover, they clearly revealed that the direction of the frequency organization of the AAF was present and is directed from the dorsorostral to ventrocaudal direction. Their study used transgenic mice, in which the calcium-

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Address for reprint requests and other correspondence: H. Tsukano, Dept. of Neurophysiology, Brain Research Institute, Niigata Univ., 1-757 Asahimachidori, Chuo-ku, Niigata 951-8585, Japan (e-mail: tsukano-nii@umin.ac.jp).

Map of the auditory cortex revealed by Stiebler et al. (1997)



Map of the auditory cortex С revealed in Issa et al. (2014)



sensitive protein GCaMP3 was initially expressed uniformly, offering much better spatial resolution regarding the auditory cortical surface than that achieved by unit recording. Principally, however, previous anatomical studies reported that frequency-organized maps in the AAF and AI reflect distinct topological projections from frequency-organized maps in the medial and lateral parts, respectively, of the ventral division of the medial geniculate body (MGv; MGB) in mice as well (Horie et al. 2013; Takemoto et al. 2014). Moreover, various mammals have multiple frequency-organized regions with distinct, unique frequency direction (Higgins et al. 2010; Kalatsky et al. 2005; Kanold et al. 2014); spectral, temporal, and spatial sensitivities (Bizley et al. 2009; Imaizumi et al. 2004; Polley et al. 2007); and thalamocortical projections (Lee et al. 2004; Storace et al. 2010, 2012) without redundancy. The mouse AI, therefore, would be exceptional if the fork-shaped frequency gradients existed inside of a single region.

In this study, we tried to clarify whether the frequency gradient inside of the AI proceeds in a straight line or shows a forked shape. For this purpose, we used a combination of flavoprotein autofluorescence imaging and anatomical techniques. Flavoproteins are endogenous fluorescent proteins in mitochondria, and fluorescence imaging of these proteins has been used to map precisely the auditory cortex (Honma et al. 2013; Horie et al. 2013; Kubota et al. 2008; Ohshima et al. 2010; Takahashi et al. 2006), visual cortex (Andermann et al. 2011; Tohmi et al. 2009, 2014; Yoshitake et al. 2013), somatosensory cortex (Komagata et al. 2011), and insular cortex (Gogolla et al. 2014). With the use of this technique, we reproduced the finding that the mouse AI is divided into two areas in response to high-frequency tones

Map of the auditory cortex B revealed by Guo et al. (2012)



Map of the auditory cortex D revealed in the present study. High - Rostral

Fig. 1. Schematic maps of the mouse auditory cortex in previous studies and the present study. A: schematic map of the auditory cortex in Stiebler et al. (1997). Frequency gradients of the anterior auditory field (AAF) and primary auditory cortex (AI) included neurons with characteristic frequencies up to 40 kHz, and neurons with characteristic frequencies higher than 50 kHz are located in the distinct ultrasonic field (UF). B: map of the auditory cortex in Guo et al. (2012). The AAF and AI had ultrasonic bands higher than 50 kHz. The UF was interpreted to be high-frequency subparts in the high-frequency area of the AAF and AI but not a distinct region. A and B: these maps were drawn according to the results obtained by unit recording. C: map of the auditory cortex in Issa et al. (2014), showing 2 streams of frequency gradients inside of the AI. The major gradient runs toward the dorsal part, including the UF area, whereas the minor gradient runs toward the secondary auditory field (AII). The direction of the frequency organization of the AAF is drawn along the ventrocaudal axis, consistent with the data reported by Horie et al. (2013). D: map of the auditory cortex reported in the present study. Newly delineated, distinct region dorsomedial field (DM) was isolated from the AI high-frequency area. The direction of the frequency organization of the AI is the one traveling toward AII. The direction of the frequency organization of the AAF matches that reported in the previous studies (Horie et al. 2013; Issa et al. 2014). The region that Stiebler et al. (1997) defined as UF [referred to as dorsoanterior field (DA)] responds to slow-frequency modulation (FM) components, regardless of tonal frequency range. C and D: these maps were drawn according to the results obtained by optical imaging. DP, dorsoposterior field.

(Issa et al. 2014; Tsukano et al. 2013a). Additionally, we used SMI-32 immunolabeling, which has been used to partition various cortical regions (Boire et al. 2005; Budinger et al. 2000; Mellott et al. 2010; Ouda et al. 2012; Paxinos et al. 2009; Rothschild et al. 2010; van der Gucht et al 2001; Wong and Kaas 2009) to investigate the cytoarchitecture in the two AI high-frequency areas. We found that the dorsal part of the AI high-frequency area is a different region from the rest of the AI, including the low-frequency area and the ventral highfrequency area, and the posterior frequency-organized region, which is referred to as the AI, is restricted to the low-frequency area and the ventral high-frequency area that has been considered as a supplemental AI area in the study by Issa et al. (2014) (Fig. 1D). Existence of multiple frequency organizations in the mouse auditory cortex may unify the auditory cortical maps of mice and other mammals.

#### METHODS

Animals. The Committee for Animal Care at Niigata University approved the experimental protocols used in this study. We used 5- to 7-wk-old C57BL/6 mice (n = 197; Charles River Japan, Yokohama, Japan), 7- to 9-wk-old Balb/c mice (n = 3; Charles River Japan), and 7- to 9-wk-old CBA/CaJ mice (n = 3; The Jackson Laboratory, Bar Harbor, ME). The animals were housed in cages with ad libitum access to food pellets and water and were kept on a 12-h light/dark cycle. Male and female mice used in the experiments (see Fig. 14) were 6-wk-old C57BL/6 mice, produced in our institute, and they were not housed with mice of the opposite sex after weaning at 3 wk old.

In vivo flavoprotein fluorescence imaging. In vivo flavoprotein fluorescence imaging was performed, as described in our previous studies (Takahashi et al. 2006; Tsukano et al. 2013b). Mice were

deeply anesthetized with urethane (1.7 g/kg ip; Wako, Osaka, Japan), and their rectal temperatures were maintained at 37°C. After local anesthesia using bupivacaine, the skin and temporal muscle over the right auditory cortex were incised. A piece of metal was attached to the skull with dental resin, and the head was fixed by screwing the metal piece onto a manipulator. The skull over the auditory cortex was removed in mice used for some experiments (see Figs. 2B, 3, 4, 6, 8, 12, and 13) to compare response patterns of the auditory cortex or further tracer-injection experiments. Transcranial imaging was performed in the other experiments. The right auditory cortex was observed unless otherwise noted. The exposed surface of the intact skull was covered with liquid paraffin (Wako) to keep the skull transparent in transcranial imaging. Cortical images ( $128 \times 168$  pixels after binning) of endogenous green fluorescence ( $\lambda = 500-550$  nm) in blue light ( $\lambda = 470-490$  nm) were recorded using a cooled chargecoupled device (CCD) camera system (AQUACOSMOS with ORCA-R2 camera; Hamamatsu Photonics, Hamamatsu, Japan). Images were taken at 9.7 Hz [54 Hz in some experiments (see Figs. 11 and 14, C-E)]. Images were averaged over 20 trials unless otherwise noted. Spatial averaging of 5  $\times$  5 pixels was applied. Images were calculated as fluorescence change  $(\Delta F)$ /baseline intensity (F<sub>0</sub>), where  $\Delta F = F - F_0$ . The F<sub>0</sub> was obtained by averaging the intensity values during the prestimulus period (~500 ms). The response amplitude was evaluated as  $\Delta F/F_0$  in a circle window with a diameter of 20 pixels. When the frequency organization was evaluated (see Figs. 5 and 7), a circle window with a diameter of 15 pixels was chosen to give the largest response amplitude, and the location of the center pixel was considered to be the frequency-specific response peak.

In vivo two-photon calcium imaging. Calcium imaging was performed using a two-photon microscope (TCS SP5 MP; Leica Microsystems, Wetzlar, Germany) with a hybrid detector (HyD; Leica Microsystems) and a Ti-Sapphire mode-locked femto second laser (Chameleon Vision; Coherent, Santa Clara, CA), as described in our previous studies (Honma et al. 2013; Tohmi et al. 2014; Yoshitake et al. 2013). Calcium-sensitive dye was prepared by dissolving Fura-2 AM (Invitrogen Life Technologies, Boston, MA) in 20% (w/v) Pluronic F-127 in DMSO (Invitrogen Life Technologies) and diluted with Ringer solution containing sulforhodamine 101 (SR-101; Invitrogen Life Technologies). After anesthetic induction with urethane (1.9 g/kg ip), craniotomy, and localization using flavoprotein fluorescence imaging, a solution of calcium-sensitive dye was pressure injected (5-20 kPa) for 5-10 min into layer II/III using glass pipettes (tip diameter:  $2-4 \mu m$ ). Astrocytes were distinguished from neurons using SR-101. After injection, the pipette was withdrawn, and the craniotomy was covered with 2% agarose (1-B; Sigma-Aldrich, St. Louis, MO) and a thin cover glass (thickness <0.15 mm; Matsunami, Osaka, Japan), which was fixed to the skull with dental cement (Sun Medical, Shiga, Japan). Excitation wavelength for Fura-2 was 800 nm, and that for SR-101 was 900–950 nm. Images ( $256 \times 256$  pixels) were recorded at 3.7 Hz in a 260  $\times$  260- $\mu$ m region.

The data were realigned using AQUACOSMOS and MATLAB software (MathWorks, Natick, MA). Data from five to six trials of the same stimulation were averaged. Size-matched regions of interest (ROIs) were chosen. Data were calculated as  $\Delta F/F_0$ , where  $\Delta F = F_0$  – F. The  $F_0$  was obtained by averaging the intensity values during the prestimulus period ( $\sim$ 3 s). The response of each neuron was defined as the maximum value in poststimulus observation windows ( $\sim 5$  s). Neurons were defined as responsive when the response peak was >4SD above the baselines during the prestimulus period to minimize errors by baseline fluctuations. The best frequency (BF) of a neuron was defined as the frequency to which the neuron had the greatest response. We examined the correlation between frequency and location and computed a regression line. The direction of the frequency organization was defined so as to make the correlation coefficient as large as possible. Then, we examined the residuals as a measure of deviation from the frequency organization. The bandwidth of tuning curves was defined as the logarithmic ratio of minimum and maximum frequencies that resulted in a response >75% of the peak amplitude.

Auditory stimuli. Tones were made by a computer using a customwritten LabVIEW program (National Instruments, Austin, TX) at a sampling rate of 500 kHz. Courtship songs of a male mouse, emitted when he was placed with a familiar female mouse in estrous in the same cage, were recorded using the recording software Avisoft-RECORDER (Avisoft Bioacoustics, Glienicke, Germany) at a sampling frequency of 250 kHz, with a microphone (CM16/CMPA; Avisoft Bioacoustics) and a preamplifier (UltraSoundGate 116; Avisoft Bioacoustics). Sounds were low-pass filtered at 150 kHz (3624; NF, Kanagawa, Japan). Pure tones at frequencies of 5-80 kHz were amplitude modulated by 20 Hz sine wave. A speaker for 5-40 kHz (SRS-3050A; Stax, Saitama, Japan) or 50-80 kHz (ES105A; Murata, Kyoto, Japan) was set 10 cm in front of the mice. Sound intensity was calibrated using the microphone (types 4135 and 2669; Brüel & Kjær, Nærum, Denmark) and the sound level meter (type 2610; Brüel & Kjær). The sound intensity was 60 dB sound pressure level (SPL) for flavoprotein fluorescence imaging and 80 dB SPL for two-photon imaging. The sound duration was 500 ms with a rise/fall time of 10 ms. The desired sound spectrum was determined using a digital spectrum analyzer (R9211A; Advantest, Tokyo, Japan) or the customwritten LabVIEW program. When the UF and DP (Stiebler et al. 1997) were activated specifically, frequency modulation (FM) direction-reversal stimulation (24 kHz/s) was used (Honma et al. 2013). The sound intensity was set to 60 dB SPL, and the band frequency was between 5 and 11 kHz.

Acoustic exposure. For acoustic exposure experiments (see Figs. 9 and 10), home cages were placed in the sound-shielded chamber and exposed to a 5- or 35-kHz sound stimulus through a speaker (SRS-3050A; Stax) placed above the cage. The exposure sound consisted of an amplitude-modulated tone with a carrier frequency of 5 or 35 kHz and modulation frequency of 20 Hz. Duration of the tones was 500 ms, and a rise/fall time was 10 ms. The sound intensity was adjusted to 70 dB SPL at the floor of the cage. This tonal stimulus was repeated at 1 Hz throughout the exposing periods (P7-P35). Mice of normal groups were reared in the normal cages. Mice of quiet groups were reared in the chamber but not exposed to any tones. Flavoprotein fluorescence imaging was performed within 1 wk after exposure was finished. When a circular window was put on the response of the dorsomedial field (DM) to evaluate response amplitudes, a window was put to make the response amplitude maximum, kept >22 pixels apart dorsal to the AI response peak, according to the data (see Fig. 2E).

Retrograde tracer experiments. To visualize neurons in the MGB projecting to each cortical region, a neural tracer was injected into the center of each region identified by flavoprotein fluorescence imaging (Horie et al. 2013). A glass capillary (tip diameter 20–30  $\mu$ m) filled with tracer solution and a platinum wire was introduced into the center of the subregion of the right auditory cortex to  $\sim$ 500  $\mu$ m below the surface. Alexa Fluor 488- or 555-conjugated cholera toxin subunit B (CTB; Molecular Probes, Eugene, OR) was used in injections (see Fig. 13). Fluorescein and Texas Red (Molecular Probes) were used in some animals, but the results were the same. Fluorescent CTB solution (0.5% in phosphate buffer) was injected iontophoretically by a 5- $\mu$ A pulse current (5 s on; 5 s off) for 15 min. In some experiments (see Figs. 8 and 12), biotinylated dextran amine (BDA; molecular weight 3,000; Molecular Probes) was injected iontophoretically by a 5- $\mu$ A pulse current (7 s on; 7 s off) for 15 min. Survival of 3 days for fluorescent CTB or 7 days for BDA was ensured until perfusion. After anesthetizing mice deeply with pentobarbital (1.0 g/kg ip), the brains were dissected and immersed in 4% paraformaldehyde overnight, and a consecutive series of 40-µm-thick coronal or horizontal sections was cut using a sliding cryotome. To observe fluorescent tracers, sections were mounted on glass slides and covered with Fluoromount (Cosmo Bio, Tokyo, Japan).

To visualize BDA, sections were rinsed initially in 20 mM PBS and incubated in PBS containing 3% hydrogen peroxide and 0.1% Triton X-100 for 15 min at room temperature. After rinsing in 20 mM PBS containing 0.1% Triton X-100 (PBST), the sections were incubated for 40 min in 20 mM PBST containing avidin-biotin peroxidase complex (Vectastain ABC kit; Vector Laboratories, Burlingame, CA). Sections were rinsed in 20 mM PBS, and BDA was visualized in a solution comprising 0.05% diaminobenzidine tetrahydrochloride and 0.003% hydrogen peroxide in 50 mM Tris-HCl buffer (pH 7.4) for 20 min. All sections were finally, thoroughly rinsed in 50 mM Tris-HCl buffer and mounted onto gelatin-coated slides. Adjacent sections were counterstained using 0.1% cresyl violet (Chroma Gesellschaft, Kongen, Germany). After the mounted sections had dried, they were dehydrated in a graded ethanol series, cleared in xylene, and coverslipped using the covering reagent Bioleit (Okenshoji, Tokyo, Japan).

The borders of the MGB subdivisions were delineated, according to the SMI-32 immunolabeling pattern (Honma et al. 2013; Horie et al. 2013; LeDoux et al. 1985, 1987) and an atlas (Paxinos 2003; Paxinos and Franklin 2001; Paxinos et al. 2009). The immunohistochemistry of SMI-32 reacts with a nonphosphorylated epitope in neurofilament M and H (NNF). Sections were rinsed and incubated in PBST containing 3% hydrogen peroxide, as described for BDA visualization. After rinsing in 20 mM PBS, the sections were incubated overnight at room temperature with the monoclonal antibody (MAb) SMI-32 (1:2,000; Covance Research Products, Berkeley, CA) (Sternberger and Sternberger 1983), diluted with 20 mM PBS containing 0.5% skim milk. Sections were then incubated in anti-mouse IgG (1:100; MBL, Nagoya, Japan) at room temperature for 2 h. The sections were rinsed in 20 mM PBS, and the immunoreactions were visualized in a Tris-HCl buffer containing 0.05% diaminobenzidine tetrahydrochloride and 0.003% hydrogen peroxide for 5 min at room temperature. After visualization, the sections were coverslipped. All sections were observed under the light microscope (Eclipse Ni; Nikon, Tokyo, Japan) and a CCD camera (DS-Fi2; Nikon). The drawings and images were prepared using CorelDRAW (E Frontier, Tokyo, Japan), Illustrator (Adobe Systems, San Jose, CA), and Photoshop (Adobe Systems) software.

AAV injection to investigate interhemispheric connectivity. To confirm that the corresponding subareas have interhemispheric connections with each other via the corpus callosum, adeno-associated virus (AAV) vector for GCaMP3 expression (Penn Vector Core, Philadelphia, PA) was injected into an area of the right AI after functional identification, which stains the axonal branches reaching the contralateral auditory cortex (see Fig. 3). A glass capillary (tip diameter: 20–30  $\mu$ m) filled with AAV solution (500  $\mu$ L) was introduced to a depth of 500  $\mu$ m from the surface and slowly pressure injected for 30 min by a custom-made pump. After injection, the hole in the skull was covered with 2% agarose (1-B; Sigma-Aldrich), and the skin was sutured. Mice were recovered from anesthesia in their home cages. Two weeks after the injection, the left auditory cortex was observed using an epifluorescence microscope [excitation (Ex), 470-490 nm; emission (Em), 500-550 nm] and a two-photon microscope (Ex, 900 nm; Em, 500-550 nm).

Statistics. The Mann-Whitney U-test or Wilcoxon signed-rank test was used to evaluate differences between unpaired or paired data from two groups, respectively. When data from three groups were compared, Tukey-Kramer test was used as a post hoc test after ANOVA. The correlation coefficients and the *P* values were calculated using Spearman rank correlation test. When the difference between two slopes was evaluated, the slope test was performed. The  $\chi^2$  test was used to evaluate the difference between two percentages. These tests were performed using SPSS (IBM, Tokyo, Japan) or MAT-LAB software. All of the data in the text and graphs are presented as means  $\pm$  SE.

#### RESULTS

Finding the new, distinct region identified from the AI. Prior imaging studies have identified distinct auditory cortical regions based on response magnitudes to tones and the topographic organization of tone-frequency responses (cochleotopy) (Harel et al. 2000; Horie et al. 2013; Kubota et al. 2008; Sawatari et al. 2011; Takahashi et al. 2006). In this study, we try to confirm frequency organizations by demonstrating distinct response magnitude peaks and mirror-reversed frequency organization in the AAF and AI using flavoprotein fluorescence imaging. In addition, we try to observe flavoprotein fluorescence responses precisely within a cortical region, typically defined as the AI in mice, according to the Paxinos anatomic atlas (Paxinos and Franklin 2001). First, we observed neural responses in the right auditory cortex of C57BL/6 mice in a 5- to 70-kHz range after craniotomy (Fig. 2A). The frequency-organized map of the AI was arranged in a caudalrostral direction. Although only one area was activated in response to a 5-kHz tone, the AI responses to a 40-kHz tone were observed in two different areas, which were dorsoventrally separated from each other, as reported previously (Fig. 2B) (Issa et al. 2014; Tsukano et al. 2013a). We also confirmed that the AI high-frequency area showed two different response peaks when stimulated by high-frequency tones using transcranial flavoprotein fluorescence imaging (Fig. 2C). Significantly separated double peaks in  $\Delta F/F_0$  were found by positioning serial ROIs across both dorsal and ventral AI highfrequency areas (Fig. 2E; P < 0.01 between ROI 6 vs. 11; P <0.05 between ROI 11 vs. 20). These data confirmed the presence of apparently separated AI high-frequency areas in mice. The regions overlapped to the places where Stiebler et al. (1997) drew the UF and DP in their auditory cortical map, hereby referred to as Stiebler's UF and DP in this report, were confirmed by several studies (Honma et al. 2013; Joachimsthaler et al. 2014; Stiebler et al. 1997), and both areas are known to be activated by directional changes of slow FM sounds (Honma et al. 2013; Tsukano et al. 2013b). Moreover, cortical responses to ultrasonic sounds are not specific enough to divide the broad area into specific subareas. Therefore, we verified whether the dorsal high-frequency area of the AI overlaps Stiebler's UF or DP using FM directional changes. The dorsal area of the AI mapped between Stiebler's UF and DP (Fig. 2D). To evaluate these data quantitatively, we positioned ROIs across Stiebler's UF and DP, and the same ROIs were placed onto the image of the responses to a 35-kHz tone obtained from the same mouse (Fig. 2F). The response peaks of the dorsal area of the AI did not overlap Stiebler's UF or DP (Fig. 2F; P < 0.05 at ROI 9; P < 0.05 at ROI 21; P < 0.05 at ROI 31). The observation that the AI was divided into two areas in the response to high-frequency tones was also made in the left auditory cortex (data not shown). We visualized the auditory cortex in CBA/CaJ and Balb/c mice as well. The structures of responses of the AAF, AI, and AII were the same among three strains, and the AI was divided into two areas in response to high-frequency tones (data not shown). These results, along with prior studies, suggest that intrinsic response peaks identify functionally distinct regions in the auditory cortex (Kalatsky et al. 2005).

It remains unclear how auditory cortical regions, defined according to sound-response magnitudes, align with traditional



Fig. 2. Two distinct responses to high-frequency tones in the AI. A: schematic drawing of the mouse auditory cortex. Auditory cortex (AC); cerebellum (Cb); medial cerebral artery (MCA); olfactory bulb (OB); rhinal fissure (rf); somatosensory cortex (SC); visual cortex (VC). B: neural responses to a 5- or 40-kHz tone obtained by flavoprotein fluorescence imaging in a mouse with craniotomy. The AI in response to a 40-kHz tone area was divided into 2 parts. Three images were obtained from the same mouse. Scale bar, 1 mm. C: neural responses to 5-70 kHz tones revealed by transcranial flavoprotein fluorescence imaging. The caudal AI low-frequency area was clearly activated. When 20-70 kHz tones were presented, the AI high-frequency area was divided into 2 parts: the dorsal part (a) and the ventral part (b), indicated by white arrows. Four images were obtained from the same mouse. D: positional relationship between the dorsal part of the AI high-frequency area and Stiebler's UF and DP. Stiebler's UF and DP were activated by slow FM directional changes (Honma et al. 2013). Three images were obtained from the same mouse. E: quantitative analysis of response slopes by a 35-kHz tone. Regions of interest (ROIs) were placed across the 2 highest peaks in the AI high-frequency areas, and the significant double peaks in fluorescence change ( $\Delta F$ )/baseline intensity ( $F_0$ ) were drawn (\*P < 0.05; \*\*P < 0.01; Wilcoxon signed-rank test, 10 mice). Each value was normalized as percent of the peak value at ROI 6, which was put on the response peak of the dorsal AI high-frequency area and used as a landmark to register across individuals. Sites a and b are equivalent to those shown in C. F: quantitative analysis of response slopes on the response in the experiment shown in D. ROIs were placed across the peaks in Stiebler's UF and DP, and the same ROIs were placed onto the image of the responses to a 35-kHz tone obtained from the same mouse (inset). The red line was obtained from the response to a 35-kHz tone, and the blue line was obtained from the response to FM directional changes in the same mouse. The response peak derived from the dorsal AI high-frequency area was placed in the middle of Stiebler's UF and DP (\*P < 0.05; Wilcoxon signed-rank test, 6 mice). Each value was normalized as percent of the peak value at ROI 30, which was put on the response peak of Stiebler's UF and used as a landmark to register across individuals. Transcranial imaging was performed in experiments C-F.

cytoarchitectonic partitions. To address this problem, we investigated the cytoarchitectural pattern of the AI low-frequency area, the ventral AI high-frequency area, and the dorsal AI high-frequency area (see Fig. 4A) after identifying the regions by flavoprotein optical imaging. We characterized these areas histologically by immunolabeling NNF using MAb SMI-32, which is widely used to partition and identify various cortical and brain regions, including the auditory cortex (Budinger et al. 2000; Mellott et al. 2010; Ouda et al. 2012; Rothschild et al. 2010), MGB (Honma et al. 2013; Horie et al. 2013; Paxinos et al. 2009), visual cortex (Boire et al. 2005; van der Gucht et al 2001; Wong and Kaas 2009), and other regions. To perform this analysis, it is necessary to determine the lowand high-frequency areas of the AI on slice sections. However, mouse monoclonal SMI-32 immunolabeling using mouse IgG as a secondary antibody gave high-background staining after marking the identified area with injection of fluorescent beads, dye, or ink. Therefore, we injected BDA into an identified area of the AI in the right hemisphere in vivo and then observed NNF cytoarchitecture in the left AI on slice sections guided by axonal branches stained with BDA projecting from neurons in the right AI. It is widely known that the corresponding areas have interhemispheric projections via the corpus callosum in mammalian brains (Budinger et al. 2000; Oviedo et al. 2010; Rouiller et al. 1991; Xiong et al. 2012), as shown in the schema of Fig. 3A. We confirmed this fact in the present study as well (Fig. 3). After we identified the precise location of the subregions in the right auditory cortex using flavoprotein fluorescence imaging, we injected AAV-GCaMP3 solution into the AI 5-kHz area (Fig. 3, B-D). This technique visualizes the axon terminals originating from the injected subarea in vivo (Glickfeld et al. 2013; Oh et al. 2014). Two weeks after injection of AAV-GCaMP3 into the AI 5-kHz area in the right hemisphere, the fluorescent axon terminals were observed in the contralateral left AI 5-kHz area (Fig. 3E). GCaMP3positive axon terminals were visualized using a two-photon microscopy in the place where an intense signal was observed in Fig. 3E (Fig. 3F), whereas no GCaMP3-positive terminals were observed outside of the AI (Fig. 3G). The large calcium responses to a 5-kHz tone derived from GCaMP3-positive terminals were observed in the left AI 5-kHz area (Fig. 3H), and the responses of the AAF and AII were close to those observed in a naïve mouse (Fig. 3D). These data confirm that it is possible to observe the NNF pattern of the identified area in the contralateral auditory cortex on slice sections, guided by axon terminals, stained by BDA injected into the right auditory cortex.

The NNF immunolabeling pattern appeared much denser in the dorsal part than in the ventral part of the AI high-frequency area or the AI low-frequency area, whereas the weak NNF immunolabeling pattern appeared in the latter two areas (Fig. 4*B*). Moreover, the dorsal part of the AI high-frequency area appeared to have the particular laminar pattern of immunolabeling. To analyze quantitatively the difference in NNF immunolabeling patterns, we first counted the number of NNF-positive neurons in each area. However, labeled neurons were not significantly different in number among the three areas (Fig. 4*C*). NNF immunolabeling clearly distinguished pyramidal from nonpyramidal neurons, the two most common neuron types. Pyramidal neurons had NNF-positive apical dendrites, reaching the superficial layer, and a densely labeled large soma

(Fig. 4D). Conversely, nonpyramidal neurons had few NNFpositive apical and basal dendrites, and their soma was labeled weakly. Therefore, the difference in the ratios of NNF-positive pyramidal neurons to nonpyramidal neurons could account for the apparent density difference with the NNF immunolabeling patterns. In layers II, III, and IV, the ratio of NNF-positive pyramidal/nonpyramidal neurons did not differ noticeably, and nonpyramidal neurons were dominant in all three areas (Fig. 4E). However, the deeper *layers V* and *VI* in the dorsal AI high-frequency area were almost fully occupied by NNFpositive pyramidal neurons. The ratio of NNF-positive pyramidal/nonpyramidal neurons was significantly higher than that observed in the other two areas [Fig. 4*F*; P < 0.05 (area a vs. c); P < 0.05 (area b vs. c)] when compared in layers V, VI, and II-VI, whereas there was no significant difference between areas a and b in any layer. Thus the presence of numerous NNF-positive pyramidal neurons in the deeper layers could explain the dense NNF immunolabeling pattern in the dorsal AI high-frequency area, since these neurons have thick NNFpositive basal dendrites and apical dendrites that reach superficial layers. These data (Figs. 2 and 4) strongly suggest that the dorsal AI high-frequency area, located between Stiebler's UF and DP, is likely a newly defined region with a different cytoarchitectural pattern. We tentatively refer to this new area as the DM (Fig. 1D).

Frequency organizations of the AI and DM. We next reevaluated the precise frequency organizations of the auditory cortex based on the finding that the dorsal AI high-frequency area is a newly defined region. We investigated frequency gradients by plotting the response peaks using flavoprotein fluorescence imaging described in our previous study (Fig. 5) (Honma et al. 2013). The posterior frequency-organized region labeled as the "AI," has a low-to-high (5-80 kHz) tonefrequency response axis directed from the dorsocaudal to the ventrorostral temporal cortex (Fig. 5, A and B). The frequency gradient of the AI ran in a mild clockwise direction. The corresponding frequency gradient for the newly identified DM was directed from ventrocaudal to dorsorostral anatomic axis (Fig. 5, A and C). Similar maps were obtained using other sound-intensity levels (for example, at 40 dB SPL; data not shown). The slopes of the AI and DM frequency gradients were not significantly different (P > 0.9) using comparison of regression coefficients. Both the AI and DM have high-frequency bands inside of their frequency gradients. The ratios of the response amplitudes of the DM and AI were plotted against the stimulus frequency to investigate whether the response property was similar in these two regions (Fig. 5, D and E). Although the AI is responsive to ultrasonic sounds, the DM is more responsive to ultrasonic sounds over 40 kHz than is the AI (Fig. 5E). These data suggest that the DM and AI have distinct frequency gradient directions for tone-response topographies and that the DM responds more vigorously to highfrequency sounds than the AI.

Differences in robustness of frequency gradients between regions have been confirmed using two-photon imaging (Issa et al. 2014). We have shown that the DM and AI have a robust, frequency-organized structure using flavoprotein fluorescence imaging. To evaluate the extent of heterogeneity in the frequency organization at the high-resolution scale, we performed in vivo two-photon imaging to determine the properties of neurons in the supragranular layers in the AI and DM (Fig. 6), Fig. 3. Interhemispheric projection between the auditory cortices. A: schematic drawing showing that a neuron in the subarea in the auditory cortex projects to the equivalent contralateral subarea. B-D: injection site of adeno-associated virus (AAV)-GCaMP3 in the right auditory cortex. The areal borders were drawn according to the flavoprotein fluorescence response to a 5-kHz tone (C). C and D: the images shown are the same except for the color range.  $\times$ , injection site. E: GCaMP3-positive axon terminals in the AI 5-kHz area in the left auditory cortex observed using a stereoscopic microscope. The top 10% pixels in intensity are colored green. F: GCaMP3-positive axon terminals visualized using a 2-photon microscopy in the place where an intense signal was observed in E. G: no GCaMP3-positive terminals found outside of the AI 5-kHz area. H: the large calcium responses to a 5-kHz tone derived from GCaMP3-positive terminals in the left AI. This response was obtained using an epifluorescent microscope under the same experimental condition as that to perform usual flavoprotein fluorescence imaging. Only the AI showed a much larger response in H due to GCaMP-stained terminals. Flavoprotein fluorescence responses, which were small compared with GCaMP signals, were observed in the 5-kHz area in the AAF and AII. Scale bars, 1 mm (B); 10 µm (F and G).



since the regional characteristic obtained by low-resolution imaging reflects an ensemble of the single neuronal properties in *layers II/III* (Andermann et al. 2011; Marshel et al. 2011; Tohmi et al. 2014). After identifying the precise location of the AI or DM using flavoprotein fluorescence imaging (Fig. 6A), we injected Fura-2 solution to observe the calcium responses in each neuron (Fig. 6B). The frequency at which the response amplitude reached the maximum in a given neuron was defined as the BF (Fig. 6C). Although the overall frequency gradients in the DM and AI had the same directions as those defined using flavoprotein fluorescence imaging, the frequency gradient in the DM neurons was less variable than that in the AI (Fig. 6, D and E). The degree of the frequency organization of

the microscopic structures was evaluated using residuals between each neuron and the regression line. The value of residuals was greater in the AI than in the DM (Fig. 6*F*; P < 0.001), implying that neuronal responses in *layers II/III* of the AI were more complex than those in the DM.

The DM was less sensitive to low-frequency tones than the AI in flavoprotein fluorescence imaging (Fig. 5*E*). This was explained by the neuronal distribution revealed by two-photon imaging; there were few neurons in the DM with BF <20 kHz (Fig. 6, *G* and *H*). In contrast, the bandwidth of the neurons in the DM and AI was similar (Fig. 6*I*). A tendency that bandwidth values obtained in two-photon studies, including this study, are smaller than those in electrophysiological study



#### NEWLY DELINEATED REGION IN MOUSE AUDITORY CORTEX

Fig. 4. Histological properties of the new region different from the AI in SMI-32 immunolabeling. A: functional location of the AI obtained using flavoprotein fluorescence imaging. Scale bar, 500 µm. B: sample images of SMI-32 immunolabeling patterns. We observed nonphosphorylated neurofilament (NNF) at sites a, b, and c, indicated in A. Scale bar, 100 µm. C: number of NNF-positive neurons in each area of the AI. Layers were identified based on Nissl staining in an adjacent section. D: typical images of an NNF-positive pyramidal and nonpyramidal neuron. E: ratio of the number of NNF-positive pyramidal/nonpyramidal neurons in the superficial layers (II, III, and IV). F: ratio of the number of NNFpositive pyramidal/nonpyramidal neurons in the deep layers (V and VI) and all of the layers (II–VI). \*P <0.05, Tukey-Kramer's test. Site a, n = 6; site b, n =6; site c, n = 7. NNF and biotinylated dextran amine (BDA) were visualized in the adjacent tissue sections.

(Guo et al. 2012) might be attributed to the property of a calcium-sensitive dye in detecting spikes.

Frequency organizations of the AAF. We also found the presence of the precise frequency organization in the AAF (Fig. 7). The neural response to a 5-kHz tone was placed at the most rostral part of the AAF. When mice heard tones at 5-80 kHz, neural responses to higher tones shifted to the ventrocaudal direction. No clear response was found near Stiebler's UF indicated by the white arrow (Fig. 7A, *inset*). The plotting of response peaks elicited by tones at 5-80 kHz also confirmed the presence of the precise octave-based frequency organization in the AAF (Fig. 7, B and C), as reported previously (Horie et al. 2013; Issa et al. 2014). Therefore, the low-high and high-low mirror-imaged frequency organizations between the

AAF and AI, reported in other species, were preserved in mice as well.

There is a discrepancy in the direction of the frequency organization of the AAF between studies using optical imaging (Fig. 7) (Honma et al. 2013; Issa et al. 2014) and studies using electrophysiology (Guo et al. 2012). The latest mapping study using electrophysiology considered the whole area, which was composed of the AAF, Stiebler's UF, and the DM in this study as a single region of the AAF, as shown by the schema in Fig. 8A (Guo et al. 2012), and the direction of the frequency organization of the AAF was drawn dorsocaudally (Fig. 1*C*). Therefore, we investigated NNF immunolabeling patterns and anatomical properties in the AAF, Stiebler's UF, and the DM (Fig. 8, B-E) to confirm our physiological mapping shown in Fig. 7.

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Fig. 5. Frequency organizations of the AI and DM revealed by flavoprotein fluorescence imaging. A: 2 frequency gradients for 5-80 kHz sounds in the AI and newly identified DM revealed by flavoprotein fluorescence imaging. The location of the 20-kHz response peak in the AI was used as the origin of coordinates on which data from several animals were superimposed. Black scale bar, 200 µm. See color-coding key on right. Top inset: response peak shifts in DM. Blue, 40 kHz; green, 60 kHz; red, 80 kHz; yellow, overlap between 60 and 80 kHz. White scale bar, 100 µm. Bottom inset: blue, 5 kHz; green, 10 kHz; yellow, 20 kHz; red, 50 kHz. White scale bar, 100 µm. B: a frequency gradient of the linear (left) and the log scales (right) in the AI. The horizontal axis is a line connecting points of 5 and 80 kHz (r = 0.85; P <0.001, Spearman's rank correlation test). The slope in the octave axis was 1.01 octave/125 µm. C: a frequency gradient in the DM (r = 0.69; P <0.001, Spearman's rank correlation test). The slope was 1.17 octave/125 µm. D: response amplitudes against each frequency in the AI (left) and DM (right). E: ratio of amplitudes of the DM to AI by each stimulus frequency (r = 0.63, P < 0.001, Spearman's rank correlation test). 5 kHz, n = 15; 10 kHz, *n* = 5; 20 kHz, *n* = 15; 30 kHz, *n* = 8; 40 kHz, *n* = 6; 50 kHz, *n* = 7; 60 kHz, *n* = 8; 70 kHz, n = 3; 80 kHz, n = 3.



First, NNF immunolabeling patterns of the AAF and Stiebler's UF were totally different (Fig. 8*C*); apparently, the gross immunolabeling pattern of Stiebler's UF was much weaker than that of the AAF (Fig. 8*C*). Although there was no difference in number of total NNF-positive neurons between the AAF and Stiebler's UF (Fig. 8*D*), Stiebler's UF lacked NNF-positive neurons in the *layer VI* (Fig. 8*E*), and the density of NNF immunolabeling within somas and dendrites per se was much weaker in Stiebler's UF, especially in the *layer III*, shown in Fig. 8*C*. Furthermore, the density of NNF immunolabeling in the DM was denser than that in the AAF (Figs. 4*B* and 8*C*), which is because the DM contained more than twice

as many NNF-positive neurons as did the AAF and Stiebler's UF (Fig. 8*D*). These data indicate that the area that was defined as the AAF in the past study (Guo et al. 2012) (Fig. 8*A*) includes three subregions with a distinct immunolabeling pattern.

The Stiebler's UF is more responsive to slow FM stimuli than to tones and does not have any frequency-organized structure (Honma et al. 2013; Stiebler et al. 1997), suggesting that the Stiebler's UF belongs to the belt region that receives thalamic inputs from the dorsal division of the MGB (MGd) (Winer and Schreiner 2011). Therefore, we investigated from which division of the MGB the Stiebler's UF receives dense





Fig. 6. Frequency organizations of the AI and DM revealed by 2-photon imaging. A: identification of subregions in the auditory cortex using flavoprotein fluorescence imaging. The image was obtained using a 35-kHz tone. Scale bar, 1 mm. B: example of staining with Fura-2 and sulforhodamine 101 in the auditory cortex. Scale bar, 50  $\mu$ m. C: sample traces of calcium response in a neuron with a best frequency (BF) of 60 kHz. Horizontal scale, 3 s; vertical scale, 2%; triangles, timing when a tone was given. A series of sounds at 80 dB sound pressure level was presented to a mouse. D: distribution of neurons in the DM. Data were obtained from 3 animals, and images were superimposed on the location of a 5-kHz response peak in the AI as the origin of coordinates obtained using flavoprotein fluorescence imaging. Color of each plot represents its BF. A significant, progressive increase in frequency was observed (P < 0.05, Spearman's rank correlation test; 163 neurons). Scale bar, 200  $\mu$ m. E: distribution of neurons in the AI. Data were obtained from 4 animals and reconstructed. A significant, progressive increase in frequency was observed (P < 0.001, Spearman's rank correlation test; 598 neurons). Scale bar, 200  $\mu$ m. E: distribution of neurons in the AI. Data were obtained from 4 animals and reconstructed. A significant, progressive increase in frequency was observed (P < 0.0001, Spearman's rank correlation test; 598 neurons). Scale bar, 200  $\mu$ m. F: comparison of the degree of the frequency organization between the AI and DM. The extent of disorder was evaluated using the distances of each numer frequency (\*\*\*P < 0.0001,  $\chi^2$  test). E: comparison of neuron bandwidths (BW) between the AI and DM, defined as the logarithmic ratio of minimum and maximum frequencies that resulted in a response >75% (P > 0.2, Mann-Whitney U-test) of the peak amplitude.

projections (Fig. 8F). We injected BDA solution into the Stiebler's UF as a retrograde tracer after identifying the region, according to the response to FM reversal stimuli (Fig. 2D), and evaluated neuronal distribution using coronal sections between 2.8 and 3.4 mm posterior to the bregma. The MGv and MGd were partitioned according to the SMI-32 immunolabeling patterns (Honma et al. 2013; Horie et al. 2013; LeDoux et al. 1985, 1987) and an atlas (Paxinos 2003; Paxinos and Franklin 2001; Paxinos et al. 2009). Whereas 64.6% neurons of BDAstained neurons were placed in the MGv, as much as 35.4% neurons were placed in the MGd (Fig. 8F). These data are clearly consistent with the results reported by Hofstetter and Ehret (1992), showing that the Stiebler's UF received robust thalamic projections from both MGd and MGv. In contrast, as for the region referred to as the AAF in the present study, 90% of the thalamic inputs originated from the MGv, and only 10% originated from the MGd (Fig. 8F), which is consistent with the fact that the AAF is one of the lemniscal core regions. Furthermore, we injected BDA solution into the DM to clarify the origin of the thalamic input. The DM presumably receives thalamic information from the MGv but not from the MGd, because the DM has the clear frequency organization shown in Fig. 5. As expected, it was revealed that as much as 97% neurons projecting to the DM were localized in the MGv, and remaining 3% neurons were located in the MGd (Fig. 8F). Overall, these data indicate that the AAF, Stiebler's UF, and DM are not combined into a single region but three different regions with distinct histological and anatomical properties. Hence, these data support our physiological mapping that reveals that the frequency gradient of the AAF travels ventro-caudally (Fig. 7).

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*Comparison of the effects of acoustic exposures between the AI and DM.* The number of cortical neurons with a particular characteristic frequency increases after animals are passively exposed for a long time to the tonal stimuli at the same frequency (de Villers-Sidani et al. 2007; Nakahara et al. 2004; Zhang et al. 2001). This plastic change can be observed, as response amplitude increases when observed by flavoprotein fluorescence imaging (Takahashi et al. 2006; Tohmi et al. 2009). The potentiation induced by the passive acoustic exposure exhibits regional and tonal specificity. Whereas the AAF is relatively insensitive to the passive acoustic exposure (Taka-



Fig. 7. Frequency organization of the AAF and AI. A: images of responses to a 5 (*left*)- and 30 (*middle*)-kHz tone and a superimposed image of these 2 images (*right*). The white arrow indicates the region defined as the high-frequency area of AAF in Guo et al. (2012). B: frequency-organized maps of the AI and AAF to 5–80 kHz sounds revealed by flavoprotein fluorescence imaging. The response peak of AI to 20 kHz is used as the origin of coordinates to superimpose the data in several animals. C: frequency gradient of the AAF. The horizontal axis is a line connecting points of 5 and 80 kHz (5 kHz, n = 15; 10 kHz, n = 5; 20 kHz, n = 15; 30 kHz, n = 8; 40 kHz, n = 6; 50 kHz, n = 7; 60 kHz, n = 8; 70 kHz, n = 3; 80 kHz, n = 3; P < 0.001).

hashi et al. 2006), the AI responses are clearly potentiated by the exposure (de Villers-Sidani et al. 2007; Nakahara et al. 2004; Zhang et al. 2001). We tested whether the plastic change to acoustic exposure was observed in the DM, which is expected to have different properties from those in the AI. We observed that when mice were reared under 5 kHz tone exposure from P7 to P35 (Fig. 9A), significant potentiation of neural responses to a 5-kHz tone occurred only in the AI  $(\Delta F/F_0 \text{ in normal environment}, 0.68 \pm 0.06\%, n = 10; quiet$ environment, 0.64  $\pm$  0.05%, n = 6; exposed, 0.87  $\pm$  0.04%, n = 14), and such potentiation was not found in the AAF or AII (Fig. 9, B and C). In the AAF, AI, AII, and DM, the responses to a 35-kHz tone were not clearly potentiated after exposure to a 5-kHz tone because of the frequency specificity in the potentiation (Fig. 10A), as reported previously (Takahashi et al. 2006). When mice were exposed to a 35-kHz tone, significant potentiation was observed only in the AI (normal environment, 0.95  $\pm$  0.08%, n = 10; quiet environment, 0.95  $\pm$ 0.05%, n = 6; exposed,  $1.09 \pm 0.03\%$ , n = 14), whereas no potentiation was observed in the DM as in the AAF or AII (Fig. 9, D and E). Although the size of responsive areas after the plasticity occurred seemed to be expanded in an experience-dependent manner, the location of the AI peak was not shifted by the plasticity. Therefore, it is unlikely that the AI and DM have merged by the plasticity. The neural responses were not potentiated in all three areas to a 5-kHz tone (Fig. 10B). Flavoprotein fluorescence imaging showed that responses of the DM to a 5-kHz tone were very weak and diffused and did not show robust, clear peaks in response to a 5-kHz tone (Fig. 2*B*). Moreover, single neuronal analysis showed that there were few low-frequency neurons in the DM (Fig. 6). Therefore, reliable analysis was not possible regarding DM responses to a 5-kHz tone, and we used only a 35-kHz tone to evaluate the effect of acoustic exposures. Overall, the resistance to plasticity of the DM indicates that the DM is a functionally distinct region from the AI.

Thalamocortical projections from the MGB to the DM. Auditory cortical regions have different latencies to auditory stimuli across species. In previous studies, neurons in the AAF were activated with shorter latencies than those in the AI (Kubota et al. 2008; Linden et al. 2003; Sawatari et al. 2011). We verified the latency of the neural response to a 35-kHz tone in the DM using flavoprotein fluorescence imaging (Fig. 11). The latency was evaluated as the time until the fluorescence signal reached 25% or 50% maximum of the peak. The latency of the AAF response to a 35-kHz tone was shorter than that of the AI, as we described previously (Fig. 11A) (Kubota et al. 2008), which is consistent with the results obtained by voltagesensitive dye imaging (Sawatari et al. 2011). The latency of the DM was also shorter than that of the AI and comparable with that in the AAF (Fig. 11A). The latencies to reach 25% maximum were 122.2  $\pm$  5.4 ms in the AAF, 120.2  $\pm$  4.5 ms in the DM, 142.0  $\pm$  4.7 ms in the AI, and 146.0  $\pm$  5.8 ms in the AII (n = 18, each; Fig. 11B). The latencies to reach 50% maximum were 185  $\pm$  4.7 ms in the AAF, 189.3  $\pm$  5.6 ms in the DM, 212.5  $\pm$  6.5 ms in the AI, and 215.6  $\pm$  7.5 ms in the AII (n = 18, each; Fig. 11B). The latencies to reach 25% and

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50% maximum had the same tendency. The latencies of the AAF and DM were significantly shorter than those of the AI and AII, and there were no significant differences between the AAF and DM or the AI and AII (Fig. 11*C*). These data also support the fact that the DM receives thalamocortical projections directly from the MGv but not via the AAF or AI.

The mouse MGv is composed of several compartments that topographically project to their corresponding subregions in the auditory cortex (Horie et al. 2013; Takemoto et al. 2014). The region that projects to the AAF or AI is localized in the middle part of the MGv. On these bases, we tried to identify the region that projected to the DM. First, we injected BDA into the AI or DM after identification, and we evaluated the locations of neurons that project to the AI or DM using coronal sections (Fig. 12). Neurons projecting to the DM were found in the ventral half of the MGv, and those projecting to the ventral AI high-frequency area were observed in a neighboring region located at the same ventrodorsal level (Fig. 12). However, we found that neurons projecting to the DM were located rostral to those projecting to the AI as a whole. Therefore, we prepared horizontal sections to evaluate the relative rostrocaudal location of neurons projecting to the DM and AI (Fig. 13). We injected Alexa Fluor-conjugated CTB into the ventral AI highfrequency area or the DM, identified using a tone at 40 kHz (Fig. 13, A and B). In horizontal sections of the MGv, the neurons projecting to the DM were located more rostrally than the neurons projecting to the AI (Fig. 13, C-F). The relative location of the neuronal population projecting to the DM was measured in reference to the averaged coordinates of neurons projecting to the AI (Fig. 13J). The neurons projecting to the DM were located significantly more rostral and more medial compared with those projecting to the AI (P < 0.001, for both).

Fig. 8. Difference in histological and anatomical properties among the AAF, Stiebler's UF, and DM. A: schematic drawing of the auditory cortical map. The area enclosed by the blue oval was the definition of the AAF proposed by Guo et al. (2012). SUF, Stiebler's UF. B: auditory cortical map revealed by flavoprotein fluorescence responses. The area enclosed by the white rectangle is equivalent to that in the blue oval in A. Scale bar, 500 µm. C: sample images of SMI-32 immunolabeling patterns in the AAF and Stiebler's UF. Scale bar, 100 µm. D: number of NNF-positive neurons in the AAF, Stiebler's UF, and DM. \*P < 0.05, Tukey-Kramer's test; AAF, n = 5; Stiebler's UF, n = 4; DM, n = 7. E: number of NNF-positive neurons in the laver VI and the others of the AAF and Stiebler's UF. \*P <0.05, Mann-Whitney U-test; AAF, n = 5; Stiebler's UF, n = 4. F: percentage of neuronal distribution in the medial geniculate body [MGB; the ventral division of MGB (MGv) or the dorsal division of MGB (MGd)] by their cortical target. \*\*P < 0.01; \*\*\*P < 0.000001,  $\chi^2$  test after Bonferroni correction; to AAF, n = 150 neurons from 3 mice; to Stiebler's UF, n = 144 neurons from 6 mice; to DM, n = 316 neurons from 7 mice.

Next, we tested whether the frequency organization in the DM reflects the distinct frequency organization in a single region within the MGv or partly overlapped regions projecting to the AI and DM, as suggested by the fork-shaped, frequencyorganized map proposed by Issa et al. (2014) (Fig. 1C). We injected fluorescent CTB into the low- and high-frequency regions of the DM. Results clearly indicated that the frequency organization in the MGv projecting to the DM was structured along a single latero-medial axis within a single compartment of the MGv (Fig. 13, G-I). The quantitative distribution map showed a significant place shift in location between the areas projecting to the low- and high-frequency areas of the DM (Fig. 13K; P < 0.001). These data indicate that neurons projecting to the DM are localized in the rostral part of the MGv with a distinct frequency organization along the lateromedial axis, and no fewer than four compartments were independent from each other within the MGv (Fig. 13L).

*Vocalization processing in the auditory cortex.* Although the frequency gradient in the AI also includes ultrasonic bands, the responses of the DM to ultrasonic tones were larger than those of the AI (Fig. 5*E*). Therefore, we verified the possibility that the DM may be involved in ultrasonic courtship songs produced by male mice for females (Fig. 14A). Although this courtship vocalization produced by male mice is one of the representative ultrasonic, communication-related sounds (Asaba et al. 2014; Hammerschmidt et al. 2009; Holy and Guo 2005), as well as isolation calls produced by pups (Ehret and Haack 1982; Thornton et al. 2005; Uematsu et al. 2007), no research has been performed about central processing of courtship songs, although several studies about pup isolation calls have been reported (Galindo-Leon et al. 2009). The courtship songs produced by male mice include characteristic features,

P7

**A** P0



С Fig. 9. Plasticity after acoustic exposure in the AI and DM. A: schedule of experiments. Imaging was per-AAF formed within 1 wk after cessation of acoustic expo-1.0 sure. P, period. B and C: response amplitudes to a 5-kHz tone in mice exposed to a 5-kHz tone (\*P <0.8  $\Delta F/F_{0}$  (%) 0.05, Mann-Whitney U-test; normal, n = 10; quiet, n =0.6 6; exposed, n = 14). Mice rearing in a normal home 0.4 cage (Normal), in the chamber without presentation of 0 2 any tones (Quiet), or in the chamber with acoustic exposure (Exposed). D and E: response amplitudes to a 35-kHz tone in mice exposed to a 35-kHz tone (\*P <0.05, Mann-Whitney U-test; normal, n = 10; quiet, n =6; exposed, n = 14).



P35

P41

such as a high-frequency band over 50 kHz, fast FM, pitch jumps, and intermittent rhythms (Fischer and Hammerschmidt 2011; Lahvis et al. 2011). The AAF, AI, AII, and DM in both sexes were clearly activated by stimulation with songs produced by males (Fig. 14*B*). Interestingly, the temporal order of the rising phase of neural responses to a male's courtship song was different from that of an artificial tone (Fig. 14, *C* and *D*). The DM was activated first, followed by simultaneous activation of the AAF and AI, and the AII was activated last. The latency to reach 25% maximum was  $143 \pm 6.2$  ms in the DM,  $170 \pm 9.4$  ms in the AAF,  $181 \pm 7.4$  ms in the AI, and  $208 \pm 13.7$  ms in the AII (Fig. 14*E*), and the latency to reach 50% maximum was  $243 \pm 6.5$  ms in the DM,  $279 \pm 8.7$  ms in the

AAF,  $291 \pm 9.3$  ms in the AI, and  $340 \pm 11.9$  ms in the AII (n = 20 in total; male, n = 7; female, n = 13; Fig. 14E). As for the latency, there were no differences between male and female mice in all of the regions. These data indicate that vocalization is similarly processed in the DM of both male and female mice regarding the latency of the DM responses to a male's courtship song. In contrast, the amplitudes of the response to a male's song were significantly larger in females than in males in the DM alone (Fig. 14F; male,  $1.61 \pm 0.10\%$ , n = 15; female,  $2.0 \pm 0.19\%$ , n = 16; P < 0.05). The response amplitudes in the AAF, AI, and AII were slightly larger in females than in males, although there were no significant differences between the sexes (AAF, P > 0.6; AI, P > 0.8;

0.2

0.2

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0.2

2912

0.2

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Fig. 10. . Frequency specificity in plasticity after acoustic exposure. A: the response amplitudes to a 35-kHz tone in mice exposed to a 5-kHz tone. There was no significant difference between normal and exposed or quiet and exposed mice. Normal, n = 10; quiet, n = 6; exposed, n = 14. B: the response amplitudes to a 5-kHz tone in mice exposed to 35-kHz. There was no significant difference between normal and exposed or quiet and exposed mice. Normal, n = 10; quiet, n = 6; exposed, n = 14.

AII, P > 0.5). The slight difference in the AAF, AI, and AII might be attributed to the significant difference in the DM, as it was reported that neural responses in a downstream region with a longer latency are affected by neural responses in an upstream region with a shorter latency in the auditory cortex (Kubota et al. 2008). As expected from this idea, there was no

difference between the sexes in response to a 70-kHz artificial tone (Fig. 14G). These data imply that ultrasonic courtship songs produced by males, mediated via a distinct rostral compartment of the MGv, reach the DM first and are processed further through pathways from the DM to other cortical regions in mice.



Fig. 11. Responses in the DM and AAF faster than those in the AI and AII. A: temporal profiles of  $\Delta F/F_0$  in the AAF, AI, and DM in response to a 35-kHz tone. Frame rate was set at 54 Hz. Horizontal black bars indicate the stimulus period. B: times required to reach 25% (*left*) and 50% (*right*) of the maximal  $\Delta F/F_0$  after the stimulus onset in the AAF, DM, AI, and AII (\*\*\*P < 0.001, \*\*P < 0.01, 1-way repeated-measures ANOVA; n = 18 each). C: schema of latencies reaching 25% maximum by color. The numbers indicate the order of response.

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#### DISCUSSION

In the present study, we observed the regional characteristics in the mouse auditory cortex using flavoprotein fluorescence imaging, two-photon calcium imaging, immunohistochemistry, and tracer experiments. The regional borders of the AI were re-delineated by isolating the DM, which has been reported to be a part of the AI, specialized for processing high-frequency sound signals in mice (Guo et al. 2012; Issa et al. 2014; Joachimsthaler et al. 2014; Sawatari et al. 2011; Stiebler et al. 1997). In the present study, we identified the DM as the fifth frequency-organized region in the cortex, in addition to the three frequency-organized regions-the AAF, AI, and AII-in the auditory cortex (Issa et al. 2014; Kubota et al. 2008) and the insular auditory field (IAF) in the insular cortex (Gogolla et al. 2014; Sawatari et al. 2011; Takemoto et al. 2014). We demonstrated that the frequency gradient of the AI ran from the dorsocaudal to ventrorostral direction, whereas the frequency gradient in the DM ran from the ventrocaudal to dorsorostral direction. The direction of the frequency organization of the AAF was from dorsorostral to ventrocaudal, consistent with recent reports (Horie et al. 2013; Issa et al. 2014; Kubota et al. 2008). The DM responses to a courtship song by males were significantly larger in female mice, which is the first evidence to suggest that the gender-specific biological importance of courtship songs might be reflected in the properties of the mouse auditory system.

Technical merits of using flavoprotein fluorescence imaging to investigate cortical functions in mice. There were several technical advantages in the present study to localize the newly found DM region. First, we used flavoprotein autofluorescence imaging to visualize the mouse auditory cortex. Flavoprotein fluorescence imaging gave us very similar results to those obtained in GCaMP3-expressing mice (Issa et al. 2014) regarding the regional borders between the DM and surrounding regions. This method detects neural activity based on activitydependent oxygen metabolism; therefore, there is no need to stain neurons using dye solutions, thus allowing uniform observability on the cortical surface (Llano et al. 2009; Shibuki et al. 2003, 2006) and allowing the delineation of regional borders, as in our previous studies (Honma et al. 2013; Kubota et al. 2008; Ohshima et al. 2010). Because of the merits of intrinsic fluorescence imaging, we were able to find a small trough in neuronal activity between the AI and DM (Fig. 2), as seen in GCaMP3-expressing mice (Issa et al. 2014). A critical requirement for delineating small cortical regions is the uniform distribution of fluorophores in the brain, which was based on endogenous flavoproteins or homogenously expressed GCaMP3. In contrast, unit recording requires the insertion of an electrode into the cortex, and therefore, the recording sites must be set at some intervals. Therefore, the regional border, such as that between the AAF and AI, must be defined as a result of the assumption that the border corresponds to the frequency-gradient reversal line based on unit recording de-



Fig. 13. Distribution of MGv neurons projecting to the DM in horizontal sections. A: injection sites in the AI and DM. Image of the brain surface and merged images obtained through a green or a red filter. The dotted lines indicate the outlines of responses to a 40-kHz tone. Scale bar, 1 mm. D, dorsal; R, rostral. B: injection site after a horizontal slice was prepared. The dotted lines indicate the outlines of the auditory cortex. *Inset*: the same image observed under bright-field microscopy. Scale bars, 500  $\mu$ m. L, lateral. C: bright-field view of the MGB in a horizontal slice. The dotted rectangle represents the window containing images of *D–F*. Scale bar, 500  $\mu$ m. LGB, lateral geniculate body. *D–F*: neurons projecting to the AI ventral high-frequency area and DM (*F*). The arrowheads indicate the lateral edge of the MGv as shown in *C*. Scale bar, 100  $\mu$ m. *G–I*: neurons projecting to the low- and high-frequency areas of the DM (*I*). Scale bar, 100  $\mu$ m. *J*: the relative coordinates of neurons projecting to the AI was set as the origin. In 6 mice, 71 neurons projecting to the AI and 103 neurons projecting to the DM were found. \*\*\*P < 0.001, Mann-Whitney U-test. *K*: the relative coordinates of a low-frequency area was set as the origin. In 3 mice, 33 neurons projecting to the low and high-frequency area were found. \*\*\*P < 0.001, Mann-Whitney U-test. *L*: 3-dimensional schematic view of the relative locations of 4 compartments within MGv. The schema of the compartment projecting to the insular auditory field (IAF), AAF, or AI was compensated, according to the elegant study by Takemoto et al. (2014) and, in part, the study by Horie et al. (2013).

scribed in previous studies. Thus it is no wonder that the small trough between the DM and the ventral AI high-frequency area was not found in previous studies using unit recording. The DM is sensitive to ultrasonic sounds over 40 kHz, and the

response of the AI is somewhat weaker than that of the DM. These factors might have led to the conclusion that a single frequency gradient extended from the AI low-frequency area to the DM in previous electrophysiological experiments (Guo et

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Fig. 14. Auditory responses to courtship male songs. *A*: recorded spectrogram of a courtship song produced by a male C57BL/6 mouse when approaching a female (*top*). The red box indicates the segment used in this study as a stimulus (*bottom*). *B*: the typical image of the response in the auditory cortex to the male's song. The dorsal anterior "UF" region identified by Stiebler et al. (1997) was not activated, probably because this region is responsive to slow FM sweeps, ~24 kHz/s, which were not included in the male's songs. The DM was identified based on the responses to a 70-kHz tone, as performed in the previous sections. The response peaks of the DM to a 70-kHz tone and a courtship song did not shift significantly. *C*: temporal profiles of  $\Delta F/F_0$  in each region in response to the male's song (n = 20). Images were obtained at 54 Hz. *D*: schema showing the latency of responses to reach 25% maximum. *E*: the time required to reach 25% (*left*) or 50% maximum (*right*); male, n = 7; female, n = 13. *Insets*: statistical significance of pair-wise comparisons between regions with respect to latency required to reach 25% maximum (*left*) and 50% maximum (*right*) of the response peak to a male's song. The sexes were mixed, because there was no difference between them. \*\*\**P* < 0.001, \**P* < 0.05, 1-way repeated-measures ANOVA; n = 20. *F*: response amplitudes to a male's song (*left* axis). The blue plots indicate the difference in averaged amplitudes between females and males (*right* axis); male, n = 15; female, n = 16. \**P* < 0.05, Mann-Whitney U-test. *G*: response amplitudes to a 70-kHz ultrasonic tone; male, n = 15; female, n = 16.

al. 2012) or that the strong frequency gradient traveled across these two different regions (Issa et al. 2014).

Another benefit of flavoprotein fluorescence imaging is that variability of the signal amplitudes in  $\Delta F/F_0$  is very small in

each mouse, especially when transcranial imaging is performed. Because the skull of a mouse is transparent, and flavoproteins exist originally in neurons, we can observe the same pattern of responses transcranially without craniotomy,

which might confound the results. Moreover, as flavoproteins are an endogenous protein in the mitochondrial electron transport chain, it is very unlikely that flavoproteins work as a calcium chelator and have some artificial effects on the calcium dynamics, which is essential for the induction of cortical plasticity (Zucker 1999).

Finally, we were able to combine flavoprotein fluorescence imaging with anatomical techniques using fluorescent traces, because the flavoprotein signals were much weaker than those from the fluorescent tracers. The combination of flavoprotein fluorescence imaging and microinjection of a retrograde fluorescent tracer could reveal multiple compartments in the MGv. Because the MGv is located deep within the brain, it is difficult to perform precise MGv experiments without anatomical studies. Direct optical imaging to observe responses in the MGv has not been reported. Although functional MRI (fMRI) imaging can be used to observe activities deep within the brain, the spatial resolution is too low to find multiple compartments in the mouse MGv. Without the fine spatial resolution achieved by anatomical studies, the precise multicompartments with a distinct frequency gradient (Fig. 13L) could not be visualized, because the general concept of one frequency organization in one region is also applicable in mice. The combination of optical imaging and localized tracer injection into functionally identified, small cortical regions is expected to be greatly advantageous for detecting fine topological organization in other sensory cortices other than the auditory cortex.

New definition of regions included in the mouse auditory cortex. Our findings suggest that the term UF might not be appropriate. Stiebler et al. (1997) defined the UF as a distinct, frequency-unorganized region, where neurons have a characteristic frequency over 50 kHz (Fig. 1A). Then, UF as a distinct region was denied, and the term UF has also been used to indicate the high-frequency area of the AAF and AI (Fig. 1B) (Guo et al. 2012) or only the dorsal AI high-frequency area (Fig. 1C) (Issa et al. 2014). However, optical imaging revealed that frequency direction of the AAF runs ventrocaudally (Issa et al. 2014) and that the AII has the distinct frequency organization (Issa et al. 2014; Kubota et al. 2008). By delineating the DM, we have identified the four distinct frequency-organized regions of the AAF, AI, DM, and AII, in total, each of which has its own high-frequency area (up to 80 kHz) corresponding to the UF. Optical imaging also revealed that a vacant region insensitive to pure tones containing vocalization-specific neurons is located between the AAF and AI dorsal high-frequency area (Issa et al. 2014), and we have already reported that the region between the AAF and DM is responsive to slow FM components, regardless of tonal frequency range (Fig. 1D) (Honma et al. 2013; Tsukano et al. 2013b). These facts indicate that the distinct FM-sensitive region exists in this area in the mouse auditory cortex, similar to that observed in bats (Suga and Jen 1976). Actually, there is a possibility that Stiebler et al. (1997) might perform recordings without distinguishing between the DM and frequency-unorganized, FM-sensitive region. They mentioned in their paper that neurons observed in the rostral part of the UF were FM sensitive but not tone sensitive (Stiebler et al. 1997). The confusion of two different regions might obscure a frequencyorganized structure and lead to the misconception that UF was not frequency organized (Stiebler et al. 1997). Overall, we propose to subdivide the dorsal part of the auditory cortex precisely and to assign new, anatomical names: the dorsoanterior field (DA) to the FM-sensitive region just dorsal to the AAF and the DM to the region newly identified in the present study (Fig. 1*D*), in addition to the classical DP named previously (Stiebler et al. 1997).

Multiple frequency organizations have been found in various mammals. By identifying the DM with a distinct frequency gradient using flavoprotein fluorescence imaging, multiple frequency organizations were revealed in the mouse auditory cortex in the present study. In primates, frequency-organized structures exist in the belt region besides the core region. With the use of fMRI, it was revealed that the caudal-medial (CM) field in macaques, which belongs to the belt, has stronger frequency gradient than do fields in the core, and other belt fields also have a frequency gradient (Petkov et al. 2006). Moreover, multiple frequency-organized structures were also found in rodents, such as guinea pigs (Nishimura et al. 2007), ferrets (Bizley and King 2009), chinchillas (Harel et al. 2000), and rats (Higgins et al. 2010; Kalatsky et al. 2005) using optical imaging. Especially the auditory cortical maps revealed in rats (Higgins et al. 2010; Kalatsky et al. 2005) are quite similar to those we revealed in mice. Several imaging studies have revealed that the AII is frequency organized in mice (Issa et al. 2014; Kubota et al. 2008). The IAF in the mouse insular cortex anterior to the auditory cortex is also frequency organized (Sawatari et al. 2011; Takemoto et al. 2014). Hence, at least five frequency-organized regions exist inside and near the auditory cortex in mice. Each frequency-organized region receives distinct thalamic information from equivalent compartments with a distinct frequency organization in the MGv (Fig. 13L) (Horie et al. 2013; Takemoto et al. 2014), and the sound responses are presumably conveyed through the course of the hierarchical, inter-regional processing in the auditory cortex (Fig. 14) (Kaas and Hackett 2000; Kubota et al. 2005).

The basic concept of "core and belt" prevails in the auditory cortex across species (Winer and Schreiner 2011). The core region contains the AAF and AI, which receive dense projections from the frequency-organized MGv and process lemniscal information from the cochlea. The belt region surrounding the core receives nonlemniscal information from the frequency-unorganized MGd across species basically (Winer and Schreiner 2011). Although the DM in mice is located dorsal to the AAF and AI and should be considered as the belt (Fig. 1D), the DM has a clear frequency gradient and receives topographic thalamic inputs directly from the MGv (Fig. 13). In rats, the ventral auditory field, which is frequency organized and placed ventral to the AAF and AI, also receives dense thalamic projections from the caudal part of the MGv (Storace et al. 2010). These findings suggest that the belt regions in rodents receive frequency-organized, lemniscal-thalamic information directly. In contrast, the belt fields with a frequency organization, including the CM, receive thalamic inputs from the frequency-unorganized MGd in marmosets (de la Mothe et al. 2012), suggesting that the frequency representation in the belt may derive from the core after intracortical interactions in primates. Although these present and prior observations suggest that rodents and primates have multiple frequency-organized belt auditory fields, it remains to be seen to what degree these fields are functionally homologous or analogous between rodents and primates.

Many animal species use ultrasonic frequencies for navigation or communication with others. Actually, rodents communicate with each other using ultrasonic frequency vocalization over 50 kHz. Ultrasonic songs produced by male mice induce exploratory behaviors in female mice (Hammerschmidt et al. 2009; Holy and Guo 2005), and isolated pups attempt to communicate with their mother by producing isolation calls (Ehret 2005; Hahn and Schanz 2005). Therefore, it is critical for mice to receive and process ultrasonic sounds. The DM has fewer neurons with low BFs and responds well to ultrasonic sounds compared with the AI (Figs. 5, D and E, and 6). Moreover, strong plasticity is not induced in the DM by acoustic exposure (Fig. 9). The degree of variability in the frequency organization in cellular resolution recording is less marked in the DM than in the AI (Fig. 6). These data imply that the DM is not dynamically regulated by learning; instead, the DM is dedicated for processing some congenitally important sounds, such as courtship chirps, as shown in Fig. 14.

The DM may play a role of recipient and relay point for processing of courtship songs. The thalamocortical auditory pathway comprises parallel pathways, and each region in the auditory cortex receives a topological projection from the corresponding subregion in the MGv (Horie et al. 2013; Takemoto et al. 2014). The DM receives dense projections directly from the rostral part of the MGv (Fig. 13) and is the first region to be activated in both sexes by a male courtship song. These data imply that the MGv determines the region of the auditory cortex to which song information will be sent. A characteristic waveform and frequency included in voices emitted under a specific condition (Hammerschmidt et al. 2009; Holy and Guo 2005; Insel et al. 1986) may selectively activate gating channels in the thalamus (Schiff et al. 2013) to choose a specific recipient in the auditory cortex. Furthermore, there was a significant difference in response amplitudes of a male courtship song in the DM between sexes (Fig. 14F), which may reflect the results of previous behavioral studies that female mice respond specifically to male courtship songs (Hammerschmidt et al. 2009). The auditory cortex is required for fear conditioning using complex sounds (Letzkus et al. 2011). Therefore, subregions of the auditory cortex may themselves have roles for evaluating the biological meanings attached to natural sounds.

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#### DISCLOSURES

The authors declare no competing interests.

#### AUTHOR CONTRIBUTIONS

Author contributions: H. Tsukano, R.H., M.K., K.T., H. Takebayashi, and K.S. conception and design of research; H. Tsukano, M.H., T.B., and A.U. performed experiments; H. Tsukano, M.H., T.B., and A.U. analyzed data; H. Tsukano, M.H., and K.S. interpreted results of experiments; H. Tsukano prepared figures; H. Tsukano drafted manuscript; H. Tsukano, M.K., H.

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**ORIGINAL** 

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# Impaired clustered protocadherin-α leads to aggregated retinogeniculate terminals and impaired visual acuity in mice

Reiko Meguro,\* Ryuichi Hishida,† Hiroaki Tsukano,† Kohei Yoshitake,†'‡ Ryota Imamura,† Manavu Tohmi,† Takashi Kitsukawa,‡'§ Takahiro Hirabayashi,‡'§ Takeshi Yagi,‡'§ Hirohide Takebayashi\* and Katsuei Shibuki†'‡

\*Division of Neurobiology and Anatomy, Graduate School of Medical and Dental Sciences, Niigata University, Niigata, Japan

†Department of Neurophysiology, Brain Research Institute, Niigata University, Niigata, Japan ‡Japan Science and Technology Agency-Core Research for Evolutional Science and Technology (CREST), Osaka University, Suita, Japan

§KOKORO-Biology Group, Graduate School of Frontier Biosciences, Osaka University, Osaka, Japan

#### Abstract

Clustered protocadherins (cPcdhs) comprising cPcdh- $\alpha$ , - $\beta$ , and - $\gamma$ , encode a large family of cadherin-like cell-adhesion molecules specific to neurons. Impairment of cPcdh- $\alpha$  results in abnormal neuronal projection patterns in specific brain areas. To elucidate the role of cPcdh- $\alpha$  in retinogeniculate projections, we investigated the morphological patterns of retinogeniculate terminals in the lateral geniculate (LG) nucleus of mice with impaired cPcdh- $\alpha$ . We found huge aggregated retinogeniculate terminals in the dorsal LG nucleus, whereas no such aggregated terminals derived from the retina were observed in the olivary pretectal nucleus and the ventral LG nucleus. These

Clustered protocadherins (cPcdhs) are the largest subgroup of the cadherin superfamily of neuronal adhesion molecules (Kohmura et al. 1998; Junghans et al. 2005; Shapiro et al. 2007; Yagi 2012). Mammalian cPcdh genes consist of three families, cPcdh- $\alpha$ , - $\beta$ , and - $\gamma$ , each of which has a clustered structure in a small genome locus on a single chromosome (Wu 2005). In normal mice, olfactory sensory neurons expressing the same odorant receptor project their axons into a specific glomerulus in the main olfactory bulb (Mori and Sakano 2011). However, these projection patterns are abnormal in cPcdh-α knockout (KO) mice, in which the constant region of cPcdh-a is deleted (Hasegawa et al. 2008). Furthermore, abnormal aggregation of serotonergic axons is observed in cPcdh-a KO mice (Katori et al. 2009), suggesting that cPcdh- $\alpha$  is important for the normal development of axonal projection patterns derived from specific types of neurons.

aggregated terminals appeared between P10 and P14, just before eye opening and at the beginning of the refinement stage of the retinogeniculate projections. Reduced visual acuity was observed in adult mice with impaired cPcdh- $\alpha$ , whereas the orientation selectivity and direction selectivity of neurons in the primary visual cortex were apparently normal. These findings suggest that cPcdh- $\alpha$  is required for adequate spacing of retinogeniculate projections, which may be essential for normal development of visual acuity.

**Keywords:** aggregated terminals, lateral geniculate nucleus, protocadherin, retinal terminals, visual acuity. *J. Neurochem.* (2015) **133**, 66–72.

In the visual system of mice, cPcdh- $\alpha$  is expressed in the optic nerve fibers, in the lateral geniculate nucleus (LG), and in the primary visual cortex (V1) for 2 weeks after birth (Morishita *et al.* 2004). This period before eye opening is very important for eye-specific segregation of the retinogeniculate fibers (Jaubert-Miazza *et al.* 2005; Guido 2008), the

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Correspondence and present address: Reiko Meguro, School of Health Sciences, Faculty of Medicine, Niigata University, 2-746 Asahi-machi, Chuo-ku, Niigata 951-8518, Japan. E-mail: meguror@clg.niigata-u.ac.jp

Abbreviations used: BDA, biotinylated dextran amine; cPcdh, clustered protocadherin; CTb, cholera toxin  $\beta$  subunit; DAB, diaminobenzidine; IGL, intergeniculate leaflet; LG, lateral geniculate nucleus; LGd, dorsal LG; LGv, ventral LG; OPT, olivary pretectal nucleus; PBS, phosphate-buffered saline; V1, primary visual cortex.

refinement of retinogeniculate synapses (Chen and Regehr 2000; Hooks and Chen 2006, 2008; Liu and Chen 2008), and the maturation of inhibitory neurons (Golding *et al.* 2014) in the dorsal LG (LGd). In the present study, we thus investigated the morphology of retinogeniculate terminals in cPcdh- $\alpha$  KO mice and found abnormally aggregated terminals mainly in LGd. These aggregated terminals found in adults were also observed at P14 but not until P10. The abnormal aggregation can result in impaired transmission of visual information through LGd. As expected, we found significantly deteriorated visual acuity in cPcdh- $\alpha$  KO mice, which was determined using a behavioral test (Prusky *et al.* 2000), whereas the orientation tuning of V1 neurons was apparently normal in cPcdh- $\alpha$  KO mice.

#### Materials and methods

#### Animal experiments

We used male and female mice of cPcdh- $\alpha$ -KO (Hasegawa *et al.* 2008) and C57BL/6J strain obtained from Charles River Japan (Tokyo, Japan). All experiments were performed in accordance with the guide for the Care and Use of Laboratory Animals of the Science Council of Japan, and were approved by the Animal Experiment Committee of Niigata University.

#### Neural tracing experiments

Five wild type (WT) mice and five cPcdh-a KO mice of aged 8-10 weeks were anesthetized intraperitoneally with a mixture of ketamine (300 mg/kg; Daiichi-Sankyo, Tokyo, Japan) and xylazine (30 mg/mL; Bayer, Leverkusen, Germany). The cholera toxin  $\beta$ subunit (CTb, 5 ng in 1 µL of saline; List Biological Laboratories, Campbell, CA, USA), an anterograde neural tracer, was injected into the left eye using a glass pipette. Five days after the injection, the mice were anesthetized with an overdose of pentobarbital (100 mg/kg, i.p.) and perfused with phosphate-buffered saline, followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2). For the developmental study, two WT and two cPcdh- $\alpha$  KO mice at each of P10, P14, and P19 were tested in a similar manner with a minimum survival time of 48 h for CTb transportation (Wu et al. 2000). The transported CTb in LG was visualized as described previously (Horie et al. 2013). Briefly, 50 µm coronal sections, prepared using a cryostat, were incubated with a goat polyclonal anti-CTb antibody (1:8000 dilution; List Biological Laboratories) overnight, followed by incubation with a biotinylated anti-goat IgG antibody (1:200 dilution; Jackson, West Grove, PA, USA) for 2 h. The sections were further incubated with an avidin-biotin complex (1:100 dilution; Vector Laboratories, Burlingame, CA, USA) and the CTb immunoreactivity was visualized with oxidized diaminobenzidine. The reacted sections were mounted on gelatin-coated glass slides and air-dried. Some serial CTb-labeled sections were counter-stained with cresyl violet. Sections were observed using a microscope (BX-50; Olympus, Tokyo, Japan) and images were acquired using a CCD camera (CS-600; Olympus). The terminal size measurement was taken using a program based on Matlab (Mathworks, Natick, MA, USA). To visualize the corticogeniculate fibers in two of the each WT and cPcdh-a mice, biotinylated dextran amine (BDA-3000; Invitrogen, Carlsbad, CA, USA) was injected into V1, as described previously (Horie *et al.* 2013; Tohmi *et al.* 2014).

#### Two-photon calcium imaging of V1 neurons

Orientation selectivity and direction selectivity in V1 neurons were tested using two-photon calcium imaging in five WT and five cPcdh-a KO mice. The two-photon calcium imaging was performed as described previously (Honma et al. 2013; Yoshitake et al. 2013; Tohmi et al. 2014). Briefly, V1 was identified using flavoprotein fluorescence imaging in mice anesthetized with urethane (1.75 g/kg, i.p.). After removing the skull above V1, a glass pipette filled with the cocktail of fura-2 AM (Invitrogen) and sulforhodamine 101 (SR-101; Invitrogen) was inserted at the center of V1 and was advanced into the supragranular layers, down to a depth of 200-300 µm from the surface. The cocktail was injected using a pressure of 4-15 kPa for 5-10 min, so that the cells in the area located 200-300 mm from the tip of the pipette were labeled with fura-2. Astrocytes labeled with SR-101 were excluded from further analysis. After the mice were set under a two-photon microscope (TCS SP5 MP; Leica Microsystems, Wetzlar, Germany), grating patterns moving in eight directions (from 0° to 315° in 45° steps) were administered to the mice and V1 neuronal responses were analyzed, as described previously (Honma et al. 2013; Yoshitake et al. 2013).

#### Behavioral assessment of visual acuity

Visual acuity of mice was measured via a visual water maze task developed by Prusky and coworkers (Prusky et al. 2000; Prusky and Douglas 2004). Ten WT and nine cPcdh- $\alpha$  KO mice (aged 7-12 weeks) were trained and tested in the visual water maze task in 32 trials every day to assess their visual acuity. During the training phase, they were trained to choose a sine-wave vertical grating pattern at a low spatial frequency (0.14 cycles/degree) from a homogeneous gray pattern with the same averaged luminous intensity. In the subsequent testing phase, the limit of their discriminatory ability was assessed at spatial frequencies higher than 0.14 cycles/degree. A preliminary grating threshold was established at which the mice achieved 70% correct choices. The spatial frequencies around the threshold were retested and the average performance was calculated to plot a frequency-of-seeing curve. The point at 70% accuracy was determined from the curve and was defined as the visual acuity.

#### Statistical tests

The Kormogorv–Smirnov test was used to evaluate the differences in the cumulative distributions of terminal area sizes between WT and cPcdh- $\alpha$  mice. For group comparisons, the Mann–Whitney *U* test was used.

#### Results

## Abnormal aggregation of retinogeniculate terminals in cPcdh- $\alpha$ KO mice

The gross anatomical features of the thalamus and the tectum were checked in WT (Fig. 1a) and cPcdh- $\alpha$  KO (Fig. 1b) mice aged 8–10 weeks. The distribution of CTb-positive terminals originating from the contralateral eye was apparently similar between the two types of mice. At a higher magnification,



**Fig. 1** Retinogeniculate axon terminals in cPcdh-α KO mice. (a and b) Gross distribution patterns of cholera toxin β subunit (CTb)-positive brown terminals derived from the contralateral eye in olivary pretectal nucleus (OPT), dorsal LG (LGd), intergeniculate leaflet (IGL; arrow head), and ventral LG (LGv) of a WT (a) and a cPcdh-α KO (b) mouse, aged 8–10 weeks. Sections were processed by Nissl staining. (c and d) Higher magnification of LGd shown in a (c) and b (d). Many aggregated terminals (arrows) were scattered in the LGd of cPcdh-α KO mice (d). (e and f) Other images of CTb-positive terminals at a higher magnification in a WT (e) and a cPcdh-α (f) mouse. These sections were not counter-stained. (g and h) Corticogeniculate terminals in LGd (arrowheads) labeled after biotinylated dextran amine (BDA) injection into the primary visual cortex (V1) of a WT (g) and a cPcdh-α KO (h) mouse. No aggregated terminals were found. Scale bars, 1 mm in (a and b), 20 μm in (c and d), and 10 μm in (e–h).

however, many aggregated terminals were found in LGd of cPcdh- $\alpha$  KO mice (Fig. 1d and f) but not in WT mice (Fig. 1c and e). Some aggregated terminals (arrows in Fig. 1d) were comparable in size to the soma of Nissl-stained LGd neurons. The abnormally aggregated terminals were intermingled with

apparently normal terminals in the LGd of cPcdh- $\alpha$  KO mice. Such abnormal terminals were also observed in the ipsilateral retinogeniculate projection, within the other side of LGd. Some aggregated terminals were also found in the intergeniculate leaflet, whereas few were observed in the olivary pretectal nucleus and in the ventral LG (LGv) in cPcdh- $\alpha$  KO and WT mice (data not shown). As a control, we tested the distribution pattern of corticogeniculate terminals, which represent another main input into the LGd. BDA-positive terminals in LGd, labeled after BDA injection into V1, were not aggregated, and were apparently similar between a WT (Fig. 1g) and a cPcdh- $\alpha$  (Fig. 1h) mouse.

## Quantitative analysis of isolated retinogeniculate terminals in cPcdh- $\alpha$ -KO mice

We arbitrarily categorized the aggregated retinogeniculate terminals into three types on the basis of their approximate sizes: large ( $\geq 100 \ \mu\text{m}^2$ ), medium (< 100 and > 40  $\ \mu\text{m}^2$ ), and small ( $\leq 40 \ \mu m^2$ ) types. The large type was distributed all over the LGd in cPcdh-a KO mice, except the area receiving CTb-negative terminals from the ipsilateral eye (Fig. 2b). In WT mice, only a few terminals of the large type appeared near the lateral surface of the LGd (Fig. 2a). The medium terminals were observed throughout the LGd in both types of mice (Fig. 2a and b), and the small terminals were as well (data not shown). A quantitative analysis of size distribution in the isolated terminals with or without aggregation revealed a significant difference between WT and cPcdh- $\alpha$  KO mice (p < 0.001, Fig. 2c). The mean size of the isolated terminals was  $13.1 \ \mu\text{m}^2$  in WT mice and 27.7  $\mu$ m<sup>2</sup> in cPcdh- $\alpha$  KO mice. This difference was statistically significant (p < 0.005, Fig. 2d). Fine terminals  $(< 13.1 \text{ }\mu\text{m}^2)$  occupied 71% of isolated terminals in WT mice and 56% of those in cPcdh-a KO mice. The density of fine terminals in cPcdh- $\alpha$  KO mice was 62.5% of that observed in WT mice; this difference was also statistically significant (p < 0.005, Fig. 2e).

## Appearance of the aggregated retinogeniculate terminals at P14 in cPcdh- $\alpha$ KO mice

We examined the post-natal development of retinogeniculate terminals in cPcdh- $\alpha$  KO mice. Gross distribution patterns were similar between WT and cPcdh- $\alpha$  KO mice at all stages after P10, including the normal completion of eye-specific segregation patterns up to P10 (data not shown). In WT mice, the retinogeniculate terminals did not change much in shape and size between P10 and P19, whereas their densities were reduced between P10 and P19 (Fig. 3a, c, and e). In cPcdh- $\alpha$  mice, the aggregated retinogeniculate terminals, which were not present at P10, were observed at P14, at the day of eye opening (Fig. 3b and d). Dark rearing had no effect on these results (data not shown), suggesting that visual experience is not required for aggregation. At P19, the aggregated terminals increased in size and were as large as those


Fig. 2 Quantitative analysis of isolated retinogeniculate terminals. (a and b) Typical schematic distribution of retinal terminals at the center level of dorsal LG (LGd), including the area that receives inputs from the ipsilateral eye. Aggregated terminals were arbitrarily categorized into large ( $\geq$  100  $\mu$ m<sup>2</sup>), medium (< 100 and > 40  $\mu$ m<sup>2</sup>), and small ( $\leq$  40  $\mu$ m<sup>2</sup>) types. Only the large and medium types are shown. The areas surrounded by a dotted line received inputs from the ipsilateral eye. (c) Cumulative distribution of area sizes. The isolated aggregated terminal size measurement was taken on the binarized digital images of cholera toxin  $\beta$  subunit (CTb)-stained sections. (d) Sizes of isolated retinogeniculate terminals. (e) Number of fine type isolated retinogeniculate terminals (< 13.1  $\mu$ m<sup>2</sup>) per mm<sup>2</sup> in LGd sections. In the analyses shown in (c–e), the areas that received inputs from the ipsilateral eye were omitted.

observed in adult mice aged 8–10 weeks (Fig. 3f). The mean size of the isolated terminals were 11.72, 12.58, 14.63  $\mu$ m<sup>2</sup> in two WT mice and 16.57, 20.63, 25.39  $\mu$ m<sup>2</sup> in two cPcdh- $\alpha$  KO mice, at P10, P14, and P19, respectively.

#### Deteriorated visual acuity in cPcdh-a KO mice

The presence of the aggregated retinogeniculate terminals suggests abnormal visual functions in cPcdh- $\alpha$  KO mice. Therefore, we performed two-photon calcium imaging of the V1 neurons. However, the orientation selectivity and direction selectivity were apparently similar between WT and cPcdh- $\alpha$ 



**Fig. 3** Ontogenetic observation of retinogeniculate terminals. (a, c, and e) Retinogeniculate terminals at P10 (a), P14 (c), and P19 (e) in WT mice. (b, d, and f) Retinogeniculate terminals at P10 (b), P14 (d), and P19 (f) in cPcdh- $\alpha$  KO mice. Scale bars, 20  $\mu$ m in (a–f).

KO mice (Fig. 4a and b), as reported previously (Yoshitake *et al.* 2013). We further tested the visual acuity of mice using a behavioral test (Prusky *et al.* 2000), and compared the results between WT and cPcdh- $\alpha$  KO mice (Fig. 4c). WT and cPcdh- $\alpha$  KO mice similarly performed low-frequency tasks at 0.14 cycles/degree during the training phase. The visual acuity of WT mice [0.56  $\pm$  0.01 cycles/degree (mean  $\pm$  SEM), n = 10] was comparable to the values reported previously (Prusky *et al.* 2000; Prusky and Douglas 2004). However, the visual acuity of cPcdh- $\alpha$  KO mice (0.41  $\pm$  0.01 cycles/degree, n = 9) was significantly deteriorated compared with that observed in WT mice (p < 0.0003; Fig. 4d and e).

#### Discussion

### Mechanisms underlying terminal aggregation in cPcdh– $\alpha$ KO mice

In the present study, we found abnormal terminal aggregation in the LGd of cPcdh- $\alpha$  KO mice. Impairment of *cPcdh*- $\alpha$ results in abnormal projection patterns from olfactory sensory neurons expressing a given odorant receptor onto a



**Fig. 4** Reduced visual acuity in cPcdh-α KO mice. (a) Sample traces of neuronal calcium response to moving grating patterns (from 0° to 315° in 45° steps) in a WT mouse (left) and a cPcdh-α KO mouse (right). (b) Cumulative distributions of the orientation selectivity index (OSI) of 481 neurons in WT mice and 423 neurons in cPcdh-α KO mice (left). The distribution of the direction selectivity index (DSI) of 197 neurons in WT mice and 164 neurons in cPcdh-α KO mice is also shown (right). DSI was estimated in neurons with an OSI > 0.4. (c) Schematic drawing of the Prusky water maze used to estimate visual acuity. (d) Examples of the results obtained for a WT and a cPcdh-α KO mice.

single glomerulus in the olfactory bulb (Hasegawa *et al.* 2008). Abnormal aggregation of serotonergic fibers is also found in cPcdh- $\alpha$  mice (Katori *et al.* 2009). The corticocortical pathways connecting the primary somatosensory cortices in both hemispheres are also impaired in cPcdh- $\alpha$  KO mice (Yamashita *et al.* 2012). These findings indicate that cPcdh- $\alpha$  is required for normal development of axonal

projection patterns derived from specific types of neurons. Although cPcdh- $\alpha$  is expressed in most neurons, impairment of cPcdh- $\alpha$  has dominant effects exclusively in specific types of neurons or synapses. The retinal fiber innervating areas, such as LGd, LGv, and the superior colliculus, also express *cPcdh-\alpha*, and almost all the optic tract fibers contain cPcdh- $\alpha$ protein during the early post-natal stage, with abnormality appearing in the LGv and the superior colliculus. These findings suggest that other cPcdhs or cell-adhesion molecules might replace cPcdh- $\alpha$  in other neurons or synapses.

Distinct subsets of the cPcdh cluster are expressed differentially in individual neurons, and enormous cell surface diversity may result from their combinatorial expression (Kohmura et al. 1998; Wang et al. 2002a; Esumi et al. 2005; Kaneko et al. 2006). The cell surface diversity attributed to cPcdhs assists the formation of specific synapses between neurons expressing similar sets of cPcdhs (Shapiro and Colman 1999; Phillips et al. 2003; Yagi 2012). Alternatively, cPcdh-y supposedly plays a role in self-avoidance between neurites that express the same sets of cPcdh- $\gamma$ (Lefebvre et al. 2012; Zipursky and Grueber 2013). The abnormal terminal aggregation observed in the LGd of cPcdh-α KO mice can be explained by either of the following mechanisms: it may be attributed to non-specific synapse formation between a single LGd neuron and many retinal ganglion cells, or the failure of avoidance between retinogeniculate terminals, which avoid one another in WT mice.

In cPcdh-a KO mice, terminal aggregation was not observed at P10, and appeared just before normal eye opening at P14; thus, cPcdh-a is likely to play an important role in the refinement process of retinogeniculate terminals between P10 and P14 (Chen and Regehr 2000; Hooks and Chen 2006, 2008; Liu and Chen 2008). The slight mean size difference of retinogeniculate terminals already appeared at P10, the starting point of the refinement process with lots of excess terminals to be eliminated. cPcdh-a might have some effects on the size of immature terminals. In contrast, cPcdhγ is required for neuronal survival and for preparing appropriate synapses in the early development of the spinal cord (Wang et al. 2002b; Prasad et al. 2008; Prasad and Weiner 2011; Chen et al. 2012). Moreover, cPcdh-y-deleted mutant mice lack voluntary movements and reflexes and die shortly after birth (Wang et al. 2002b; Chen et al. 2012). Therefore, cPcdh- $\alpha$  and cPcdh- $\gamma$  may play distinct roles in the neural circuit formation at different developmental stages.

### Relationship between terminal aggregation and impaired visual acuity in cPcdh- $\alpha$ KO mice

In the present study, we observed aggregation of retinogeniculate terminals and impaired visual acuity in cPcdh- $\alpha$  KO mice. However, the relationship between the two phenomena remains unclear, because there could be other abnormalities in retinal circuits, in which cPcdh- $\gamma$  plays an important role (Lefebvre *et al.* 2012), or in circuits higher than LGd, such as geniculocortical projections, intracortical circuits in V1 or higher visual areas. To date, orientation selectivity, direction selectivity, and ocular dominance plasticity in V1 are apparently normal in cPcdh-a KO mice (Fig. 4 and Yoshitake et al. 2013). The estimation of visual acuity required visual discrimination learning, in which no apparent difference was found between WT and cPcdh-a KO mice when a grating pattern of 0.14 cycles/degree was used. However, V1 plasticity induced by spatial mismatches between whisker and visual inputs is impaired in cPcdh-a KO mice, suggesting the presence of some abnormalities in higher areas of these mice (Yoshitake et al. 2013). The possibility of retinal circuit and geniculocortical circuit abnormality also needs to be considered. However, an important finding in the present study is that the magnitude of impaired visual acuity in cPcdh- $\alpha$  KO mice was comparable to that of the aggregated retinothalamic terminals. We investigated visual acuity in the present study, since the aggregated retinogeniculate terminals had suggested impaired visual acuity in cPcdh-a KO mice. However, we cannot exclude the possibility that other parameters such as the size of receptive field are abnormal in cPcdh-a KO mice.

Normal retinogeniculate synapses are highly efficient in signal transmission (Budisantoso et al. 2012), and aggregation of terminals is unlikely to further assist signal transmission. Instead, non-specific mixing of the information conveyed by each retinal ganglion axon at the aggregated terminals probably impairs signal transmission. Therefore, the fine spatial information conveyed through LGd may be mediated mainly via fine terminals (< 13.1  $\mu$ m<sup>2</sup>). The density of fine terminals in LGd was reduced to 62.5% in cPcdh-a KO mice compared with WT mice. Assuming that retinogeniculate terminals with spatial information were distributed in the grid, the visual acuity discriminating two vertical (or horizontal) lines are inversely proportional to the grid intervals, or proportional to the square root of the terminal density. When the visual acuity determined using grating patterns would be proportional to the square root of the fine-terminal density, it would deteriorate to 79% in cPcdh-α KO mice. Because the visual acuity observed in WT mice was 0.56 cycles/degree, the expected visual acuity in cPcdh-α KO mice could be 0.44 cycles/degree. This expected value is very close to 0.41 cycles/degree, which was the value obtained in behavioral experiments. This simplified argument suggests that the aggregation of retinogeniculate terminals in cPcdh-a KO mice explains a considerable part of the deteriorated visual acuity in cPcdh- $\alpha$  KO mice, although the possibility that other abnormalities may contribute to the observed visual impairment cannot be excluded (Katori et al. 2009; Lefebvre et al. 2012; Yoshitake et al. 2013). Therefore, morphological and functional studies on the retinogeniculate synapses in mice with genetically manipulated cPcdhs may be useful for elucidating the functions of cPcdhs in the future.

### Acknowledgments and conflict of interest disclosure

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All experiments were conducted in compliance with the ARRIVE guidelines.

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# Somatic Mutations in the MTOR Gene Cause Focal Cortical Dysplasia Type IIb

Mitsuko Nakashima, MD, PhD,<sup>1a</sup> Hirotomo Saitsu, MD, PhD,<sup>1a</sup>

Nobuyuki Takei, PhD,<sup>2</sup> Jun Tohyama, MD, PhD,<sup>3</sup> Mitsuhiro Kato, MD, PhD,<sup>4</sup>

Hiroki Kitaura, DDS, PhD,<sup>5</sup> Masaaki Shiina, MD, PhD,<sup>6</sup> Hiroshi Shirozu, MD, PhD,<sup>7</sup>

Hiroshi Masuda, MD,<sup>7</sup> Keisuke Watanabe, PhD,<sup>8</sup> Chihiro Ohba, MD, PhD,<sup>1</sup>

Yoshinori Tsurusaki, PhD,<sup>1</sup> Noriko Miyake, MD, PhD,<sup>1</sup> Yingjun Zheng, MD, PhD,<sup>5</sup>

Tatsuhiro Sato, PhD,<sup>9</sup> Hirohide Takebayashi, MD, PhD,<sup>8</sup>

Kazuhiro Ogata, MD, PhD,<sup>6</sup> Shigeki Kameyama, MD, PhD,<sup>7</sup>

Akiyoshi Kakita, MD, PhD,<sup>5b</sup> and Naomichi Matsumoto, MD, PhD<sup>1b</sup>

**Objective:** Focal cortical dysplasia (FCD) type IIb is a cortical malformation characterized by cortical architectural abnormalities, dysmorphic neurons, and balloon cells. It has been suggested that FCDs are caused by somatic mutations in cells in the developing brain. Here, we explore the possible involvement of somatic mutations in FCD type IIb. **Methods:** We collected a total of 24 blood-brain paired samples with FCD, including 13 individuals with FCD type IIb, 5 with type IIa, and 6 with type I. We performed whole-exome sequencing using paired samples from 9 of the FCD type IIb subjects. Somatic *MTOR* mutations were identified and further investigated using all 24 paired samples by deep sequencing of the entire gene's coding region. Somatic *MTOR* mutations were confirmed by droplet digital polymerase chain reaction. The effect of *MTOR* mutations on mammalian target of rapamycin (mTOR) kinase signaling was evaluated by immunohistochemistry and Western blotting analyses of brain samples and by *in vitro* transfection experiments.

**Results:** We identified four lesion-specific somatic *MTOR* mutations in 6 of 13 (46%) individuals with FCD type IIb showing mutant allele rates of 1.11% to 9.31%. Functional analyses showed that phosphorylation of ribosomal protein S6 in FCD type IIb brain tissues with *MTOR* mutations was clearly elevated, compared to control samples. Transfection of any of the four *MTOR* mutants into HEK293T cells led to elevated phosphorylation of 4EBP, the direct target of mTOR kinase.

**Interpretation:** We found low-prevalence somatic mutations in *MTOR* in FCD type IIb, indicating that activating somatic mutations in *MTOR* cause FCD type IIb.

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Focal cortical dysplasia (FCD) is a cortical malformation frequently associated with drug-resistant epilepsy that requires surgical treatment.<sup>1,2</sup> FCD can be classified into three types (I, II, and III) based on its clinicopathological features.<sup>3</sup> Type I refers to abnormalities in cortical architecture without cellular anomalies,<sup>3</sup> whereas type III

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<sup>a</sup>These authors contributed equally to this work.

<sup>b</sup>These are co-last authors.

Address correspondence to Dr Naomichi Matsumoto, Department of Human Genetics, Yokohama City University Graduate School of Medicine, 3–9 Fukuura, Kanazawa-ku, Yokohama 236-0004, Japan. E-mail: naomat@yokohama-cu.ac.jp

From the <sup>1</sup>Department of Human Genetics, Yokohama City University Graduate School of Medicine, Yokohama, Japan; <sup>2</sup>Department of Molecular Neurobiology, Brain Research Institute, Niigata University, Niigata, Japan; <sup>3</sup>Department of Child Neurology, Nishi-Niigata Chuo National Hospital, Niigata, Japan; <sup>4</sup>Department of Pediatrics, Showa University School of Medicine, Tokyo, Japan; <sup>5</sup>Department of Pathology, Brain Research Institute, University of Niigata, Japan; <sup>6</sup>Department of Biochemistry, Yokohama City University Graduate School of Medicine, Yokohama, Japan; <sup>and</sup> Pepartment of Publicate, Japan; <sup>6</sup>Department of Biochemistry, Yokohama City University Graduate School of Medicine, Yokohama, Japan; <sup>and</sup> Pepartment of Functional Neurosurgery, Epilepsy Center, Nishi-Niigata Chuo National Hospital, Niigata, Japan; <sup>8</sup>Division of Neurobiology and

Anatomy, Graduate School of Medical and Dental Sciences, Niigata University, Niigata, Japan; and <sup>9</sup>Division of Biochemistry, School of Pharmaceutical Sciences, Kitasato University, Tokyo, Japan.

Additional Supporting Information can be found in the online version of this article.

involves cortical lamination abnormalities associated with a principal lesion, which are usually adjacent and affect the same cortical area or lobes.<sup>3</sup> FCD type II is characterized by severe architectural abnormalities and dysmorphic neurons and can be further subdivided into types IIb (with balloon cells) and IIa (without balloon cells).<sup>3,4</sup>

It has been suggested that FCDs are likely to arise from somatic mutations in cells in the developing brain, but that the molecular basis of the three types of FCD can differ.<sup>2,4</sup> Several studies have demonstrated that the phosphatidylinositol 3-kinase (PI3K)/AKT3/mechanistic target of rapamycin (mTOR)–signaling pathway is activated in FCD type IIb.<sup>5–11</sup> This pathway regulates cell proliferation, metabolism, autophagy, and apoptosis in developing cerebral cells. Furthermore, some cortical malformations are demonstrated to possess somatic mutations in genes of the PI3K/AKT3/mTOR-signaling pathway.<sup>12–19</sup> In this study, we analyzed paired brain lesions and blood leukocytes (or saliva) from individuals with FCDs who underwent surgery to evaluate the possible involvement of somatic mutations in the disease.

#### **Subjects and Methods**

#### Study Subjects and Samples

Thirteen individuals with FCD type IIb, 5 with type IIa, and 6 with type I were investigated in this study. All participants underwent preoperative clinical evaluations, including seizure charts, electroencephalography, and neuroimaging with magnetic resonance imaging (MRI), followed by surgical treatment. Subjects or their families provided us with written informed consent for participation in this study. The institutional review boards of Yokohama City University, Nishi-Niigata Chuo National Hospital, Yamagata University, and the University of Niigata approved this study. Surgically operated brain tissues (lesions) and peripheral blood leukocytes (or saliva) (normal tissues) were obtained from all participants. Fresh surgical specimens were immediately cut into 5-mm blocks in the operating room, and some of the blocks were fixed with 20% buffered formalin and embedded in paraffin wax. Serial 4-µm-thick sections were then cut and stained with hematoxylin-eosin or Klüver-Barrera stain. Histopathological diagnosis was performed according to the recent classification system.<sup>3</sup> As controls for immunohistochemistry (IHC) and Western blotting analyses, we selected 6 subjects (ID/age [years]/sex = 15473/18/M, 15732/38/F, 15294/26/M, 16080/43/M, 16312/12/M, and 16337/12/M) who had suffered from localized neocortical seizures for years and underwent surgery to treat epilepsy. In all cases, histopathological evaluation of resected brain lesions revealed none of the obvious abnormalities in cortical layering that are required for a diagnosis of FCD type I.<sup>3</sup> These cases could thus be regarded as mild malformations of cortical development.3

#### **DNA** Preparation

Fresh frozen brain tissues were dissolved in sodium dodecyl sulfate (SDS)/sodium chloride–based lysis solution with proteinase K and incubated at 55°C for 1 hour, then 37°C overnight. Genomic DNA was extracted from peripheral blood leukocytes and saliva using QuickGene-610L (Fujifilm, Tokyo, Japan) and Oragene (DNA Genotek Inc., Kanata, Ontario, Canada), respectively, according to the manufacturer's instructions.

# Somatic Variant Calling Using Paired Whole Exome Sequencing Data

Paired brain lesions and blood leukocytes (or saliva) from nine individuals with FCD Type IIb were analyzed by whole-exome sequencing (WES). DNA was captured using a SureSelect Human All Exon V5 Kit (Agilent Technologies, Santa Clara, CA) and sequenced on an Illumina HiSeq 2500 (Illumina, San Diego, CA) with 101-bp paired-end reads. Read bases below the Phred quality score of 20 were trimmed from the 3' end of reads. These cleaned reads were aligned to the human reference genome sequence (UCSC hg19, NCBI build 37) using Novoalign (Novocraft Technologies, Petaling Jaya, Malaysia). Paired WES data (from brain and blood/saliva) were analyzed by MuTect<sup>20</sup> and VarScan 2<sup>21</sup> to detect de novo somatic singlenucleotide variants (SNVs) in brain tissues. For MuTect, we carefully examined both filter-passed and -rejected variants to avoid false-negative results, considering the cellular complexity in brain tissues. For Varscan 2, minimum read depth for somatic variant calls in normal and tumor samples was set to 12. Exclusion criteria were: (1) variants on mutant alleles with <2 reads in the brain and  $\geq 2$  reads in leukocytes (or saliva); (2) variants registered in dbSNP 137, except for clinically associated single-nucleotide polymorphisms (flagged); (3) variants registered in 6,500 exomes of the National Heart, Lung, and Blood Institute Exome Sequencing Project Exome Variant Sever and the 1000 Genomes database<sup>22,23</sup>; (4) variants observed in our 575 in-house control exomes; and (5) synonymous variants. Called variants were annotated using ANNO-VAR software.<sup>24</sup>

#### Validation of Candidate Somatic Variants

Candidate somatic variants extracted from WES data were validated by polymerase chain reaction (PCR)-based deep sequencing using specific primers. Sequencing libraries were prepared using the SureSelect XT Library Prep Kit (Agilent Technologies) and sequenced on a MiSeq (Illumina) with 150-bp pairedend reads. Trimming and alignment of reads were performed as described above. Allele counting was performed using Integrative Genomics Viewer software (IGV).<sup>25</sup>

#### Somatic Mutation Screening of MTOR

A total of 16 primer sets covering the entire coding region and intron-exon boundaries of *MTOR* (MIM 601231; RefSeq accession number: NM\_004958.3) were used to amplify DNA from brain lesions and blood leukocytes (or saliva). Sequence libraries were prepared with the Nextera DNA Sample Preparation Kit (Illumina). An Agilent DNA 1000 Kit (Agilent Technologies) and KAPA Library Quantification Kits (Kapa Biosystems, Wilmington, MA) were used to check size distribution and DNA concentration of the libraries, respectively. Libraries were sequenced on an MiSeq (Illumina). Trimming and alignment of reads were performed as described above. Variants were called by MuTect. All mutations were visually reviewed by IGV.

#### Droplet Digital PCR

Somatic MTOR mutations were confirmed by droplet digital PCR (ddPCR). Custom locked nucleic acid probes for each wild-type (WT) and mutant allele- and region-specific primers were purchased from Integrated DNA Technology (Coralville, IA). A 20-µl assay mix containing 50ng of genomic DNA, ddPCR Supermix for Probes (No dUTP; Bio-Rad, Hercules, CA), 250nM of WT and mutant probes, and 900nM of PCR primers were emulsified into ~20,000 droplets using a QX100 Droplet Generator (Bio-Rad). Target-specific primers and probes are shown in Supporting Table 1. In preliminary experiments, appropriate Tm values and thresholds were examined for each WT and mutant allele using cloned DNAs to minimize background. These experiments revealed that virtually no falsepositive droplets were observed in the optimized experimental conditions. We tested six paired samples with MTOR mutations using mutation-specific primers and probes sets. PCR was performed using the following cycles: 10 minutes at 95°C; 40 cycles of 30 seconds at 94°C and 2 minutes at 59°C c.6644C>T) (c.6644C>A, or 53°C (c.4376C>A, c.4379T>C); and 10 minutes at 98°C. Droplet reading and data analysis were performed using QX200 Droplet Reader and QuantaSoft software (Bio-Rad), respectively.

### Statistical Analysis of Correlation Between MTOR Mutations and Clinical Features

We assessed the relevance between MTOR mutations and clinical features, including seizure onset age, seizure frequency, neurological findings, IQ at pre- or postsurgery, and lesion volume, by Wilcoxon rank-sum or Fisher exact test (p < 0.05 was considered as significant). Lesion volume in each subject was calculated from MRI proton density weighted images (PDWIs) using OsiriX MD (Pixmeo, Geneva, Switzerland). In brief, the margin of high-intensity signals in PDWIs, which indicates FCD, obtained just before surgery was delineated manually as a region of interest (ROI) area in both axial and coronal views, and the ROI area in each slice was calculated. Then, the volume of the lesion was automatically calculated based on each area and slice thickness. In subjects 14434, 17424, and 11683, lesion volume was manually calculated. All analyses were performed using R software (version 3.1.0; R Foundation for Statistical Computing, Vienna, Austria).

#### Immunohistochemistry

Paraffin-embedded sections were immunostained with rabbit polyclonal antibody against the ribosomal protein, phospho-S6 (Ser235/236; diluted 1:1,000; Cell Signaling Technologies, Danvers, MA), as previously described.<sup>26</sup>



FIGURE 1: Flow chart of somatic mutation analysis in subjects with focal cortical dysplasia (FCD). The flow of analysis in this study is outlined. ddPCR = droplet digital polymerase chain reaction; PCR = polymerase chain reaction.

#### Structural Analysis of mTOR

FoldX software (version  $3.0\beta3$ ) was used to calculate free energy changes after mutation using the mTOR crystal structure (amino acids [aa] 1,376-2,549)/mLST8 complex (Protein Data Bank [PDB] 4JSN).<sup>27</sup> The calculation was repeated three times, and the resultant data are presented as an average value with a standard deviation.

#### Western Blotting Analysis

Fresh-frozen tissues of surgical specimens taken from the 5 subjects with MTOR mutations (ID: 16964, 17424, 11683, 14434, and 16578) and the six controls (described above). Fresh-frozen tissues of a patient with an MTOR mutation (ID: 15622) were not available for Western blotting. These were lysed with SDS sample buffer containing protease (Complete; Roche Diagnostics, Mannheim, Germany) and phosphatase inhibitor cocktails (PhosSTOP; Roche Diagnostics). Protein concentration was determined using BCA protein assay reagent (Thermo Fisher Scientific, Waltham, MA). Equal amounts of protein (40 $\mu$ g/lane for phospho and total S6 and 2 $\mu$ g/lane for actin) were separated by SDS polyacrylamide gel electrophoresis (PAGE) and analyzed by Western blotting with rabbit polyclonal antibodies against total S6 (Cell Signaling Technologies) and phospho-S6 (Ser235/236), and with a mouse monoclonal antibody against actin (Merck Millipore, Billerica, MA).

Subject	Mean Depth <sup>b</sup> (Brain/Blood)	%_bases_above_ 20× (Brain/Blood)	Program	<i>De novo</i> SNV Call (Filter Passed)	Common Variants in Two Programs	Candidate Gene(s) Common in Two or More Cases
11683	193.7/178.4	95.0/94.3	MuTect	422 (13)	13	UNC79
			Varscan 2	35		
15035 <sup>a</sup>	192.9/194.5	94.9/94.7	MuTect	446 (6)	25	
			Varscan 2	74		
15622	146.3/121.3	95.6/94.6	MuTect	428 (10)	11	NME5
			Varscan 2	28		
16129	109.2/67.37	93.8/88.4	MuTect	318 (10)	30	NME5
			Varscan 2	148		
16578	117.8/143.2	94.5/95.4	MuTect	357 (12)	56	NBPF8, UNC79, <u>Mtor</u> , KAT6B
			Varscan 2	232		
17562	158.2/166.2	95.8/95.8	MuTect	348 (8)	20	NBPF8
			Varscan 2	79		
14434	154.2/259.4	92.8/95.5	MuTect	376 (9)	102	<u>MTOR</u> , KAT6B, CES4A
			Varscan 2	706		
15381	127.5/108.6	94.8/93.7	MuTect	460 (10)	23	
			Varscan 2	43		
16325	131.0/141.2	93.7/94.0	MuTect	319 (6)	22	NBPF8, CES4A
			Varscan 2	68		

<sup>b</sup>Against protein coding sequences of RefSeq genes.

FCD = focal cortical dysplasia; SNV = single-nucleotide variant.

Human WT MTOR complementary DNA (cDNA; NM\_004958.3) and mutant MTOR cDNA (p.Ala1459Asp, p.Leu1460Pro, p.Ser2215Phe and p.Ser2215Tyr) were cloned in pEF-BOS-FLAG vector.<sup>28</sup> Human EIF4EBP1 (NM\_004095.3) was introduced into pCMV-FLAG.<sup>29</sup> HEK293T cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and penicillin/streptomycin at 37°C in an atmosphere of 5% CO2. HEK293T cells were cotransfected with FLAG-4EBP and WT or mutant MTOR plasmids using Fugene6 (Roche Diagnostics), according to the manufacturer's instructions. To assess the activity of MTOR mutants, cells were starved for serum and amino acids in Hank's balanced salt solution for 1 hour. Cells were then lysed and incubated with anti-DYKDDDDK antibody beads (WAKO, Osaka, Japan) at 4°C for 4 hours. Immunoprecipitates were analyzed by SDS-PAGE and Western blotting with anti-phospho-4EBP (Thr37/46; Cell Signaling Technologies) or anti-DYKDDDDK (WAKO), as previously described.30

#### Results

#### Somatic Mutation Screening and Validation

A flow chart of our analysis is illustrated in Figure 1. Using MuTect<sup>20</sup> and VarScan 2,<sup>21</sup> we detected a number of possible somatic variants in WES data of nine paired samples (Table 1). To quickly narrow down candidate variants, we first searched for those variants commonly detected by the two programs, then focused on candidate genes whose variants were found in two or more cases (Table 1). Among six candidate genes, *MTOR* is of interest. It encodes the serine/threonine kinase mTOR, a key regulator of the PI3K/AKT3/mTOR-signaling pathway,<sup>31,32</sup> and an *MTOR* somatic mutation has been reported in hemimegalencephaly.<sup>13</sup> Considering the possible involvement of the mTOR pathway in FCD, we investigated variants in candidate genes involved in the mTOR pathway (Supporting Table 2). We only detected

TABLE 2	Summ	ary of Deep Sequ	encing of N	1TOR in Indiv	viduals With	FCD					
		Whole-Exc	ome Sequen	cing	Variant-T.	argeted Deep { (Validation)	Sequencing <sup>c</sup>	LW	OR-Targeted Deep	) Sequencing	
			Brain	Blood		Brain	Blood			Brain	Blood
Subject	Type	Variant in <i>MTOR</i> by MuTect	Mut/Ref Counts (% <sup>b</sup> )	Mut/Ref Counts (% <sup>b</sup> )	Mutation	Mut/Ref Counts (% <sup>b</sup> )	Mut/Ref Counts (% <sup>b</sup> )	Mean depth <sup>d</sup> (Brain/Blood)	Mutation	Mut/Ref Counts (% <sup>b</sup> )	Mut/Ref Counts (% <sup>b</sup> )
11683	IIb	c.6644C>A (p.Ser2215Tyr)	4/357 (1.11)	0/318 (0.00)	True	406/28,719 (1.39)	9/31,396 (0.03)	1,968/1,335	c.6644C>A (p.Ser2215Tyr)	38/2,426 (1.54)	0/1,242 (0.00)
15035 <sup>a</sup>	IIb		I					1,619/1,189	1		
15622	IIb	c.4376C>A (p.Ala1459Asp)	3/221 (1.34)	0/187 (0.00)	True	406/28,605 (1.40)	18/38,815 (0.05)	860/3,017	c.4376C>A (p.Ala1459Asp)	33/1,970 (1.65)	6/10,394 (0.06)
16129	IIb	c.1897T>C (p.Ser633Pro)	2/67 (2.90)	0/97 (00.0)	False	24/26,447 (0.09)	29/35,285 (0.08)	2,546/2,611		I	1
16578	IIb	c.4379T>C (p.Leu1460Pro)	4/199 (1.97)	0/174 (0.00)	True	623/28,382 (2.15)	52/103,377 (0.05)	3,195/15,181	c.4379T>C (p.Leu1460Pro)	150/9,303 (1.59)	26/42,551 (0.06)
17562	IIb	[	I				I	2,198/2,851	[		
14434	IIb	c.6644C>T (p.Ser2215Phe)	6/314 (1.88)	0/502 (0.00)	True	160/6,876 (2.27)	1/17,069 (0.01)	1,148/3,373	c.6644C>T (p.Ser2215Phe)	24/747 (3.11)	0/2,556 (0.00)
15381	IIb		I	I			I	2478/2,215			
16325	IIb	c.1820C>T (p.Ala607Val)	2/59 (3.28)	0/59 (0.00)	False	20/11,990 (0.17)	74/42,140 (0.17)	1,224/1,422		I	
15524	IIb	ND	ND	ND	ND	ND	ND	3,168/5,350			
16964	IIb	ND	ND	ND	ND	ND	ND	2,606/6,393	c.4379T>C (p.Leu1460Pro)	111/2,167 (4.87)	4/8,255 (0.05)
17424 <sup>a</sup>	lIIb	ND	ND	ND	ND	ND	ND	1,483/4,723	c.6644C>A (p.Ser2215Tyr)	74/721 (9.31)	3/3,665 (0.08)
18437	IIb	ND	ND	ND	ND	ND	ND	2,003/1,9375	Ι	Ι	
<sup>a</sup> DNA fro <sup>b</sup> Percent c <sup>c</sup> Variants <sup>d</sup> <sup>d</sup> Against F FCD = fo	im saliva i of mutant were calle- protein co cal cortice	instead of blood was allele was calculated d by MuTect, and re ding sequences of M il dysplasia; Mut = $m$	used. by mutant al. ads were man TOR. nutant allele; )	lele reads/(mu ually counted N.D. = whole-	tant allele read by Integrative exome sequen	s + reference allt Genomics View cing and variant	ele reads). er software (IGV -targeted sequenc	). Sing were not perfor	med; Ref = reference	allele.	



FIGURE 2: Somatic *MTOR* mutations in individuals with focal cortical dysplasia (FCD) type IIb. (A) Schematic of the mammalian target of rapamycin (mTOR) protein with HEAT repeats, FAT domain, kinase domain comprising N- and C-lobes, FRB domain, and FATC domain. All four identified missense mutations occurred at evolutionarily conserved amino acids in the FAT domain (n = 2; Ala1459 and Leu1460) or N-lobe of the kinase domain (n = 2; Ser2215). Multiple amino acid sequences of mTOR proteins were aligned with tools available on the CLUSTALW website (http://www.genome.jp/tools/clustalw/). (B) Percentage of mutant alleles of *MTOR* in blood leukocytes and brain lesions in FCD type IIb. All samples showed both results analyzed by targeted amplicon deep sequencing (NGS) and ddPCR. ddPCR = droplet digital polymerase chain reaction; NGS = next-generation sequencing.

variants in six mTOR-related genes (*MTOR*, *AKT3*, *TSC1*, *TSC2*, *PIK3*, and *PTEN*) by MuTect and subsequently examined by targeted deep sequencing. Four *MTOR* mutations were validated: c.4376C>A (p.Ala1459Asp); c.4379T>C (p.Leu1460Pro); c.6644C>A (p.Ser2215Tyr); and c.6644C>T (p.Ser2215Phe; Table 2). No variants in other genes were validated because they were absent in targeted deep sequencing, suggesting that they were false positive. We also examined germline or somatic mutations in candidate genes involved in the mTOR pathway (Supporting Table 2) using nonbrain Varscan 2 data, but we could not detect any pathogenic variants in these genes.

We then screened the entire *MTOR* coding region using deep sequencing of PCR amplicons from 13 individuals with FCD type IIb, including the 9 analyzed by WES. In addition, to explore possible involvement of somatic *MTOR* mutations in other types of FCD, we also screened 5 subjects with FCD type IIa and 6 with type I. We identified two recurrent mutations (c.4379T>C and c.6644C>A) in an additional two individuals with FCD type IIb. A total of four lesion-specific somatic *MTOR* mutations were verified in 6 individuals with FCD type IIb (6 of 13 [46%]; Fig 2A; Table 2). No candidate mutations were identified in individuals with FCD type I or IIa (Supporting Table 3). Mutant allele frequencies in brain lesions were very low (range, 1.54–9.31%; average, 3.67%) but were clearly higher than those in blood leukocytes or saliva (all <0.1%; Table 2), excluding possible PCR or sequencing errors. Furthermore, we verified the mutant allele frequencies by ddPCR. Fractional abundance in each sample was nearly consistent with mutant allele frequencies from the deep



FIGURE 3: Brain magnetic resonance imaging (MRI) and histopathological features of subjects with somatic *MTOR* mutations. Coronal or axial brain MRI of subjects with somatic *MTOR* mutations. T2-weighted (A, D, G, J, M, P) and proton-density-weighted (B, E, H, K, N, Q) images show abnormal configurations of cerebral gyri and high signal, respectively. Yellow arrowheads indicate focal cortical dysplasia (FCD) lesions. Photomicrographs of hematoxylineosin-stained histology sections (C, F, I, L, O, R) demonstrating characteristic dysplastic features with dysmorphic neurons (arrowheads) and balloon cells (arrows) in surgical specimens taken from 6 individuals with somatic *MTOR* mutations. Bar =  $20\mu$ m.



FIGURE 4: Expression of phospho-S6 in focal cortical dysplasia (FCD) type IIb. Immunohistochemical features (A–D) and Western blotting (E) for phospho-S6 in brain tissues taken from subjects with FCD type IIb and controls. (A–C) Many dysmorphic neurons and balloon cells in an FCD type IIb brain section with an *MTOR* mutation (A: 16578, B: 15622; Table 2 and Supporting Table 3) and without a mutation (C: 15381; Table 2 and Supporting Table 3) showed intense immunoreactivity to phospho-S6. (D) Neurons in the control (#16080) showed no or very faint reactivity. Bar =  $120\mu$ m. (E) Expression of phospho-S6 (Ser235/ 236), total S6 protein, and actin are depicted in the upper, middle, and lower panel, respectively. Western blotting showed a larger amount of phosphorylated S6 in brain tissues of FCD type IIb (lanes 1–5: 16964, 17424, 11683, 14434, and 16578, respectively), compared to controls (lanes 1–6: 15473, 15732, 15294, 16080, 16312, and 16337, respectively). Equal amounts of protein (40µg/lane for phospho and total S6 and 2µg/lane for actin) were applied.

sequencing data (Fig 2B). Fractional abundance in blood was extremely low or zero (0–0.034%; Fig 2B), being equivalent to no-template control. Low-prevalence mosaic rates for all mutations were reproducible by ddPCR.

### Correlation Between MTOR Mutations and Clinical Features

Correlation of *MTOR* mutations with clinical phenotypes was statistically analyzed. Clinical features of the 13 individuals with FCD type IIb are described in detail (Supporting Table 4). Brain MRI showed severe architectural abnormalities in 6 individuals with somatic *MTOR* mutations (Fig 3), as well as in 7 without *MTOR* mutations (data not shown). We found no statistical difference for any clinical feature between mutation-positive and -negative groups.

# Phosphorylation of Ribosomal Protein S6 in FCD Samples

At primary histopathological examination, all specimens with FCD type IIb (Table 2 and Supporting Table 4)



FIGURE 5: Mammalian target of rapamycin (mTOR) signaling activation by MTOR mutations. Structural consideration of the four mutations. (A) Crystal structure of mTOR (residues 1,376-2,549) in complex with mLST8, ATP<sub>2</sub>S, and magnesium ions (Protein Data Bank [PDB] code 4JSP). FAT domain, N- and C-lobes of the kinase domain, and the FRB domain in the N-lobe are colored blue, magenta, cyan, and light blue, respectively. Nonhydrolyzed ATP analog, ATP<sub>γ</sub>S, and magnesium ions are shown as gray sticks and orange balls, respectively. Residues at mutation sites are shown as van der Waals spheres in red. The secondary structure assignment is according to published nomenclature. (B and C) Close-up of regions around the mutation sites from the front and back. Side chains of some residues near the mutation sites are represented as transparent van der Waals spheres. (D) Free energy change upon amino acid mutations, estimated from calculations using FoldX software. (E) HEK293T cells were transfected with FLAG-MTOR or pcDNA3.1-MTOR hyperactive mutant and FLAG-4EBP plasmids. Cell lysates were immunoprecipitated with anti-Flag antibody beads and then probed with anti-phospho-4EBP (Thr36/47; left, upper panel), anti-Flag for mTOR (left, middle panel), and 4EBP (left, lower panel). Three separate experiments were performed. Transfection efficacy was almost the same in each transfection. Note that all the mutants found in this study led to significant activation of the mTOR pathway, and the mutants are comparable with the hyperactive mutant (right panel). n = 3 for WT and mutants. \*p<0.0001 by analysis of variance. Western blotting were quantified by densitometric analysis using ImageJ software (NIH, Bethesda, MD). Bars represent means ± standard error of the mean. ATP = adenosine triphosphate; FCD = focal cortical dysplasia.

TABLE 3. Prediction of Somatic MTOR Mutation Pathogenicity									
Subject	Mutation	SIFT	PolyPhen2	Mutation Taster					
11683 and 17424	c.6644C>A (p.Ser2215Tyr)	0	Possibly damaging (0.876)	Disease-causing (0.999)					
15622	c.4376C>A (p.Ala1459Asp)	0	Benign (0.133)	Disease-causing (0.999)					
16578 and 16964	c.4379T>C (p.Leu1460Pro)	0	Benign (0.352)	Disease-causing (0.999)					
14434	c.6644C>T (p.Ser2215Phe)	0	Possibly damaging (0.824)	Disease-causing (0.999)					
SIFT (http://sift.jcvi.or genetics.bwh.harvard.ec ing). Mutation Taster ( disease-causing or poly	g/): scores of less than 0.05% indica du/pph2/): HumVar scores are evalu (http://www.mutationtaster.org/): rap morphisms	ate substitu ated as 0.0 oid evaluat	ations that are predicted as intoler 000 (most probably benign) to 0.9 ion of DNA sequence alterations.	rant. PolyPhen-2 (http:// 999 (most probably damag- Alterations are classified as					

showed numerous dysmorphic neurons and balloon cells regardless of the presence or absence of mutations (Fig 3C, F, I, L, O, R). We then examined the activation of the mTOR pathway in FCD type I, IIa, and IIb using IHC for phospho-S6, which is a downstream phosphorylated protein in the mTOR pathway. In FCD type IIb specimens with or without MTOR mutations, almost all dysmorphic neurons and balloon cells were distributed in both the cortex and subcortical white matter and showed intense reactivity for phospho-S6 (Fig 4A-C). A small proportion of normal-looking pyramidal neurons distributed in the cortex also showed a variable intensity of reactivity for phospho-S6. On the other hand, neurons showed no or faint reactivity for phospho-S6 in all control specimens (Fig 4D). We also confirmed the expression level of phospho-S6 by Western blotting of proteins extracted from lesion-specific brain sections. The ratio of phosphorylation (the ratio of intensities of phospho-S6/ total-S6) in each FCD type IIb individual with an MTOR mutation (range, 0.72-1.24; average = 1.17) was much higher than that in the control specimens (range, 0.22-0.63; average = 0.51; Fig 4E). Note that the lowest ratio (0.72) in FCD type IIb was still greater than the highest ratio (0.63) in the control. These results indicated that mTOR signaling was increased in FCD type IIb cases of MTOR mutations.

#### MTOR Mutations Activate the mTOR Pathway

mTOR (aa 1,376–2,549) consists of Huntingtin, elongation factor 3, a subunit of protein phosphatase 2A, and TOR1 (HEAT) repeats, a FRAP, ATM, and TRAP (FAT) domain, a kinase domain comprising N- and Clobes that form a catalytic cleft, a FKBP12/rapamycin binding (FRB) domain, and a FRAP, ATM, TRRAP, and C-terminal (FATC) domain (Figs 2A and 5A).<sup>31</sup> The neighboring p.Leu1460Pro and p.Ala1459Asp mutations are located in the FAT domain, whereas p.Ser2215Tyr and p.Ser2215Phe are at the same residue within the kinase domain (Figs 2A and 5A). All altered amino acids are evolutionarily well conserved (Fig 2A), and the mutations were predicted to be deleterious by at least two online programs (Table 3). To evaluate the effects of missense mutations on mTOR function from a structural viewpoint, we mapped their location on the mTOR crystal structure (aa 1,376-2,549) in complex with mLST8, ATPyS, and magnesium ions.<sup>31</sup> The FRB domain in the N-lobe and helix 9b in the C-lobe hinder substrate access to the active site, negatively regulating mTOR kinase activity.33-35 Because residues at sites Ala1459 and Leu1460 are located in an N-terminal helix of the FAT domain and are involved in a hydrophobic core (Fig 5A, C), mutations here would destabilize the local structure of the FAT domain and impair its interaction with the kinase domain. This could release it from the restriction of active site accessibility, thereby hyperactivating mTOR kinase activity as reported previously.33 Ser2215 is located in helix 3b, which is adjacent to the structural frame involving closely located helices 3, 9, and 9b (Fig 5A, B). Thus, mutations of Ser2215 to Tyr and Phe are likely to destabilize the structural frame and affect helix 9b conformation, restricting active site accessibility, and leading to mTOR kinase hyperactivation. Thus, all four somatic MTOR mutations were predicted to induce mTOR pathway hyperactivation. The free energy change calculated by FoldX software revealed a remarkable increase after all mutations, supporting our structural predictions (Fig 5D). Moreover, the MTOR mutations found in FCD type IIb were analyzed in cultured cells (in vitro). Plasmids carrying WT and mutant MTORs (p.Ala1459Asp, p.Leu1460Pro, p.Ser2215Phe, and p.Ser2215Tyr) were transfected into HEK293T cells together with FLAG-4EBP, the best-known substrate for mTOR complex 1 (mTORC1). Mutant MTOR-transfected cells exhibited significantly strong phosphorylation of 4EBP, compared to WT MTOR-transfected cells, indicating that all MTOR mutants are constitutively active



FIGURE 6: Schematic presentation of the mammalian target of rapamycin (mTOR)-signaling pathway. Upstream regulators of mTOR complex 1 (mTORC1), after stimulation with growth factors, are PI3K, Akt, TSC1/2, and Rheb. PTEN suppresses Akt activity. Another regulatory pathway that is activated by amino acids is Rags/Ragulator signaling. Gator1 suppresses mTORC1 activity. Activated mTORC1 directly phosphorylates 4EBP, p70S6K, and ULK1. Focal cortical dysplasia is caused by gain-of-function mutations (red arrows) or loss-of-function mutations (blue arrows) in mTOR-related genes. Black arrows and cross-end bars indicate activation of phosphorylation and inhibition of phosphorylation, respectively.

(Fig 5E). Signal intensities of all *MTOR* mutants were comparable with that of the plasmid carrying a hyperactive mutation of rat mTOR, which was used as a positive control.<sup>34</sup>

#### Discussion

We found that the low-prevalence somatic MTOR mutations in 6 of 13 individuals with FCD type IIb (46%; Table 2 and Supporting Table 4). While this article was being reviewed, Lim et al also reported that somatic MTOR mutations caused FCD type IIb.36 These results corroborated that low-prevalence somatic MTOR mutation is one of the genetic causes in FCD type IIb. However, there may be mutations in up- or downstream genes in the mTOR cascade in the other subjects having no MTOR mutation (Fig 6). Indeed, somatic mutations in PTEN and germline mutations in DEPDC5, encoding a component of Gator1, were reported in FCD type IIb.<sup>16–18,37</sup> PTEN is one of the tumor-suppressor genes, encoding plasma membrane lipid phosphatase, which antagonizes PI3K-Akt signaling.<sup>38</sup> Gator1 has GTPaseactivating activity for RagA/B and thus suppresses mTORC1 activity in static and low-amino-acid conditions.<sup>39</sup> Therefore, loss of function in PTEN and Gator1

may cause mTOR activation. Similarly, mutations in other genes involved in the mTOR cascade are involved in tuberous sclerosis (with mutations in *TSC1* and *TSC2*) and hemimegalencephaly (with mutations in *AKT3*, *DEPDC5*, *MTOR*, *PIK3CA*, and *PTEN*), which share histopathological similarities.<sup>12–17,19,37</sup> These findings strongly suggest that unidentified mutations in related molecules of the mTOR pathway may possibly be the pathogenesis of FCD type IIb.

Somatic MTOR mutations in FCD type IIb are likely to cause hyperactivation of the mTOR-signaling pathway, which is involved in growth, migration, and the maturation of neurons and glial cells.8 All individuals with MTOR mutations showed distinctive balloon cells in their brain tissues (Fig 3 C, F, I, L, O, R). Such cells are also observed in tuberous sclerosis and hemimegalencephaly, in which the mTOR-signaling pathway is activated,<sup>2,8,10,13,32</sup> raising the possibility that they are markers of aberrant mTOR signaling. Our IHC and Western blotting results clearly demonstrated high levels of phospho-S6, the well-known hallmark of mTOR cascade activation, in FCD type IIb lesions with MTOR mutations, specifically in dysmorphic neurons and balloon cells (Fig 4A, B, E). We failed to detect apparent differences on IHC expression of phospho-S6 between FCD type IIb with and without mutations (Fig 4A-C). In both cases, a large proportion of the abnormal cells (dysmorphic neurons and balloon cells) were phospho-S6-positive. Furthermore, we found no clinical differences between FCD type IIb individuals with or without MTOR mutations. These findings supported the hypothesis that MTOR-mutation-negative subjects may have mutations in other molecular members of the pathway, which lead to net activation of the mTOR signaling.<sup>19</sup>

We also investigated MTOR mutations in limited numbers of brain samples with FCD type I and IIa (six in type I and five in type IIa) and found no associated variants (Supporting Table 3). IHC examination of FCD type IIa revealed that many dysmorphic neurons showed phospho-S6-immunoreactivity as those noted in FCD type IIb (data not shown). In our series of FCD type IIa, both the number of dysmorphic neurons and intensities of phospho-S6-immunopositivity differed markedly among cases. Interestingly, Lim et al also reported somatic MTOR mutations in FCD type IIa.<sup>36</sup> Therefore, a further study with a large number of FCD type IIa cases would be necessary for better understanding of the molecular profiles and precise pathomechanisms underlying FCD type IIa. In control neocortical tissues, we found no dysmorphic neurons, balloon cells, or apparent cytoarchitectural abnormalities without any trace of aberrant mTOR signaling (Fig 4D, E). Consistent with this,

we observed no apparent phospho-S6 immunoreactivity in FCD type I (data not shown).mTOR activity is controlled by various extracellular cues, such as nutrients and growth factors in the brain.8 In a culture system, all of the mutants found in this study were constitutively active (Fig 5E), regardless of any extracellular stimuli. Therefore, these somatic mutations could induce dysregulation of growth of neurons and glia, or presumably of their progenitors during brain development. A large number of somatic mutations in cancer cells are clustered in the FAT domains or the kinase domains, and most activate the mTOR-signaling pathway.<sup>6,33</sup> The mTOR protein can form two distinct complexes-mTORC1 and mTOR complex 2 (mTORC2)-and involvement of these different complexes results in divergence of the signaling pathway into two distinct channels. mTORC1 is composed of mTOR, mLST8, and Raptor and phosphorylates the downstream proteins, S6K1 and 4EBP, which regulate cell growth, proliferation, ribosome biogenesis, and autophagy (Fig 6).<sup>32,40</sup> mTORC2 is composed of mTOR, mLST8, Rictor, and mSin1 and phosphorylates Akt, PKC, and SGK, which are involved in cell cycle, cell survival, and actin organization and morphology.<sup>32,40,41</sup> Rapamycin, an allosteric inhibitor of mTOR, binds to mTOR with FKBP12 and inhibits kinase activity of mTORC1. mTORC1 is known to play a pivotal role in cerebral cortical development, though little is known about mTORC2.<sup>8,9</sup> The mutations, p.Ser2215Tyr and p.Leu1460Pro, lead to the constitutive activation of mTORC1 signaling, but not that of mTORC2, and both mutations are sensitive to rapamycin.<sup>6,42</sup> These findings imply that mTOR inhibitors would be able to alleviate intractable epilepsy in FCD type IIb.

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#### Authorship

M.K., A.K., and N.Ma. conceived the study. M.N., H.Sa., and N.Ma. designed and performed analyses. M.N., Y.T., N.Mi., and H.Sa. performed exome sequencing. M.N. and C.O. performed targeted amplicon sequencing. M.N. performed statistical analysis and ddPCR. M.S. and K.O. performed protein structural analyses. J.T., M.K., H.Sh., H.M., and S.K. evaluated patients and provided samples. H.K, K.W., H.T., Y.Z., and A.K. processed tissue samples and performed pathological evaluation. N.T. and T.S. validated mTORC1 signaling. M.N., H.Sa., N.T., A.K., and N.Ma. wrote the manuscript. M.K., A.K., and N.Ma. obtained research funding.

#### **Potential Conflicts of Interest**

Nothing to report.

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### Original Article

### Immunohistochemical profiles of IDH1, MGMT and P53: Practical significance for prognostication of patients with diffuse gliomas

### Ryosuke Ogura,<sup>2</sup> Yoshihiro Tsukamoto,<sup>2</sup> Manabu Natsumeda,<sup>1</sup> Mizuho Isogawa,<sup>1</sup> Hiroshi Aoki,<sup>1</sup> Tsutomu Kobayashi,<sup>1</sup> Seiichi Yoshida,<sup>1</sup> Kouichiro Okamoto,<sup>1</sup> Hitoshi Takahashi,<sup>2</sup> Yukihiko Fujii<sup>1</sup> and Akiyoshi Kakita<sup>2</sup>

Departments of <sup>1</sup>Neurosurgery and <sup>2</sup>Pathology, Brain Research Institute, University of Niigata, Niigata, Japan

Genetic and epigenetic status, including mutations of isocitrate dehydrogenase (IDH) and TP53 and methylation of O<sup>6</sup>-methylguanine-DNA methyltransferase (MGMT), are associated with the development of various types of glioma and are useful for prognostication. Here, using routinely available histology sections from 312 patients with diffuse gliomas, we performed immunohistochemistry using antibodies specific for IDH1 mutation, MGMT methylation status, and aberrant p53 expression to evaluate the possible prognostic significance of these features. With regard to overall survival (OS), univariate analysis indicated that an IDH1-positive profile in patients with glioblastoma (GBM), anaplastic astrocytoma (AA), anaplastic oligoastrocytoma and oligodendroglioma, or a MGMT-negative profile in patients with GBM and AA were significantly associated with a favorable outcome. Multivariate analysis revealed that both profiles were independent factors influencing prognosis. The OS of patients with IDH1-positive/MGMTnegative profiles was significantly longer than that of patients with negative/negative and negative/positive profiles. A p53 profile was not an independent prognostic factor. However, for GBM/AA patients with IDH1negative/MGMT-negative profiles, p53 overexpression was significantly associated with an unfavorable outcome. Thus, the immunohistochemical profiles of IDH1 and MGMT are of considerable significance in gliomas, and a combination of IDH1, MGMT and p53 profiles may be useful for prognostication of GBM/AA.

**Key words:** glioma, IDH1, immunohistochemistry, MGMT, p53.

#### INTRODUCTION

Glioma is the most common type of primary brain tumor. Recent molecular studies of various histopathological types of glioma<sup>1</sup> have revealed several genetic pathways critical for the development of astrocytic and oligodendroglial tumors.<sup>2,3</sup> For prognostication of patients with diffuse gliomas, it has been shown that the molecular profiles of isocitrate dehydrogenase 1/2 (IDH1/2) mutation,  $O^6$ -methylguanine-DNA methyltransferase (MGMT) promoter methylation, 1p19q co-deletion, and glioma CpG island methylator phenotype (G-CIMP) are possible factors, whereas the impact of TP53 and EGFR mutation has remained unclear.<sup>4</sup> Moreover, a recent genetic and epigenetic study has revealed that a combination of IDH1 mutation and MGMT methylation status outperforms information about either IDH1 or MGMT alone in predicting the survival of patients with glioblastoma (GBM).<sup>5</sup> Thus, in clinical practice, it is important to have information on these molecular profiles. However, identification of molecular profiles by DNA sequencing requires intensive laboratory work by trained personnel using specialized equipment, and therefore the methodology is not always available for daily clinical use at every medical center.

On the other hand, immunohistochemistry is a technique that can be applied to routinely available histology sections. For detection of *IDH1* mutations, an IDH1-R132H mutation-specific antibody is available, and the results show concordance with DNA sequencing without any cross-reactivity with the wild-type or other mutant IDH1 proteins.<sup>6,7</sup> Moreover, immunohistochemistry with a mono-clonal antibody MT3.1 against MGMT protein has been

Correspondence: Akiyoshi Kakita, MD, PhD, Department of Pathology, Brain Research Institute, University of Niigata, 1-757 Asahimachi, Chuo-ku, Niigata 951-8585, Japan. Email: kakita@ bri.niigata-u.ac.jp

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**Table 1** Clinical profiles of patients in each group

Group	Grade IV	Gr	ade III	Gra	de II
	GBM	AA	AOA/AOD	DA	OA/OD
No. of patients	165	61	40	19	27
Age (years)					
Median	64	58	59	38	45
Range	18-85	24-89	22-81	24-67	25-74
Age (years), $n$ (%)					
<50	25 (15.1)	23 (37.7)	8 (20)	12 (63.2)	15 (55.6)
50–59 years	26 (15.8)	10 (16.4)	11 (27.5)	5 (26.3)	4 (14.8)
60–69 years	60 (36.4)	7 (11.5)	10 (25.0)	2 (10.5)	6 (22.2)
≥70	54 (32.7)	21 (34.4)	11 (27.5)	0	2 (7.4)
Gender, $n$ (%)			× /		
Male	93 (56.3)	33 (54.1)	26 (65)	9 (47.4)	14 (51.9)
Female	72 (43.6)	28 (45.9)	14 (35)	10 (52.6)	13 (48.1)
KPS, <i>n</i> (%)					
100	4 (2.4)	11 (18.0)	4 (10.0)	12 (63.2)	9 (33.3)
70–90	95 (57.6)	32 (52.5)	19 (47.5)	7 (36.8)	17 (63.0)
<70	66 (40.0)	18 (29.5)	17 (42.5)	0	1 (3.7)
Surgery, $n$ (%)					
Total/Subtotal	72 (43.6)	12 (19.6)	21 (52.5)	12 (63.2)	17 (63.0)
Partial	53 (32.1)	27 (44.3)	19 (47.5)	7 (36.8)	9 (33.3)
Stereo biopsy	38 (23.0)	19 (31.2)	0	0	1 (3.7)
Others	2 (1.2)	3 (4.9)	0	0	0
Initial treatment, $n$ (%)					
None	8 (4.8)	2 (3.3)	0	12 (63.2)	11 (40.7)
RT alone	61 (37.0)	21 (34.4)	8 (20.0)	7 (36.8)	16 (59.3)
CRT	95 (57.6)	36 (59.0)	32 (80.0)	0 `	0 ` ´
Others	1 (0.6)	2 (3.3)	0	0	0

GBM, glioblastoma; AA, anaplastic astrocytoma; AOA, anaplastic oligoastrocytoma; AOD, anaplastic oligodendroglioma; DA, diffuse astrocytoma; OA, oligoastrocytoma; OD, oligodendroglioma; KPS, Karnofsky performance status; RT, radiotherapy; CRT, chemoradiotherapy.

employed to assess *in vivo MGMT* methylation status, and a significant negative association of MGMT with survival has been detected in patients with WHO grades II and IV astrocytomas.<sup>8</sup> Furthermore, immunohistochemical detection of overall p53 protein expression may be an independent indicator of shorter survival in patients with astrocytic tumors, even though overexpression of p53 is not always associated with point mutations in the *TP53* gene.<sup>9</sup> In the present study, we performed immunohistochemistry using antibodies specific for IDH1 mutation, MGMT status, and aberrant p53 expression in order to evaluate the possible prognostic significance of these features and their combinations in patients with diffuse gliomas.

#### **MATERIALS AND METHODS**

#### **Patients**

We reviewed the medical records of 312 consecutive patients (175 male, 137 female: age at surgery, mean  $\pm$  SD = 58.4  $\pm$  15.6 years) who were admitted to the University of Niigata Hospital, Japan, between 2000 and 2013, and were diagnosed pathologically as having diffuse gliomas, including diffuse astrocytoma (DA), oligodendroglioma (OD), oligoastrocytoma (OA), anaplastic astrocytoma

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(AA), anaplastic oligodendroglioma (AOD), anaplastic oligoastrocytoma (AOA) and GBM. The study was approved by the hospital Ethics Committee, and written informed consent to use resected tissues for research purposes was obtained from all patients. First, in accordance with the histopathological diagnosis of diffuse gliomas,<sup>1</sup> the patients were divided into five groups: GBM, AA, AOA/AOD, DA and OA/OD. Table 1 summarizes the clinical profiles of the patients in each of these histologically defined groups.

#### Histology and immunohistochemistry

The surgical specimens were fixed with 20% buffered formalin and embedded in paraffin. Histopathological examination was performed on 4-µm-thick sections stained with HE. The paraffin-embedded sections were processed for immunohistochemistry using methods previously described.<sup>10</sup> Primary monoclonal antibodies against the following antigens were used: human IDH1 R132H (clone H09; Dianova, Hamburg, Germany; dilution 1:100), MGMT (clone MT3.1; Chemicon International, Temecula, CA, USA; 1:50), and human p53 (clone DO-7; Dako, Glostrup, Denmark; 1:50). Immunohistochemical features were evaluated by two persons (RO and YT) independently without information on the patients' profiles or histological grades, and then were confirmed by a pathologist (AK).

## *IDH1* mutations: direct DNA sequencing and immunohistochemical evaluation

Genomic DNA was extracted from paraffin-embedded sections, as described previously,<sup>11</sup> and PCR amplification was performed using primer sets (forward: 5'-CGGTCT TCAGAGAAGCCATT-3'; and reverse: 5'-TTCATACC TTGCTTAATGGGTGT-3') for exon 4 of the *IDH1* gene around the R132 residue. The PCR products were then sequenced on a 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) with a Big Dye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems) in accordance with the manufacturer's instructions.

IDH1 immunoreactivity was judged as "positive" when a large proportion of tumor cells showed strong cytoplasmic reactivity (Fig. 1A), whereas it was regarded as "negative" if weak or no reactivity was observed (Fig. 1B). Labeling of macrophages was ignored.

To compare the results of both direct DNA sequencing and immunohistochemistry, we preliminarily applied both methods to specimens taken from 37 patients with GBM. All four patients with an IDH1-immunopositive profile showed an identical heterozygous mutation in IDH1 (CGT $\rightarrow$ CAT) (Fig. 1C) resulting in an Arg $\rightarrow$ His (R132H) amino acid substitution. The other 33 patients with an IDH1-immunonegative profile showed the wild-type IDH1 gene. Furthermore, direct DNA sequencing using samples taken from areas with sparsely infiltrating tumor cells failed to detect the mutation, although in one patient with anaplastic astrocytoma immunohistochemistry demonstrated a small number of positive cells in this type of area (Fig. 1C). Thus, application of immunohistochemistry for detection of IDH1 R132H mutation was shown to be reliable and of practical use.

#### **Evaluation of MGMT and p53**

MGMT immunoreactivity was evaluated in representative areas of the tumors showing the characteristic features defining their histological grades.<sup>1</sup> Individual tumor nuclei were regarded as positive when they showed an intensity similar to that of vascular endothelial cells used as an internal positive control (Fig. 1D,E). In accordance with a previous report,<sup>12</sup> we determined percentages of MGMTpositive tumor cells in individual cases by continuously counting over 400 nuclei observed in high-magnification views. We carefully excluded possible positive staining of non-neoplastic cells, including oligodendrocytes and infiltrating inflammatory cells.<sup>13,14</sup> For individual cases, when the proportion of labeled nuclei/all tumor nuclei was 30% or more, the case was regarded as "positive", whereas a case was considered "negative" when the proportion was less than 30% (Fig. 1D,E). Indeed, there was a clear difference between the numbers of "negative" and "positive" cases (Fig. 1F), which enabled us to judge with confidence. Immunonegativity for MGMT may represent the methylation status of the MGMT gene, and is positively linked to the response of a tumor to chemotherapy.<sup>15</sup>

Evaluation of p53 expression was performed in a semiquantitative manner by continuously counting over 1000 tumor cells in the areas of highest expression. According to the percentage of tumor cells showing reactivity, we distinguished four groups: many (>50%), several (10–50%), rare (<10%), or no cells positive (Fig. 1G–J).<sup>16</sup> In accordance with previous reports,<sup>9,17</sup> we considered cases in the many and several groups as "positive" and those in the rare and negative groups as "negative".

#### Statistical analysis

Data analysis was carried out using the SPSS ver17.0 software package (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 5.0 statistical software (http://graphpad-prism.software.informer.com/5.0/). Overall survival (OS) and progression-free survival (PFS) were calculated from the time of surgery until death, disease progression, or last follow-up according to the Kaplan-Meier method with log-rank test for comparison between groups. Univariate and multivariate analysis with the Cox proportional hazards model was used to assess prognostic factors. Results are expressed as relative risk with the 95% confidence interval (95% CI).

#### RESULTS

### Significance of immunohistochemical profiles of IDH1, MGMT and p53

Tables 2 and 3 summarize the immunohistochemical profiles and their prognostic values in terms of OS and PFS for patients included in each of the histologically defined groups. Figure 2 shows the Kaplan-Meier curves of the OS rate and median OS (mOS) for patients with grades III/IV malignant astrocytomas (AA/GBM).

The numbers of patients with IDH1-positive profiles were significantly higher and lower than those of patients with IDH1-negative profiles for grade II (DA, P = 0.003; OA/OD, P = 0.004) and grades III (AA, P = 0.007)/IV (GBM, P < 0.001) gliomas, respectively, and there were no significant differences in the numbers of patients with both profiles in the AOA/AOD group (P = 0.114). The numbers of patients with MGMT-negative profiles in the oligodendroglial tumor groups (OA/OD, P = 0.001; AOA/AOD,



**Fig. 1** Evaluation of isocitrate dehydrogenase 1 (IDH1), O<sup>6</sup>-methylguanine-DNA methyltransferase (MGMT) and p53 status. Immunohistochemistry for IDH1 (A, B), MGMT (D, E), and p53 (G–J). Examples of IDH1 positivity (A), IDH1 negativity (B), MGMT positivity (D), and MGMT negativity (E). Arrowheads in D and E indicate endothelial cells used as an internal positive control. Examples of p53 many (G), several (H), rare (I), and no (J) cells positive. (C) Demonstration of IDH1 immunohistochemistry and direct DNA sequencing of *IDH1* in a patient with anaplastic astrocytoma. In the tumor center (left panels), many IDH1-positive cells and heterozygous mutation of *IDH1* R132H (arrow) are evident, whereas in the infiltrating area (right panels), scattered IDH1-positive cells but no mutations are demonstrated. (F) A histogram showing the proportion of the MGMT-labeled nuclei/all tumor nuclei for all 165 cases of glioblastoma. A clear numerical distinction is evident between less than 30% (regarded as "negative") and 30% and more ("positive"). Scale bars = 50 µm.

Group	Total		IDH1			MGMT		p5	3
		Positive	Negative	P value	Negative	Positive	P value	Negative	P value
GBM									
No. of patients (%)	165	6 (4)	159 (96)	< 0.001	89 (54)	76 (46)	0.312	62 (38)	0.001
mOS (months)	14	66	14	0.006	18	12	< 0.001	13	0.921
mPFS (months)	5	59	4	0.005	7	3	< 0.001	5	0.671
AA									
No. of patients (%)	61	20 (33)	41 (67)	0.007	33 (54)	28 (46)	0.522	28 (46)	0.522
mOS (months)	22	46	15	0.013	30	15	0.009	18	0.967
mPFS (months)	5	22	4	0.002	11	3	< 0.001	6	0.847
AOA/AOD									
No. of patients (%)	40	15 (38)	25 (62)	0.114	32 (80)	8 (20)	< 0.001	19 (47)	0.752
mOS (months)	80	ŇŔ	25	0.025	80	21	0.206	25	0.522
mPFS (months)	24	NR	10	0.001	28	7	0.160	20	0.470
DA									
No. of patients (%)	19	16 (84)	3 (16)	0.003	11 (58)	8 (42)	0.491	14 (74)	0.039
mOS (months)	NR	NR	ND	_	NR	ND	_	ND	-
mPFS (months)	NR	NR	51	_	44	NR	_	51	-
OA/OD									
No. of patients (%)	27	22 (81)	5 (19)	0.004	21 (78)	6 (22)	0.001	23 (85)	< 0.001
mOS (months)	NR	ŇŔ	40	0.100	NR	NR	0.381	NR	0.021
mPFS (months)	NR	NR	19	0.080	NR	19	0.142	NR	0.031

**Table 2**Prognostic value of mOS and mPFS of patients included in each histologically defined group, in accordance with immunohis-<br/>tochemical profiles of IDH1, MGMT and p53 of the gliomas

P-values were calculated by the log-rank test and Chi-square test.

mOS, median overall survival; mPFS, median progression free survival; NR, not reached; ND, not determined. GBM, glioblastoma; AA, anaplastic astrocytoma; AOA, anaplastic oligoastrocytoma; AOD, anaplastic oligodendroglioma; DA, diffuse astrocytoma; OA, oligoastrocytoma; OD, oligodendroglioma

P < 0.001) were significantly higher than those of patients with MGMT-positive profiles, whereas the numbers of patients with either profile in the astrocytic tumor groups (DA, P = 0.491; AA, P = 0.522; GBM, P = 0.312) were almost equal. The proportion of patients with a p53positive profile in each histologically defined group appeared to increase in accordance with malignancy grading (OA/OD 15%, DA 26%, AOA/AOD 53%, AA 54% and GBM 62%). The number of patients with p53positive profiles was significantly higher than that of patients with p53-negative profiles for grade IV (GBM, P = 0.001) only.

With regard to OS and PFS, univariate analysis indicated that either an IDH1-positive profile for patients with GBM (OS and PFS: P = 0.014 and P = 0.015, respectively), AA (P = 0.020 and P = 0.004) (Fig. 2A for GBM/ AA) and AOA/AOD (P = 0.035 and P = 0.003), or a MGMT-negative profile for patients with GBM (both P < 0.001) and AA (P = 0.016 and P = 0.006) (Fig. 2B for GBM/AA) was significantly associated with a favorable outcome. Multivariate analysis revealed that both an IDH1-positive profile (P = 0.031 and P = 0.013) and a MGMT-negative profile (both P < 0.001) were independent factors affecting prognosis for patients with GBM (Table 3). For patients with grade II gliomas (DA and OA/OD), the prognostic value of IDH1 and MGMT status was less significant.

On the basis of the IDH1 and MGMT profiles, a total of 226 patients with grades III/IV malignant astrocytomas (165 and 61 patients with GBM and AA, respectively) were divided into four groups. The mOS of patients with IDH1-positive/MGMT-negative (n = 18), positive/positive (n = 8), negative/negative (n = 104), and negative/positive (n = 96) profiles was 56, 22, 17 and 12 months, respectively (Fig. 2C). The mOS of patients with positive/negative profiles was significantly longer than that of patients with negative/negative (P = 0.002) and negative/positive (P < 0.001) profiles. Similarly, the mOS of patients with positive/positive and negative/negative profiles was significantly longer (P = 0.008 and P < 0.001, respectively) than that of patients with a negative/positive profile. Thus, a combination of both IDH1 and MGMT profiles was able to accurately predict outcome in patients with malignant atrocytomas.

On the other hand, a p53 positive/negative profile was not an independent factor affecting prognosis for patients with malignant astrocytomas (Table 3) when we defined the many and several groups (p53 >10%) (Fig. 2D) or the many group only (p53 >50%) (Fig. 2E) as "positive". Next, we examined the effects of combinations of the IDH1, MGMT and p53 profiles (Fig. 2F–I). For patients with IDH1-positive profiles (Fig. 2F,G), neither p53 >10% (Fig. 2F) nor p53 >50% (Fig. 2G) were a prognostic factor. Similarly, for patients with IDH1-negative profiles

#### IDH1/MGMT/p53 immunoprofiles and glioma prognosis

Table 3	Prognostic univariate	e and multivariate	Cox regression	analyses of	patients included in	each histologically	defined group
				/			

Univariate			OS			PFS	
		HR	95% CI	P-value	HR	95% CI	P-value
GBM							
IDH1	Pos vs. neg	0.230	0.071-0.742	0.014	0.228	0.069-0.751	0.015
MGMT	Neg vs. pos	0.463	0.323-0.665	< 0.001	0.503	0.347-0.729	< 0.001
p53	Pos vs. neg	1.014	0.711-1.445	0.940	1.073	0.740-1.556	0.709
KPS	≧70 vs. <70	0.614	0.431-0.876	0.007	0.717	0.493-1.043	0.082
Age	≧65 vs. <65	1.632	1.142-2.332	0.007	1.226	0.848-1.774	0.278
Total/subtotal	Yes vs. no	0.530	0.367-0.765	0.001	0.605	0.420-0.872	0.007
CRT	Yes vs. no	0.446	0.313-0.638	< 0.001	0.585	0.400-0.854	0.005
AA							
IDH1	Pos vs. neg	0.459	0.239-0.884	0.020	0.373	0.189-0.736	0.004
MGMT	Neg vs. pos	0.473	0.257-0.872	0.016	0.416	0.221-0.780	0.006
p53	Pos vs. neg	1.066	0.583-1.951	0.835	0.987	0.530-1.839	0.968
KPS	$\geq 70 \text{ vs.} < 70$	0.334	0.169-0.660	0.002	0.413	0.204-0.835	0.014
Age	≧60 vs. <60	3.120	1.651-5.898	< 0.001	1.964	1.001-3.850	0.050
Total/subtotal	Yes vs. no	0.382	0.173-0.844	0.017	0.370	0.160-0.858	0.021
CRT	Yes vs. no	0.565	0.305-1.046	0.069	0.883	0.461-1.692	0.707
AOA/AOD							
IDH1	Pos vs. neg	0.291	0.092-0.916	0.035	0.238	0.093-0.611	0.003
MGMT	Neg vs. pos	0.521	0.184–1.474	0.219	0.529	0.212-1.320	0.172
p53	Pos vs. neg	0.742	0.294-1.874	0.528	0.760	0.356-1.622	0.477
KPS	$\geq 70$ vs. $< 70$	0.339	0.122-0.945	0.039	0.394	0.173-0.896	0.026
Age	$\geq 60$ vs. < 60	2.067	0.777-5.501	0.146	3.267	1.407-7.584	0.006
Total/subtotal	Yes vs. no	0.260	0.092-0.737	0.011	0.399	0.184-0.866	0.020
CRT	Yes vs. no	0.560	0.198-1.578	0.272	0.836	0.333-2.096	0.702
DA	100 101 110	01000	011/0 110/0	01272	01020		01702
IDH1	Pos vs. neg	NR			1.014	0.117-8.774	0.990
MGMT	Neg vs. pos	NR			2.276	0.384–13.497	0.365
n53	Pos vs. neg	6.329	0.569-70.412	0.133	0.645	0.075-5.544	0.690
Age	$\geq 45$ vs < 45	2 171	0 131-35 996	0.588	0.400	0.046-3.451	0.405
Total/subtotal	Yes vs. no	0.812	0.066-9.975	0.871	0.714	0.140-3.643	0.685
RT	Yes vs no	NR	01000 91970	01071	1 290	0 255-6 525	0.758
OA/OD	105 (5.110	1.11			1.270	0.255 0.525	0.750
IDH1	Pos vs. neg	0.246	0.040 - 1.498	0.128	0.306	0.075-1.239	0.097
MGMT	Neg vs pos	0.458	0.076-2.746	0 393	0 399	0 112-1 420	0.156
n53	Pos vs. neg	6.407	1.052-39.020	0.044	4.155	1.025-16.839	0.046
Age	$\geq 45$ vs < 45	1 504	0.251-9.006	0.655	2 299	0 547-9 657	0.255
Total/subtotal	Yes vs no	0.345	0.058-2.068	0.244	0.321	0.076–1.348	0.121
RT	Yes vs. no	1.829	0.204–16.389	0.589	3.236	0.397-26.349	0.272
Multivariate	100 101 110	11025	05	0.000	01200	PES	
withivallate			05% CI	Dyvalue		05% CI	D voluo
		ПК	95% CI	<i>P</i> -value	пк	95% CI	<i>P</i> -value
GBM				0			
IDH1	Pos vs. neg	0.265	0.079-0.886	0.031	0.209	0.061-0.715	0.013
MGMT	Neg vs. pos	0.457	0.315-0.663	< 0.001	0.483	0.329-0.710	< 0.001
KPS	≧70 vs. <70	0.694	0.481-1.002	0.051	0.667	0.454-0.980	0.039
Age	≧65 vs. <65	1.207	0.814-1.791	0.349	0.982	0.657–1.469	0.931
Total/subtotal	Yes vs. no	0.582	0.402-0.841	0.004	0.618	0.427-0.895	0.011
CRT	Yes vs. no	0.580	0.390-0.864	0.007	0.675	0.446-1.023	0.064

*P* values were calculated by Cox proportional hazard models.

CI, confidence interval; HR, hazard ratio; neg, negative; pos, positive; GBM, glioblastoma; AA, anaplastic astrocytoma; AOA, anaplastic oligoastrocytoma; AOD, anaplastic oligodendroglioma; DA, diffuse astrocytoma; OA, oligoastrocyotma; OD, oligodendroglioma; KPS, Karnofsky performance status; RT, radiotherapy; CRT, chemoradiotherapy

(Fig. 2H,I), p53 >10% (Fig. 2H) was not a prognostic factor, and a MGMT-negative profile appeared to operate as a favorable factor. However, the OS of patients with p53 >50% who were MGMT-negative (n = 28) was significantly shorter than that of patients with p53  $\leq 50\%$ /MGMT-

negative profiles (n = 76, P = 0.044) (Fig. 2I). Thus, for patients with IDH1-negative/MGMT-negative profiles, p53 >50% was associated with a poor outcome.

Based on combinations of the IDH1, MGMT and p53 profiles, three groups of patients with malignant

astrocytomas were distinguishable in terms of outcome (Figs 2J and 3). Group C (n = 124) showed significantly the poorest outcome (mOS = 12 m) relative to Group B (n = 76, mOS = 18 m, P < 0.001) and Group A (n = 18, mOS = 56 m, P < 0.001). Group B also showed a poorer outcome than Group A (P = 0.005).

#### **Clinical factors and prognosis**

For patients with malignant astrocytomas, several clinical characteristics, including Karnofsky performance status score, age, total/subtotal removal of the tumor, and use of chemoradiotherapy (CRT: yes vs. no, n = 131 vs. 95; Fig. 2K) showed a significant relationship with OS (Table 3). Multivariate analysis indicated that total/subtotal removal of the tumor and use of CRT were independent factors significantly associated with a favorable outcome (Table 3).

For patients with malignant astrocytomas who received CRT (n = 131; Fig. 2L), the mOS of those with a MGMT-negative profile (n = 77, 24 months) was significantly (P < 0.001) longer than that of patients with a MGMT-positive profile (n = 54, 15 months).

#### DISCUSSION

Recent molecular analyses of glioma tissue have revealed that several profiles, including *IDH1/2* mutation, *TP53* mutation, *TERT* promoter mutation, *ATRX* mutation, 1p/19q co-deletion, and *MGMT* methylation status, are associated with the development of various histological types of glioma. In the present study, we performed immunohistochemistry to evaluate subsets of these molecular profiles using routinely available histology sections (Fig. 1). This revealed that the significance of the profiles differed in accordance with the histopathological classification of the gliomas, and that combination of the profiles was of prognostic value (Tables 2 and 3, and Figs 2 and 3).

Patients with *IDH1*-mutated malignant glioma may survive longer than those with wild-type *IDH1* glioma.<sup>18-20</sup> Recently, a genetic and epigenetic study has indicated a possible mechanism underlying this phenomenon, in which IDH1 mutations induce the CpG island methylator phenotype, a factor associated with biologically benign behavior of the glioma cells.<sup>21</sup> Therefore, in clinical practice, detection of IDH1 mutations is important, and for this purpose direct DNA sequencing is frequently employed. However, tissue samples from diffuse gliomas are usually an admixture of tumor cells and pre-existing brain parenchyma; therefore the sensitivity of this method for detection of a mutant sequence within wild-type sequences depends on the ratio of the two tissue components, and is estimated to be 20% or more.<sup>22</sup> Pyrosequencing analysis is a much more sensitive method, capable of detecting a minimum of 5% mutant alleles.<sup>22,23</sup> Moreover, the use of BEAMing (beads, emulsion, amplification, magnetics) PCR and droplet digital PCR can detect a mutant allele in a tissue sample with a sensitivity of 0.01%.<sup>24</sup> Thus, advances in methodology have improved the sensitivity of mutation detection; on the other hand, these techniques require specific equipment and skill, and as a consequence they are sometimes unavailable in a standard clinical laboratory. Immunohistochemistry is a technique that can be performed simultaneously with practical histopathological diagnosis of tumors. This method has apparently high sensitivity for detection of IDH1 mutations.7 Consistent with this, our preliminary attempt to perform both immunohistochemistry and direct DNA sequencing on tissues showing glioma infiltration showed that mutation was detectable by immunohistochemistry only (Fig. 1C).

Immunohistochemistry with the IDH1-R132H antibody is highly specific for detecting the most common mutation in the gene.<sup>7</sup> The sensitivity of this method is estimated to be 94%,<sup>25</sup> but other *IDH1* mutations, including R132C, R132S, R132G, R132L, R132V and R132P,<sup>6,7,20,26-30</sup> or *IDH2* mutations<sup>31</sup> are not recognized by this antibody. Therefore, even though such *IDH1/2* mutations are rare in practice, for IDH1-immunonegative cases, direct DNA sequencing for *IDH1/2* may provide more detailed information on the genes.<sup>32–34</sup>

The frequencies of patients with IDH1-immunopositive profiles in the present study (Table 2) were similar to those reported previously, where direct DNA sequencing identified *IDH1* mutations in 70%-80% of diffuse gliomas, including DA (72.51%), OD (77.81%), OA (81.43%), AA (48.71%), AOA (66.40%), AOD (32.79%) and primary

**Fig. 2** Kaplan-Meier curves. Overall survival rate (OS) for patients with grades III/IV malignant astrocytomas (anaplastic astrocytoma and glioblastoma) (A–J). Median OS (months: *m*) for patients with (A) isocitrate dehydrogenase 1 (IDH1), (B) O<sup>6</sup>-methylguanine-DNA methyltransferase (MGMT), (C) the IDH1/MGMT combination, (D) p53 (>10%: many and several groups, or  $\le 10\%$ ), (E) p53 (>50%: many groups only, or  $\le 50\%$ ), (F) in patients with IDH1 positivity, combination of p53 (>/ $\le 10\%$ )/MGMT, (G) in patients with IDH1 positivity, combination of p53 (>/ $\le 10\%$ )/MGMT, and (I) in patients with IDH1 negativity, combination of p53 (>/ $\le 50\%$ )/MGMT, and (I) in patients with IDH1 negativity, combination of p53 (>/ $\le 50\%$ )/immunopositive (*pos*) or negative (*neg*) profiles are described. (J) OS for patients in Groups A, B and C. The groups were defined as depicted in Figure 3. (K) OS for patients with (*yes*) or without (*no*) chemoradiotherapy (CRT). (L) For the patients with CRT, a MGMT-negative profile was significantly associated with a favorable outcome. *P*-values were calculated by the log-rank test.



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Fig. 3 Schematic representation of Groups A, B and C distinguishable on the basis of combinations of the isocitrate dehydrogenase 1 (IDH1), O<sup>6</sup>-methylguanine-DNA methyltransferase (MGMT) and p53 immunoprofiles. The median overall survival (mOSs) of the three groups differ significantly. Eight patients with IDH1-positive and MGMT-positive profiles were excluded. Note that for the patients with IDH1-negative and MGMT-negative profiles, the p53 profile (> or  $\leq$ 50%) was a useful factor for prognostication.

GBM (4.43%).<sup>31</sup> In grade II gliomas, it is still unclear whether *IDH1* mutations influence outcome.<sup>35–38</sup> In the present study, IDH1 immunoprofiles showed no significant association with the outcome of patients with grade II gliomas (Tables 2 and 3). On the other hand, for patients who were diagnosed histopathologically as having grade III or IV glioma, IDH1 positivity was a factor significantly predictive of a favorable outcome (Tables 2 and 3).

At present, *MGMT* status is recognized to be a prognostic factor and a powerful predictor of the response to temozolomide in patients with GBM.<sup>15</sup> Based on several clinical studies involving patients with GBM, it has been shown that *MGMT* promoter methylation assessed by methylation-specific PCR or pyrosequencing is a favorable prognostic factor.<sup>12,39,40</sup> MGMT status can be regulated by several mechanisms, including histone modification<sup>41–43</sup> and post-transcriptional regulation with miRNA,<sup>44</sup> and for this reason MGMT status is not always identical at the DNA, RNA and protein levels. For example, it has been shown that mRNA expression is transcriptionally regulated, resulting in a low level in approximately 20% of patients, in whom the *MGMT* promoter is unmethylated.<sup>45,46</sup> Moreover, it has been demonstrated that variation in *MGMT* promoter methylation can occur within the same tumor after treatment.<sup>47</sup> Furthermore, a previous study employing multivariate analysis has indicated that expression of MGMT protein, rather than *MGMT* status, was correlated with the survival of patients with GBM.<sup>48</sup> Thus, immunohistochemical evaluation of MGMT protein status in glioma tissue may provide useful prognostic information.

Recent molecular studies have provided more details of the complex mechanisms that determine *MGMT* methylation status. For example, it seems plausible that *IDH* mutations can lead to *MGMT* methylation, as gliomas with *IDH* mutation show a reduction of  $\alpha$ -ketoglutarate and accumulation of 2-hydroxyglutarate, resulting in genome-wide histone and DNA methylation.<sup>49</sup> Moreover, *TP53* mutation may also influence MGMT methylation status, in association with TNF- $\alpha$ -induced NF- $\kappa$ B activation and chronic inflammation, which can induce DNA methylation.<sup>50-52</sup> Thus, *IDH1* and *TP53* mutations may alter *MGMT* methylation status.

There are some technical issues concerning MGMTimmunohistochemistry, including intra- and inter-observer variability in the assessment of immunoreactivity,8,12,53 the need for exclusion of non-neoplastic cells,13 variability among antigen retrieval methods, the antibodies employed, and the cutoff values adopted.<sup>53</sup> According to our criteria for judgment of MGMT positivity or negativity (Fig. 1D–F), we found that a MGMT-negative profile was associated with a favorable outcome for patients with malignant atrocytomas (Tables 2 and 3, and Fig. 2B). Consistent with this evidence, a recent study of both protein expression and DNA methylation found that patients with high protein expression had a poor outcome, even though the promoter was methylated,<sup>41</sup> and that patients with low protein expression and a methylated promoter had a favorable outcome.54,55

We therefore evaluated the association between CRT and MGMT profiles and survival in patients with malignant astrocytomas, and found that a MGMT-negative profile predicted a favorable outcome for patients who received CRT (Fig. 2L), suggesting that glioma cells with low MGMT expression may be much more sensitive to CRT. The proportions of patients with MGMT-negative profiles in the oligodendroglial (OA/OD and AOA/AOD) groups were high (Table 2). This seems to reflect the fact that *MGMT* promoter methylation is frequently evident in oligodendroglial tumors.<sup>56</sup>

It has been recognized that the p53 pathway plays a role in the pathogenesis of gliomas. However, at present it remains controversial whether *TP53* mutation can be regarded as a true prognostic factor. Immunoreactivity of

p53 may be associated with *TP53* mutation, LOH of *TP53*, and aberrant expression of various p53-regulatory genes, including MDM2 and p14<sup>ARF,1,3,57-59</sup> In the present study, as the grade of malignancy increased, the proportion of patients with p53-positive profiles tended to increase; however, expression of p53 in patients with any tumor grade showed no significant association with outcome (Tables 2 and 3).

In the present study, we found that a combination of both IDH1 and MGMT profiles would be useful for prognostication (Fig. 2C). Therefore, after diagnosis of GBM and AA, on the basis of immunohistochemistry for both IDH1 and MGMT, it would be possible to estimate the mOS of the patients. Moreover, if patients were found to have a MGMT-negative profile, CRT could be considered. Consistent with this, a recent molecular study has revealed that the combination of *IDH1* mutation and *MGMT* methylation status outperforms information on either *IDH1* or *MGMT* alone for predicting the survival of patients with GBM: patients with IDH1 mutations/MGMT methylation survived longer, whereas those with no IDH1 mutations/no MGMT methylation showed shorter survival.<sup>5</sup>

Furthermore, we found that p53 overexpression in patients with IDH1-negative and MGMT-negative profiles could be a useful prognostic factor (Fig. 2I). Therefore, based on a combination of the IDH1, MGMT and p53 profiles, three groups were distinguishable, and their mOSs differed significantly (Figs 2J and 3). Clinicopathologically, these findings appear to have potential importance for predicting the outcome of patients with malignant astrocytomas.

In conclusion, the immunohistochemical profiles of IDH1, MGMT and p53 are of significant importance for prognostication in patients with malignant astrocytomas. A combination of IDH1 positivity and MGMT negativity may indicate a favorable prognosis, whereas a combination of IDH1 negativity and MGMT positivity may be unfavorable. For patients with IDH1 negativity and MGMT negativity, the p53 profile may have a crucial prognostic impact, overexpression conferring an unfavorable prognosis. If information on these profiles were immediately available following histopathological diagnosis, it would be useful for prompt and accurate decision-making with regard to treatment.

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### Original Article

# Characteristic expression of p57/Kip2 in balloon cells in focal cortical dysplasia

### Tadashi Kimura,<sup>1,7</sup> Hiroki Kitaura,<sup>1</sup> Hiroshi Masuda,<sup>2</sup> Shigeki Kameyama,<sup>2</sup> Yuko Saito,<sup>3</sup> Kenji Sugai,<sup>3</sup> Taisuke Otsuki,<sup>3</sup> Atsuko Nakazawa,<sup>4</sup> Nobuhito Morota,<sup>4</sup> Takamichi Yamamoto,<sup>5</sup> Kouji Iida,<sup>6</sup> Masanori Nakagawa,<sup>7</sup> Toshiki Mizuno,<sup>7</sup> Hitoshi Takahashi<sup>1</sup> and Akiyoshi Kakita<sup>1</sup>

<sup>1</sup>Department of Pathology, Brain Research Institute, University of Niigata, <sup>2</sup>Department of Functional Neurosurgery, Epilepsy Center, Nishi-Niigata Chuo National Hospital, Niigata, <sup>3</sup>National Center of Neurology and Psychiatry, <sup>4</sup>National Medical Center for Children and Mothers, National Center for Child Health and Development, Tokyo, <sup>5</sup>Comprehensive Epilepsy Center, Seirei Hamamatsu General Hospital, Hamamatsu, <sup>6</sup>Department of Neurosurgery, University of

Hiroshima, Hiroshima and 7Department of Neurology, Graduate School of Medical Science, Kyoto Prefectural

University of Medicine, Kyoto, Japan

Balloon cells are a pathognomonic cellular feature of various cortical malformations, including focal cortical dysplasia type IIb (FCD IIb), cortical tubers of tuberous sclerosis (TSC) and hemimegalencephaly (HME). In the present study, we investigated the immunohistochemical expression of p57/Kip2, a member of the Cip/Kip family of cyclin-dependent kinase inhibitory proteins, in balloon cells in surgical specimens taken from 26, 17 and six patients with FCD IIb, TSC and HME, respectively. Characteristic dotlike reactivity with a faint, intense, reticular and process-like pattern was confined to the proximal portion of the cytoplasmic processes of the cells. Immunoelectron microscopy revealed the p57/Kip2 reactivity on intermediate filaments in the proximal portion of the processes. The immunohistochemical profile appeared similar to that of CD34; however, a double immunofluorescence study demonstrated that no cells showed reactivity for both p57/Kip2 and CD34. The frequencies of the p57/Kip2-positive cells in FCD IIb and HME were significantly higher than those in TSC, suggesting that the balloon cells may be heterogeneous. These findings suggest some functional significance of the protein

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on the cytoplasmic processes of balloon cells and appear consistent with the notion that the cells are abnormally differentiated progenitor cells.

**Key words:** balloon cell, focal cortical dysplasia, hemimegalencephaly, p57/Kip2, tuberous sclerosis.

#### **INTRODUCTION**

Balloon cells are a pathognomonic cellular feature of various cortical malformations, including focal cortical dysplasia type IIb (FCD IIb),<sup>1</sup> cortical tubers of tuberous sclerosis (TSC)<sup>2</sup> and hemimegalencephaly (HME).<sup>3</sup> Patients with these malformations often suffer intractable epilepsy requiring surgical resection of the lesions. The origin and identity of these cells are uncertain. However, a large number of studies have indicated that subsets of balloon cells express various proteins characteristic of glial, neuronal and stem cell/progenitor cells, including GFAP, vimentin, nestin,4,5 neurofilament, neuronal specific nuclear protein (NeuN), microtubule associated protein 2 (MAP2),6 class III beta-tubulin (TuJ1),<sup>7</sup> glutamate receptor,<sup>8</sup> CD133, CD34,9 and minichomosome maintenance complex component 2 (Mcm2).<sup>10</sup> With regard to the origin of balloon cells, an in vitro study of isolated balloon cells taken from patients with FCD IIb and TSC has indicated that such cells are abnormal stem cells that contribute to the pathogenesis of these disorders.<sup>11</sup> Furthermore, an immunohistochemical study using a panel of several antibodies specific for certain progenitor cell types, including brain lipid-binding protein (BLBP), Otxl, GFAP-δ and Pax6, has indicated that balloon cells are derived from radial glial progenitor cells.<sup>5</sup> Another morphological and electrophysiological study of FCD IIb

Correspondence: Akiyoshi Kakita, MD, PhD, Department of Pathology, Brain Research Institute, University of Niigata, 1-757 Asahimachi, Chuo-ku, Niigata 951-8585, Japan. Email: kakita@ bri.niigata-u.ac.jp

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has also proposed that balloon cells represent radial glial cells that have failed to undergo postnatal degeneration.<sup>12</sup> Moreover, an immunohistochemical study examining the expression of cell cycle proteins, including non-phosphorylated retinoblastoma protein, cdk4 and p53, in balloon cells in FCD IIb has hypothesized that these cells are the remnants of early cortical cells, which have failed to undergo differentiation or elimination during development and have undergone cell cycle arrest.<sup>13</sup> Thus, dysregulation of cell cycle proteins may be a pivotal mechanism underlying the dysplastic features of balloon cells, including abnormal cell fate determination, cell immaturity, and subsequent abnormal morphology.

In the present study, we examined the immunohistochemical expression of p57/Kip2 and p27/Kip1, members of the Cip/Kip family of cyclin-dependent kinase (CDK) inhibitory proteins, in balloon cells in surgical specimens of FCD IIb, TSC and HME taken from patients with epilepsy. Recent biological studies have provided evidence that these proteins play important roles in cell cycle regulation, proliferation, differentiation and migration of various types of cancer cells<sup>14</sup> and of cortical precursors in the developing brain.<sup>15</sup> We found that various proportions of balloon cells in the malformed brain tissue showed reactivity for p57/Kip2, but not for p27/Kip1.

#### **MATERIALS AND METHODS**

#### Subjects

The study was approved by the various institutional ethics committees, and all patients provided consent to use their resected tissues for research purposes. We examined surgical specimens taken from 26 patients with FCD IIb (14 male, 12 female: 15 right, 11 left: age at resection mean  $\pm$  SD = 16.4  $\pm$  12.9 years), 17 patients with TSC (nine male, eight female: seven right, 10 left: age  $6.29 \pm 7.6$  years) and six patients with HME (three male, three female: four right, two left: age  $1.00 \pm 1.2$  years), who had undergone resection of neocortical tissue because of intractable epilepsy (Table 1). Five of the patients with TSC were confirmed as harboring mutations in the TSC1 or TSC2 gene, as previously reported <sup>16</sup> (Table 1). The surgical specimens were cut into slices and fixed with phosphate-buffered 20% formalin and embedded in paraffin wax. Serial 4-µm-thick sections were then cut, and subjected to HE and KB staining. Diagnoses of FCD IIb,1 cortical tubers of TSC2 and HME,3 were made on the basis of histopathological features, clinical information, images and prognosis.

The term "balloon cells" is used for the abnormal cells observed in FCD IIb and HME, whereas "giant cells" is the term sometimes applied for those in cortical tubers. These cells are morphologically indistinguishable. For clarity, the term "balloon cells" is used here for all cell populations.<sup>2,11</sup>

### Immunohistochemistry, cell counting and statistical analysis

The paraffin-embedded sections were processed by the avidin-biotin-peroxidase complex (ABC) method with a Vectastain ABC kit (Vector, Burlingame, CA, USA). As the primary antibodies, we used a rabbit polyclonal antibody against p57/Kip2 (Abcam, Cambridge, MA, USA; 1:200), and mouse monoclonal antibodies against p27/Kip1 (clone 57, BD Biosciences Pharmingen, Franklin Lakes, NJ, USA; 1:200) and CD34 (clone Qbend 10, Immunotech, Monrovia, CA, USA; 1:200). Pretreatment was performed by heat/autoclaving for 10 min at 121°C in 10 mmol/L sodium citrate buffer.

For cell counting, a single p57/Kip2-immunostained section from each case was used. The sections were observed under a light microscope (BX53, Olympus, Tokyo, Japan) and images were captured by a digital camera (DP73, Olympus). Balloon cells were identified as bizarre cells with abundant cytoplasm and eccentric nuclei (Fig. 1A). The numbers of the p57/Kip2-positive and -negative balloon cells in the images were counted manually using the "Count tool" of the Adobe Photoshop software package.

Statistical significance of differences in the frequency of p57/Kip2-positive balloon cells in any of the FCD IIb, TSC or HME cases was analyzed with nonparametric Kruskal-Wallis test and Mann-Whitney *U*-test at a significance level of P < 0.01. The results are shown as mean  $\pm$  SD. Data analysis was carried out using the IBM SPSS statistics version 20 software package (IBM SPSS Inc., Chicago, IL, USA).

#### **Double immunofluorescence**

A double-labeling immunofluorescence study was performed on several sections from eight patients with FCD IIb using the rabbit polyclonal anti-p57/Kip2 antibody (1:100) and the mouse monoclonal anti-CD34 antibody (1:100). The secondary antibodies used were Alexa Fluor 555 goat anti-mouse IgG and Alexa Fluor 488 goat antirabbit IgG (both Molecular Probes, Eugene, OR, USA; 1:1000). Slides were treated with Autofluorescence Eliminator Reagent (Millipore, Bellerica, MA, USA), and then mounted with glass coverslips using VECTAshield mounting medium with 4,6-diamidino-2-phenylindole (DAPI) nuclear stain (Vector Laboratories, Burlingame, CA, USA). A laser-scanning confocal microscope (Carl Zeiss LSM-700, Jena, Germany) was used to visualize immunoreactivity.

Case ID	Sex	Age (y)	Onset (y)	Location	Side	Pathology	Number of balloon cells	Number of p57/Kip2 positive cells	Percentages
1	F	2	1	Т	R	FCD IIb	95	67	70.53
2	F	2	_	Р	L	FCD IIb	69	50	72.46
3	Μ	3	0	F	L	FCD IIb	78	39	50.00
4	М	7	6	F	R	FCD IIb	51	50	98.04
5	М	8	5	F	R	FCD IIb	120	97	80.83
6	М	8	7	F	L	FCD IIb	65	42	64.62
7	F	8	4	F	L	FCD IIb	163	80	49.08
8	F	9	5	Р	R	FCD IIb	13	11	84.62
9	М	10	1	F	R	FCD IIb	19	12	63.16
10	F	10	3	Р	R	FCD IIb	85	64	75.29
11	F	10	0	F	L	FCD IIb	154	102	66.23
12	М	11	9	0	R	FCD IIb	21	16	76.19
13	М	11	5	Р	L	FCD IIb	73	62	84.93
14	Μ	12	6	F	R	FCD IIb	33	5	15.15
15	М	12	5	F	L	FCD IIb	63	31	49.21
16	F	12	0	T	R	FCD IIb	157	73	46.50
17	F	17	7	F	L	FCD IIb	66	50	75.76
18	F	18	5	Р	R	FCD IIb	81	66	81.48
19	M	20	10	0	L	FCD IIb	50	26	52.00
20	F	20	4	F	R	FCD IIb	58	39	67.24
21	F	23	7	P	R	FCD IIb	84	70	83.33
22	M	26	6	P	R	FCD IIb	109	94	86.24
23	М	34	10	F	R	FCD IIb	162	95	58.64
24	M	38	1	M	Ĺ	FCD IIb	42	36	85.71
25	F	41	5	Р	R	FCD IIb	78	40	51.28
26	M	54	3	F	L	FCD IIb	31	21	67.74
27	F	0	0	F	Ē	TSC	340	1	0.29
28	F	Ő	0	F	R	TSC	140	0	0.00
29	M	1	_	F	R	TSC	70	18	25 71
30	M	2	0	F	Ī.	TSC	70	2	2.82
31	M	2	0	F	R	TSC	18	6	33 33
32**	F	2	0	Т	I	TSC	81	14	17.28
33	F	2	0	Ť	Ĩ.	TSC	117	1	0.85
34	M	3	1	F	R	TSC	37	22	59.46
35	M	3	2	F	Ī.	TSC	52	4	7 69
36	F	3	0	T	Ē	TSC	43	0	0.00
37	F	4	2	P	Ī	TSC	37	1	2 70
38	F	5	0	P	Ľ.	TSC	14	0	0.00
30*	F	9	0	F	Ī	TSC	10	1	10.00
40**	M	11	0	F	R	TSC	0	1	11.11
41	M	11	_	F	I	TSC	24	5	20.83
42*	M	24	10	F	R	TSC	18	15	83 33
43**	M	25	6	F	R	TSC	30	4	13 33
44	M	0	0	P	I	HME	23	4	17 39
45	M	0	0	F	Ľ	HME	94	82	87.23
46	F	0	0	F	R	HME	58	30	51 72
47	м	1	0	F	R	HME	140	7	5 00
48	F	2	0	F	R	HME	21	/ 1Q	58.06
49	M	3	0	T	R	HME	150	91	60.67

Table 1 Clinical and pathological profiles of the patients with FCD, TSC and HME

F, female; M, male; F, frontal; T, temporal; P, parietal; O, occipital; R, right; L, left; FCD, focal cortical dysplasia; TSC, tuberous sclerosis; HME, hemimegalencephaly; y, year(s). Mutations in \* *TSC1* and \*\* *TSC2* genes.

#### Immunoelectron microscopy

The ultrastructural localization of p57/Kip2 was examined using surgical specimens taken from five patients with FCD IIb, employing the post-embedding method previously described.<sup>17</sup> Small tissue blocks were prepared from

formalin-fixed tissue, and washed with PBS. The tissue blocks were then washed with gradually increasing concentrations of dimethylformamide, and embedded in LR White resin (London Resin Company, Berkshire, UK). Ultrathin sections were cut, incubated with the anti-p57/ Kip2 antibody (1:25) for 36 h, and reacted with 15-nm gold

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**Fig. 1** p57/Kip2 immunohistochemical features. Balloon cells in focal cortical dysplasia (FCD) type IIb (A-F), tuberous sclerosis (TSC) (G), and hemimegalencephaly (HME) (H), and dysmorphic neurons in FCD type IIb (I, J). (A, B) Low-power magnification views of white matter of a sample demonstrating several balloon cells with abundant cytoplasm and eccentric nuclei (A), and dot-like immunoreactivity around the cytoplasm of the balloon cells (B). (C-F) Higher-magnification views of balloon cells showing faint (C), intense (D), reticular (E) and process-like (F) fashion. (G) No balloon cells with immunoreactivity are seen. (H) Many balloon cells showing similar immunoreactivity. (J) No dysmorphic neurons with immunoreactivity are seen. (A, I) Hematoxylin and eosin stain. (B-H, J) Immunostained and then counterstained with hematoxylin. Images of A and B, and those of I and J are taken from serial sections. Bars =  $50 \mu m$  for A, B, G and H, and  $20 \mu m$  for C-F, I and J.

colloidal particle-conjugated anti-rabbit IgG (British BioCell, Cardiff, UK, 1:30). The sections were then stained with lead citrate, and examined with a Hitachi H-7700 digital electron microscope at 75 kV.

#### RESULTS

#### p57/Kip2 immunohistochemistry

In FCD IIb, balloon cells showed characteristic p57/Kip2 immunoreactivity (Fig. 1B) in the form of dot-like profiles around the abundant cytoplasm, showing faint (Fig. 1C), intense (Fig. 1D), reticular (Fig. 1E) and process-like (Fig. 1F) patterns. These profiles appeared to be confined to the cytoplasmic membrane and proximal processes of the cells. No apparent immunoreactivity was evident within the perikaryon proper. Balloon cells with faint or intense p57/Kip2 immunoreactivity were found predominantly within the white matter, whereas those with the reticular and process-like patterns were scattered in deeper layers of the cortex and at its boundary with the white matter. Interestingly, a large proportion of balloon cells observed in TSC were p57/Kip2-negative (Fig. 1G). On the other hand, balloon cells in HME (Fig. 1H) showed immunoreactivity similar to that in FCD IIb.

The median frequencies of the p57/Kip2-positive balloon cells were 69.1% in FCD IIb (range, 51.8 to 81.9), 54.9% in HME (range, 17.4 to 60.7), and 10.0% in TSC (range, 0.9 to 20.8) (Table 1). The frequencies in FCD IIb and HME were higher than those in TSC ( $\chi^2$  (2, n = 49) = 22.9, P < 0.001). A post-hoc test revealed a significant difference between FCD IIb and TSC (P < 0.001), but not between HME and TSC.

We found no morphological differences between balloon cells with p57/Kip2 immunoreactivity and those without the immunoreactivity. No dysmorphic neurons (Fig. 1I) or other neurons with normal morphology showed reactivity for p57/Kip2 (Fig. 1J).

With regard to p27/Kip1, no labeling of balloon cells or dysmorphic neurons was evident in FCD IIb, TSC or HME (data not shown).

#### **Double immunofluorescence**

As the expression pattern of p57/Kip2 in balloon cells (Fig. 1B) appeared similar to that of CD34,<sup>9</sup> we performed double immunofluorescence for detection of both p57/Kip2 and CD34. Surprisingly, we encountered no balloon cells that were immunoreactive with both of the antibodies used (Fig. 2).

#### **Electron microscopy**

Ultrastructurally, the balloon cells exhibited abundant cytoplasm containing numerous intermediate filaments,

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and short cytoplasmic processes with abundant filaments (Fig. 3A, C), as previously reported.<sup>18,19</sup> Immunogold labeling for p57/Kip2 was observed mainly on the filaments at the peripheral cytoplasm and proximal portion of the processes (Fig. 3B, D, E).

#### DISCUSSION

In the present study, for the first time, we have demonstrated the expression of p57/Kip2 in balloon cells (Fig. 1). The characteristic immunohistochemical features (Figs 1,3) suggest some significant role of the protein in the processes. Furthermore, we have shown that in comparison with balloon cells in FCD IIb and HME, those in TSC express p57/Kip2 much more rarely (Table 1), indicating that the roles of the protein in balloon cells may differ somewhat between FCD IIb/HME and TSC.

The CDK inhibitors of the Cip/Kip family, including p57/Kip2 and p27/Kip1, are known to regulate the cell cycle. Recent understanding of the roles of the family members in development of the CNS has been expanding, and it is now known to be involved in multiple aspects of neurogenesis through cell cycle arrest, specific cell-type differentiation and neuronal migration.<sup>15,20,21</sup> Recently, it has been revealed that the members of this family differentially regulate neuroglial fate determination depending on environmental signals and developmental stage.<sup>22</sup> Balloon cells are considered to be the consequence of aberrant differentiation of radial glial cells, a type of progenitor cell, to neurons and glia.<sup>5</sup> Therefore, expression of p57/Kip2 in balloon cells may be associated with abnormal differentiation of the progenitor cells. It seems unlikely that the p57/Kip2 profiles observed in the present study reflected aberrant neurogenesis or neuronal migration, because neurons with normal or dysplastic morphologies were distributed irregularly within the cortex and white matter of FCD IIb/TSC/HME, but they showed no p57/Kip2 immunoreactivity.

It has been hypothesized that balloon cells may be arrested in cell cycle G1, based on the findings that they frequently express G1 markers in their nuclei.<sup>13</sup> Concerning the cell cycle, a biological study demonstrated that p57/Kip2 was strongly expressed in association with the late G1 phase of developing cells of the CNS.<sup>20</sup> Therefore, the p57/Kip2 selected expression in the balloon cells in the present study appears compatible with the notion that they are arrested in G1. However, we could not consider close association between the p57/Kip2 expression and the cell cycle of the balloon cells, because G1 associated proteins usually localize within the nucleus, but p57/Kip2 was observed at the cytoplasmic processes, rather than the nucleus, of the balloon cells.



**Fig. 2** Double immunofluorescence. Immunofluorescence with the p57/Kip2 (green, A, D) and CD34 (red, B, E) signals to demonstrate the spatial relationship of the two antigens. (C, F) Merged image of both signals. Nuclei are labeled with 4,6-diamidino-2-phenylindole (DAPI) (blue). High-power views demonstrating a single balloon cell with two nuclei (A-C) and lower-power views showing two cells (arrow and arrowhead) (D-F) in FCD type IIb. Note the cells show either signal. No co-localized signals are observed. Bars = 10  $\mu$ m for A-C, and 20  $\mu$ m for D-F.

TSC is an autosomal-dominant disease resulting from mutations of either TSC1 or TSC2, encoding hamartin and tuberin, respectively. The TSC gene products form a functional heterodimer, which negatively regulates the activities of mammalian target of rapamycin (mTOR),<sup>23</sup> a serine/ threonine kinase known to be a major effector of cell growth. In the CNS, activation of mTOR signaling may cause abnormal development of neurons and glia.<sup>24</sup> On the other hand, recent genetic studies have revealed somatic mutations in the PIK3CA, AKT3 and MTOR genes in brain tissue of patients with HME, indicating aberrant activation of mTOR signaling.<sup>25,26</sup> The molecular genetic mechanisms underlying FCD IIb have remained uncertain. FCD IIb, TSC and HME often share a number of histopathological features, including disruption of cortical cytoarchitecture, dysmorphic neurons and balloon cells. On the other hand, based on our experience of routine histopathological diagnoses of these developmental disorders, we have formed an impression that calcification and astrocytosis are much

more remarkable in TSC than in FCD IIb and HME. Moreover, the frequency of dysmorphic neurons appears to be much higher in HME than in FCD IIb and TSC. In a subset of patients with HME, balloon cells were not observed. Furthermore, a previous biochemical analysis demonstrated somewhat differential Pi3K-pathway activation in FCD IIb and TSC.27 Another electrophysiological study demonstrated that FCD IIb and TSC display differences in the topography of abnormal cells, excitatory and inhibitory synaptic network properties, and GABA(A) receptor sensitibity.28 Accordingly, certain differences in the pathomechanisms of these disorders may be associated with differences in the histological picture and expression of p57/Kip2 in balloon cells (Table 1). In the present study, we found no clear association between genetic profiles and the frequencies of p57/Kip2-positive balloon cells in patients with TSC. For example, the frequency in two patients with TSC1 mutations (cases #39 and #42 in Table 1) were considerably differed.



**Fig. 3** Ultrastructural localization of p57/Kip2. (A) Low-power view of a balloon cell showing eccentric nucleus, abundant cytoplasm with filaments and cell organelle, and short cytoplasmic processes. (B) A higher magnification view of the short processes. The area indicated by a square in A. Note the immunoreactivity in the proximal portion of the cytoplasmic processes (arrows). (C-E) Another example of balloon cell with abundant intermediate filaments. (D, E) Higher magnification views of the areas indicated by square 1 rotated by 45° and square 2 in C, respectively, showing the immunoreactivity on the intermediate filaments. Bars = 10  $\mu$ m for A, 500 nm for B, D and E, and 5  $\mu$ m for C.

CD34, a stem cell/progenitor cell marker, is well known to be expressed in balloon cells in FCD IIb, TSC and HME, where the immunolabeled cells are exclusively located within the deep white matter or close to the gray matter boundary, but not in the cortex.<sup>9</sup> In the present study, we also detected several CD34-labeled balloon cells; however, double-labeling immunofluorescence revealed distinct populations of balloon cells showing immunoreactivity for either CD34 or p57/Kip2 (Fig. 2). Even though these populations were located closely to each other within the white matter, we found no balloon cells that expressed both proteins (Fig. 2). CD34 is known to be expressed during neural tube formation.<sup>29</sup> Moreover, it has been reported that CD34-labeled progenitor-like cells are sometimes observed in ganglioglioma and other epilepsy-associated brain

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tumors taken from patients with epilepsy.<sup>30</sup> However, the significance of CD34, and also p57/Kip2, in balloon cells has remained uncertain. It seems unlikely that certain cellular mechanisms would induce some sort of conflict in the expression of CD34 and p57/Kip2. Therefore, we speculate that the available evidence points to heterogeneity of the cells.

We were able to observe the ultrastructural localization of p57/Kip2 on intermediate filaments, presumably mainly vimentin filaments and possibly glial filaments as reported previously,<sup>19</sup> in the proximal parts of the cytoplasmic processes (Fig. 3). This ultrastructural localization appeared to be compatible with the immunohistochemical picture (Fig. 1). In the perikarya of balloon cells, the intermediate filaments are loosely arranged and intermingled with intracellular organelles, whereas in the proximal processes, the filaments show a tendency to be much more densely packed and aligned in the direction of extension of the processes (Fig. 3D, E). Up to now, the significance of p57/Kip2 localization in balloon cells has been uncertain. However, recent biochemical studies have provided evidence for a critical role of p57/Kip2 in the regulation of actin cytoskeletal dynamics, and thus migration ability, in cancer cells.<sup>31</sup> Therefore, there is a possibility that p57/Kip2 may be involved in the intermediate filament assembly that is necessary for extension of the cytoplasmic processes of balloon cells.

In conclusion, we have reported aberrant expression of p57/Kip2 in balloon cells in various types of brain malformation. Our observations appear to be consistent with the notion that these cells represent abnormal differentiation of progenitor cells. Characteristically, the immunoreactivity appears to be localized in the proximal portion of cytoplasmic processes, implying that the protein has functional significance in the processes. The significant difference in the frequency of immunoreactive cells between FCD IIb and TSC suggests that the function of the protein may differ between these malformations.

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#### DISCLAIMER

The authors report no conflicts of interest concerning the materials or methods used in this study or the findings presented.

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### RESEARCH ARTICLE

# p62 Deficiency Enhances α-Synuclein Pathology in Mice

Kunikazu Tanji<sup>1</sup>\*; Saori Odagiri<sup>2</sup>\*; Yasuo Miki<sup>1</sup>; Atsushi Maruyama<sup>3</sup>; Yoshikazu Nikaido<sup>4</sup>; Junsei Mimura<sup>3</sup>; Fumiaki Mori<sup>1</sup>; Eiji Warabi<sup>5</sup>; Toru Yanagawa<sup>5</sup>; Shinya Ueno<sup>4</sup>; Ken Itoh<sup>3</sup>; Koichi Wakabayashi<sup>1</sup>

<sup>1</sup> Department of Neuropathology, <sup>3</sup> Department of Stress Response Science, <sup>4</sup> Department of Neurophysiology, Hirosaki University Graduate School of Medicine, Hirosaki, <sup>2</sup> Department of Neuroanatomy, Cell Biology and Histology, <sup>5</sup> Majors of Medical Sciences, University of Tsukuba, Tsukuba, Japan.

### Keywords

α-synuclein, Lewy body disease, p62/Sequestsome 1/SQSTM1, Parkinson's disease, proteolysis, stress.

### Corresponding author:

Kunikazu Tanji, PhD, Department of Neuropathology, Hirosaki University Graduate School of Medicine, 5 Zaifu-cho, Hirosaki 036-8562, Japan (E-mail: *kunikazu@cc.hirosaki-u.ac.jp*)

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\* These authors contributed equally to the manuscript.

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# INTRODUCTION

Lewy body disease (LBD) including dementia with LBs and Parkinson's disease (PD) is pathologically characterized by the presence of intracellular inclusions called LBs.  $\alpha$ -Synuclein has been identified as a component of LBs (41), and the duplication and triplication of the  $\alpha$ -synuclein gene are found in both sporadic and early onset forms of PD (40). Mutations (A30P and A53T) in the  $\alpha$ -synuclein gene are linked to autosomal dominant forms of PD (20, 31). Originally,  $\alpha$ -synuclein is a proteinase K (PK)-soluble protein that localizes at presynaptic terminals; however,  $\alpha$ -synuclein becomes resistant to PK and widely deposited throughout the brain of patients with LBD (19, 42). These findings suggest that  $\alpha$ -synuclein is significantly involved in the pathogenesis of both familial and sporadic cases of LBD.

 $\alpha$ -Synuclein is physiologically processed by two intracellular degradation systems, including the ubiquitin–proteasome and autophagy–lysosome systems. In case of  $\alpha$ -synuclein overload, the autophagy–lysosome system, including chaperone-mediated autophagy, predominantly aids in the degradation of excess  $\alpha$ -synuclein (6, 22, 46). Thus, it is possible that dysfunction of intracellular degradation system results in the up-regulation of

# Abstract

In Lewy body disease (LBD) such as dementia with LBs and Parkinson's disease, several lines of evidence show that disrupted proteolysis occurs. p62/SQSTM1 (p62) is highly involved with intracellular proteolysis and is a component of ubiquitin-positive inclusions in various neurodegenerative disorders. However, it is not clear whether p62 deficiency affects inclusion formation and abnormal protein accumulation. To answer this question, we used a mouse model of LBD that lacks p62, and found that LB-like inclusions were observed in transgenic mice that overexpressed  $\alpha$ -synuclein (Tg mice) with or without the p62 protein. p62 deficiency enhanced  $\alpha$ -synuclein pathology with regard to the number of inclusions and staining intensity compared with Tg mice that expressed p62. To further investigate the molecular mechanisms associated with the loss of p62 in Tg mice, we assessed the *mRNA* and protein levels of several molecules, and found that the neighbor of the brca1 gene (*NBr1*), which is functionally and structurally similar to p62, is increased in Tg mice without p62 compared with control Tg mice. These findings suggest that p62 and NBR1 affect the pathogenesis of neurodegenerative diseases through the cooperative modulation of  $\alpha$ -synuclein aggregation.

 $\alpha$ -synuclein expression and contributes to abnormal protein accumulation. Indeed, several lysosomal-related genes were identified as a causative mutation in familial PD, including leucine-rich repeat kinase 2 and adenosine-3-phosphate 13A2. Furthermore, PD has been genetically linked to rare lysosomal storage diseases, including Gaucher's disease (25) and Sanfilippo syndrome (47).

p62/SQSTM1/sequestosome 1 (referred to as p62) is a multifunctional protein that is strongly associated with the intracellular degradation system. P62 knockout (KO) mice exhibit matureonset obesity, insulin and leptin resistance (37). Pathologically, loss of p62 results in the accumulation of hyperphosphorylated tau and insoluble K63-linked polyubiquitin chains (33, 48). p62 contains a ubiquitin-associated (UBA) domain at the C-terminus that enables its interaction with ubiquitinated and misfolded proteins. Additionally, p62 possesses a Phox and Bem1p (PB1) domain at the N-terminus and a LC3 interacting region, suggesting that p62 is able to interact with proteasome components and autophagosomal membranes (29, 38). Thus, it has been suggested that p62 can efficiently degrade ubiquitinated and misfolded proteins through the proteasome and autophagy-lysosome systems. It has been reported that p62 is an inducible protein that easily aggregates under several pathological conditions, such as oxidative stress and neurodegeneration (1, 11, 27). Accordingly, dysfunction of the intracellular degradation systems induces p62 aggregation *in vivo* (2, 16). Furthermore, loss of p62 suppressed ubiquitinpositive inclusions in neurons of brain-specific autophagydeficient mice (17). Additionally, ubiquitin- and p62-positive protein aggregates were abrogated in Atg8 and p62 double-mutant flies (26). These findings suggest that p62 may be responsible for the formation of cytoplasmic inclusions and abnormal protein accumulation.

In this study, we used transgenic (Tg) mice overexpressing  $\alpha$ -synuclein with a A53T mutation as a model for LBD. We crossed the Tg mice with p62 KO mice to examine the involvement of p62 in abnormal  $\alpha$ -synuclein pathology. Immunohistochemical analyses showed that p62 deficiency enhanced  $\alpha$ -synuclein pathology, as shown by an increase in inclusion number and staining intensity. We assessed several genes and proteins related to stress response and proteolysis. These data revealed that the expression of neighbor of brca1 gene (NBR1), which is a functional homologue to p62, was increased in p62-deficient mice.

# **MATERIALS AND METHODS**

### Animals and experimental design

α-Synuclein Tg mice have been widely used as an animal model for LBD (7, 14, 21, 23, 24, 34, 36, 45). To create this LBD model in a p62-deficient background, we used mice overexpressing human α-synuclein with the A53T mutation under the prion promoter (Jackson Laboratories, Bar Harbor, ME, USA) (7) and p62 KO mice with exon 1–4 deleted as previously described (17). The p62 KO mice lacked abnormal tau pathology. α-Synuclein Tg and p62 KO mice were backcrossed with C57BL/6J mice for at least 10 generations. First, heterozygous α-synuclein Tg mice were bred with p62 KO mice to generate α-synuclein<sup>+/-</sup>/p62<sup>+/-</sup> mice. Second, α-synuclein<sup>+/-</sup>/p62<sup>+/-</sup> mice were inbred to generate wild type, p62 KO, α-synuclein<sup>+/-</sup>/p62<sup>+/+</sup> and α-synuclein<sup>+/-</sup>/p62<sup>+/+</sup> mice (Figure 1A). Hereafter, wild-type, p62 KO, α-synuclein<sup>+/-</sup>/p62<sup>+/+</sup> and α-synuclein<sup>+/-</sup>/p62<sup>-/-</sup> mice are simply referred to as WT, KO, Tg and Tg/KO mice, respectively. All comparisons were made



**Figure 1.** Characterization of p62 protein deficiency in an animal model of Lewy body disease. **A.** Breeding strategies to generate p62 deficiency in  $\alpha$ -synuclein transgenic (Tg) mice. Initially, heterozygous  $\alpha$ -synuclein Tg and homozygous p62-knockout (KO) mice were crossed. Next, littermates and heterozygous p62-deficient mice were mated to generate Tg mice without p62 (Tg/KO), of which four groups were used in this study (black circles). **B.** Immunoblot analysis confirmed that  $\alpha$ -synuclein was overexpressed in Tg and Tg/KO mice and that p62



signals were diminished in KO and Tg/KO mice (9 weeks of age, n = 6 per group). The molecular mass is indicated on the left side of the panel. β-Actin was used as a loading control. **C.** A quantitative analysis shows that human  $\alpha$ -synuclein is expressed in Tg and Tg/KO mice and that p62 is absent in KO and Tg/KO mice. The values of Tg mice are defined as 100%. **D.** The weight changes of Tg (black circle) and Tg/KO mice (grey circle) are shown (mean ± standard deviation, n = 6–8 per group).

among littermates to minimize confounding effects by different genetic backgrounds. Mice were housed with a light/dark cycle of 12 h and were given food and water ad libitum. The experimental protocol was approved by the Institutional Animal Care and Use Committee at the Hirosaki University Graduate School of Medicine in Japan. Tg mice were genotyped using real-time polymerase chain reaction (PCR) analysis (forward primer, 5'-TGT AGG CTC CAA AAC CAA GG-3'; reverse primer, 5'-TAT GCC TGT GGA TCC TGA CA-3'), and verified by backcrossing. Conventional PCR was used for p62 genotyping (primer pair for wild type, forward, 5'-CTT ACG GGT CCT TTT CCC AAC-3'; reverse, 5'-TCC TCC TTG CCC AGA AGA TAG-3'; primer for p62 KO, forward; 5'-CTG CAT GTC TTC TCC CAT GAC-3'; reverse, 5'-TAG ATA CCT AGG TGA GCT CTG-3'). Mice were transcardially perfused with phosphate-buffered saline. The brain was removed, and the right hemisphere was fixed with 4% paraformaldehyde for 48 h. After dehydrating through a graded ethanol series, the right hemisphere was embedded in paraffin and cut into 4- $\mu$ m thick sections. The left hemisphere was frozen at -80°C for subsequent biochemical analyses.

### Antibodies and immunohistochemistry

Rabbit antibodies against Keap1 (ProteinTech Group, Inc., Chicago, IL, USA), p62 (MBL, Nagoya, Japan), NBR1 (Sigma, St. Louis, MO, USA and Santa Cruz Biotechnology, Santa Cruz, CA, USA), NAD(P)H quinone oxidoreductase 1 (NQO1) (Sigma), LC3 (Sigma and MBL), ubiquitin (DAKO, Glostrup, Denmark), UBQLN1 (Lifespan Biosciences, Seattle, WA, USA), phosphorylated  $\alpha$ -synuclein (Abcam, Cambridge, UK) and  $\beta$ -actin (Sigma) were used in this study. Mouse antibodies against p62 (BD Biosciences, Franklin Lakes, NJ, USA), SNAP25 (Chemicon, Temecula, CA, USA), synaptophysin (DAKO), human  $\alpha$ -synuclein (LB509; Zymed, South San Francisco, CA, USA), human and mouse  $\alpha$ -synucleins (4D6; GeneTex, Irvine, CA, USA) and phosphorylated  $\alpha$ -synuclein (pSyn#64; Wako, Osaka, Japan) were also used.

The sections were dehydrated and pretreated with heat retrieval using an autoclave for 10 minutes in 10 mM citrate buffer (pH 6.0) for rabbit anti-Keap1 and anti-NBR1 antibodies. The sections were then subjected to immunohistochemical processing using the avidin-biotin-peroxidase complex method with diaminobenzidine (Sigma). In addition, the sections were counterstained with hematoxylin. For the staining of presynaptic PK-resistant  $\alpha$ -synuclein, sections were pretreated with PK (Gibco BRL, Gaithersburg, MD, USA; 50 µg/mL) in a PK buffer containing 10 mM Tris-HCl, pH 7.8, 100 mM NaCl, 0.1% Nonidet-P40 at 37°C for 5 minutes. The total number of inclusions immunostained with anti-phosphorylated  $\alpha$ -synuclein was quantified in contiguous sections. Immunohistochemical studies were performed at 9 weeks of age (n = 6 per group).

# Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA was extracted from the right hemisphere of the brain using the RNeasy lipid tissue mini kit (Qiagen, Hilden, Germany) at 9 weeks of age (n = 3 per group). cDNA was synthesized from 1  $\mu$ g of total RNA using the PrimeScript<sup>®</sup> II first-strand cDNA

synthesis kit (Takara Bio Inc., Otsu, Japan). An aliquot of cDNA was used for gene expression analysis with the SYBR® Premix Ex Taq<sup>TM</sup> II (Perfect Real Time) (Takara Bio Inc.) and CFX Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) using the following primer sets: heme oxygenase-1 (Ho-1) (5'-CCA GCA ACA AAG TGC AAG ATT C-3'; 5'-TCA CAT GGC ATA AAG CCC TAC AG-3'), Ngo1 (5'-GTC ATT CTC TGG CCA ATT CAG AGT-3'; 5'-TTC CAG GAT TTG AAT TCG GG-3'), glutamate-cysteine ligase catalytic subunit (Gclc) (5'-AAA ATG CGG AGG CAT CAA-3'; 5'-ATA TGC TGC AGG CTT GGA AT-3'), p62 (5'-AGC TGC CTT GTA CCC ACA TC-3'; 5'-CAG AGA AGC CCA TGG ACA G-3'), Cyclophilin A (5-ATG CTG GAC CCA ACA CAA AT-3'; 5-TCT TTC ACT TTG CCA AAC ACC-3'), Keap1 (5'-CAC AGC AGC GTG GAG AGA-3'; 5'-CAA CAT TGG CGC GAC TAG A-3'), Lamp1 (5'-CCT ACG AGA CTG CGA ATG GT-3'; 5'-CCA CAA GAA CTG CCA TTT TTC-3'), Cathepsin D (5'-CCC TCC ATT CAT TGC AAG ATA C-3'; 5'-TGC TGG ACT TGT CAC TGT TGT-3'), transcription factor EB (TfEB) (5'-GAG CTG GGA ATG CTG ATC C-3'; 5'-GGG ACT TCT GCA GGT CCT T-3'); Rab7l1 (5'-GCT GCA GCT CTG GGA TAT TG-3'; 5'-TAG TAG AGT CGT GTC ATG GAT GTG-3') and Nbr1 (5'-TCA ACA GGA CTC GCA AAC AG-3'; 5'-ATG CTG CTC CCA TTG TGG-3'). Cyclophilin A was used for normalization.

### **Immunoblot** analysis

Western blot analysis was performed as previously described (43). For total cell lysate, we used a lysis buffer with 4% sodium dodecyl sulfate (SDS; 75 mM Tris-HCl, pH 6.8, 4% SDS, 25% glycerol, 5%  $\beta$ -mercaptoethanol) and passed sample through 21 gauge needle attached on a 1 mL syringe. For an experiment using insoluble sample of detergent, samples were weighted and lysed with 10-fold volume of Tris-based buffer (pH 7.4) containing 0.1% Triton X-100 on ice. After homogenization with a pestle 20 times, they were passed 10 times through 21 gauge needle attached on a 1 mL syringe. Lysates were incubated for 5 minutes on ice, and centrifuged at 12 000  $\times$  g for 10 minutes. Supernatant was used as a soluble fraction. The pellets were resuspended with 8 M urea and sonicated (insoluble fraction). Signal detection was performed according to the protocol provided with the ECL or ECL prime detection systems (Amersham Pharmacia Biotech, Piscataway, NJ, USA). We performed each immunoblot analysis a minimum of three times, and all data were quantified and collected.

### Animal behavioral testing

### The Morris water maze

Spatial learning was assessed in a round tank of water (0.95 m in diameter) at 30°C. An escape platform (10 cm in diameter) was placed 1 cm below the water surface. A camera (Primetech Engineering Corp., Tokyo, Japan) was mounted above the maze and attached to a computer running the Smart software (Primetech Engineering Corp.). The training paradigm for the hidden platform version of the Morris water maze consisted of two trials per day for five consecutive days. The time taken to reach the platform (latency to escape) was recorded for each trial. The time limit was 120 s, and the intertrial interval was 1 h. If the animal could not

find the platform, it was placed on the platform for 20 s. After removing the platform, the probe trial was carried out 2 h after the completion of training on the fifth day. The latency to reach the former location of the platform and the percentage of total time spent in each quadrant were recorded.

### Forced swim test

Immobility time was analyzed using a forced swim test. Animals were individually placed in a transparent acrylic cylindrical beaker (height: 25 cm, diameter: 18 cm) containing 4600 mL of clear water at  $25 \pm 1^{\circ}$ C for 6 minutes. A mouse was judged to be immobile when it remained passively floating in the water for more than 2 s. Immobility time was quantified using a Forced Swim Scan software (Clever Sys Inc., Reston, VA, USA).

### Quantitative analysis and statistical analysis

A semi-quantitative analysis of protein levels was performed using the ImageJ software provided by the NIH. All data were represented as the mean + standard deviation. The statistical significance was evaluated using one-way analysis of variance (ANOVA) with Bonferroni's post hoc test to analyze four genotypes and Student's *t*-test to analyze two genotypes. A probability value of less than 0.05 (P < 0.05) was considered to be significant.

### RESULTS

# Characterization of $\alpha$ -synuclein Tg mice with or without p62

To test the possibility that p62 is responsible for the formation of cytoplasmic inclusions and abnormal protein accumulation, we generated mice that overexpressed human  $\alpha$ -synuclein (Tg) on a p62-deficient background (Figure 1A). First, we crossed Tg mice with p62 KO mice. Next, littermates with or without p62 and/or human  $\alpha$ -synuclein were selected by genotyping and crossed to generate Tg mice lacking p62. Consequently, littermates with or without endogenous p62 and/or human  $\alpha$ -synuclein expression were born at the expected Mendelian ratio. For our studies, we used WT, KO, Tg and Tg/KO mice.

We confirmed that  $\alpha$ -synuclein was robustly expressed in the brains of Tg mice and Tg/KO mice (Figure 1B). We used a human α-synuclein-specific antibody, LB509, to confirm that human α-synuclein expression was present only in Tg and Tg/KO mice. There were no differences in the endogenous and human  $\alpha$ -synuclein levels between the Tg and Tg/KO mice. We also confirmed that p62 protein levels were diminished in the brains of KO and Tg/KO mice. Interestingly, the amount of p62 was slightly higher in Tg mice than it was in WT mice (Figure 1C). An increase of p62 was also supported by immunohistochemical studies that showed an increase in p62 immunoreactivity in Tg mice compared with WT mice (Figure 2A). α-Synuclein expression was mainly observed in the presynapses in the brains of WT and p62 KO mice; however, additional staining of  $\alpha$ -synuclein was observed in the cytoplasm and presynapses in the brains of Tg and Tg/KO mice (Figure 2B). Consistent with previous papers (28, 37), KO mice exhibited mature-onset obesity. As they aged, Tg/KO mice had a heavier average body weight than did Tg mice (Figure 1D). The majority of Tg mice remained healthy until at least 70 weeks of age. Tg and Tg/KO mice were behaviorally indistinguishable and displayed lower food intake and activity at the end stage of the disease.

# Tg/KO mice exhibit an increase in phosphorylated $\alpha$ -synuclein staining and inclusion number compared with Tg mice

Similar to the human pathological conditions, there are two types of abnormal  $\alpha$ -synuclein in the brains of Tg mice (7, 42), including phosphorylated  $\alpha$ -synuclein (P-syn) and PK-resistant  $\alpha$ -synuclein (PK-syn). Immunohistochemical analyses showed that P-syn is observed in both Tg/KO and Tg mice (Figure 3A). We compared the number of P-syn-positive inclusions in the thalamus of Tg and Tg/KO mice. Quantitative data indicated that the number of inclusions was higher in Tg/KO mice compared with Tg mice (Figure 3B). Furthermore, the intensity of P-syn staining was increased in the hippocampus and cerebral cortex of Tg/KO mice compared with Tg mice (Figure 3C). Unlike human pathological conditions, p62 was not localized in the cytoplasmic inclusions in the brains of Tg mice. Immunohistochemical studies demonstrated that PK treatment abolished normal a-synuclein immunoreactivity, and PK-syn was found in the presynapses of the brain of both Tg and Tg/KO mice (Figure 3D). Western blot analysis verified that P-syn signal intensity was higher in Tg/KO than Tg mice using two kinds of antibodies against P-syn (Figure 3E). Furthermore, we fractionated samples of Tg and Tg/KO mice by buffer with 0.1% Triton X-100 detergent, and found that insoluble P-syn level was increased in Tg/KO compared with Tg mice (Figure 3F). Thus, p62 deficiency modulates  $\alpha$ -synuclein pathology with regard to P-syn staining intensity, the number of P-syn inclusions and solubility.

# Behavioral tests revealed a longer immobility time for p62-deficient mice

Given the presynaptic aggregation of PK-syn in the hippocampus of Tg and Tg/KO mice, we sought to determine whether memory function was also affected in these mice. We performed the Morris water maze test using mice at a younger age (9 weeks old) to exclude differences in body weight. The average weight was comparable between Tg and Tg/KO mice (21.1 g in Tg, 22.0 g in Tg/KO) at 9 weeks of age. During the training phase of the Morris water maze test, WT and Tg mice showed a gradual decrease in escape latency over time; however, KO and Tg/KO mice exhibited longer escape latencies (Supporting Information Video Clip S1 and S2). When the platform was removed, 80% of WT mice and 70% of Tg mice found the platform location. In contrast, less than 50% of KO and Tg/KO mice found the platform location. KO and Tg/KO mice took a longer time to reach to the platform location (Figure 4A) and spent less time in the target quadrant (Figure 4B) than did WT mice. The lower rate of platform crossing in the KO mice was due to their immobility (Figure 4C,D), which is consistent with previous results showing that KO mice exhibited immobility during training and probe trials (33). During only the first minute of a forced swim test, KO mice showed a significantly increased immobility that lasted longer than 2 s when compared with WT mice (Figure 4E,F). Thereafter, the time course for



**Figure 2.** *p62* and  $\alpha$ -synuclein staining in wild-type (WT), knockout (KO), Tg and Tg/KO mice. **A.** Immunohistochemical analysis shows that p62 immunoreactivity is observed in WT and Tg mice but not in KO mice (9 weeks of age, n = 6 per group). Bar = 20 µm. **B.** Human and mouse  $\alpha$ -synuclein is strongly expressed in the presynapse and cytoplasm of cortical neurons in Tg and Tg/KO mice. Bar = 10 µm.

floating behavior (the percentage of immobility) was similar between groups. There was no significant difference between Tg/KO and WT mice. These results suggest that p62 plays a role in maintaining neurological functions, such as stress responses and motivation to escape.

# Increased levels of the functional homologue, NBR1, in p62 KO and Tg/KO mice

To analyze the molecular mechanisms associated with the loss of p62 on Tg mice, we performed quantitative RT-PCR analysis using

primers for genes related to the stress response and proteolysis (Figure 5). Consistent with the genotype results, the *p62 mRNA* level was diminished in KO and Tg/KO mice. Keap1 is a binding partner of p62 and functions as a sensor for noxious stimuli such as oxidants and electrophiles. The *mRNA* level of *Keap1* appeared to be different between the four groups; however, the data were not statistically significant (P = 0.069). Previous papers have reported that autophagy-deficient mice display a higher expression of detoxifying enzymes, such as *Ho-1*, *Nqo1* and *Gclc* (18). There were no differences in the *mRNA* levels of these enzymes among the four groups. Recent evidence indicates that  $\alpha$ -synuclein

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**Figure 3.** The effect of p62 deficiency on abnormal  $\alpha$ -synuclein expression. **A.** Cytoplasmic inclusions are positive for phosphorylated  $\alpha$ -synuclein (P-syn) in the thalamus of Tg and Tg/knockout (KO) mice. Bar = 20  $\mu$ m. **B.** A quantitative analysis shows that the number of cytoplasmic inclusions is significantly increased in Tg/KO mice when compared with Tg mice (9 weeks of age, n = 6 per group). The groups differed significantly [analysis of variance (ANOVA), *F*(3, 11) = 160.81, *P* < 0.01]. **C.** P-syn staining is observed in the neurons of the cerebral cortex and hippocampus in Tg and Tg/KO mice. An increased staining intensity is observed in Tg/KO mice. Bar = 500  $\mu$ m. **D.** No

obvious differences in proteinase K-resistant  $\alpha$ -synuclein (PK-syn) are found between Tg and Tg/KO mice. Bar = 250 µm. **E.** P-syn level is significantly increased in Tg/KO mice compared with Tg mice. Ratio of P-syn to  $\beta$ -actin was calculated, and the values of Tg mice are defined as 1.0. The groups differed significantly [ANOVA, *F*(3, 11) = 147.1, *P* < 0.01]. **F.** Triton X-100 soluble and insoluble samples were prepared from Tg and Tg/KO mice (9 weeks of age, n = 2 per Tg and Tg/KO groups). Insoluble P-syn level is increased in Tg/KO mice compared with Tg mice. P-syn levels were normalized by total synuclein, and the values of Tg mice were defined as 1.0 in a soluble or insoluble sample.

overexpression causes dynamic changes in the autophagy– lysosomal system. Therefore, we assessed levels of *TfEB*, a major transcriptional regulator for this system (39), lysosomal enzymes (*Lamp1* and *cathepsin D*), molecules responsible for membrane trafficking (*Rab711*) and selective autophagy markers (*Nbr1*). Among these genes, only the *Nbr1 mRNA* levels were significantly different (P < 0.01) between the four groups. Consistent with this result, the NBR1 protein levels were significantly increased in mice lacking p62 compared with mice with p62 (P < 0.05) (Figure 6A,B). Additionally, Keap1 protein levels were also significantly different among the four groups at the protein level. There were no alterations in NQO1, synaptic proteins and proteolysis-related molecules, such as ubiquitin and LC3, which are essential to autophagosomal formation (13). Based on the



Figure 3. Continued.

increased NBR1 levels in mice lacking p62, we compared the distribution patterns of p62 and NBR1 in the mouse brain. Interestingly, immunoblotting showed that p62 and NBR1 are similarly distributed in distinct regions of the mouse brain (Figure 7A,B). NBR1 was mainly localized in the cytoplasm of neurons, and its intensity was higher in Tg/KO than in Tg mice (Figure 7C). These data are consistent with the qRT-PCR and immunoblotting analyses.

# DISCUSSION

p62 is an inducible protein that easily aggregates under pathological conditions, such as oxidative stress and disrupted proteolysis, and it is localized in cytoplasmic inclusions in LBD and other neurodegenerative diseases, suggesting that p62 contributes to inclusion formation. Moreover, p62- and ubiquitin-positive inclusions in the neurons of brain-specific Atg7-deficient mice disappear with the loss of p62 (17). Based on these findings, we initially predicted that p62 deficiency would lead to a decrease in the number of inclusions in Tg mice that overexpressed  $\alpha$ -synuclein. However, our data suggest that p62 deficiency results in an exaggeration of  $\alpha$ -synuclein pathology with regard to P-syn staining intensity and inclusion number. Consistent with our findings, Doi *et al* demonstrated that a loss of p62 exacerbated neuropathological outcomes (5) in a mouse model of spinal and bulbar muscular atrophy, which is one of polyglutamine diseases. Our

energy intake. Interestingly, lack of autophagic activity in POMC neurons caused higher post-weaning body weight and p62/ ubiquitin aggregation (4, 32). Furthermore, leptin signaling is also disrupted in these mice. This may have broad implications for the pathophysiology of p62 KO mice. Because p62 helps shuttle insoluble and ubiquitinated proteins into autophagosomes, disruption of autophagic flux or loss of p62 gives rise to the accumulation

pathological data showed that the number of P-syn-positive inclu-

sion increased by 1.5-fold in Tg/KO mice compared with Tg mice.

Consistently, this was supported by Western blot analyses showing

that P-syn level was higher in Tg/KO mice than Tg mice using two kinds of antibodies against P-syn. Considering that increased

P-syn is mainly resistant to detergent of Triton X-100, it is possible

that biochemical property of  $\alpha$ -synuclein is altered and leads to

more aggregation in Tg/KO mice. Although it remains controversial whether the formation of cytoplasmic inclusions exerts a ben-

eficial or toxic effect on cells, our findings strengthen the idea that p62 can modulate  $\alpha$ -synuclein aggregation and the pathogenesis of diseases. Consistent with previous results (28, 37), a p62 deficiency resulted in mature-onset obesity in mice. Recent evidence indicates that hyperphagia is the primary cause of obesity in p62deficient mice due to the disruption of leptin signaling (9). Accordingly, p62 is highly expressed in hypothalamic neurons, including proopiomelanocortin (POMC) neurons in the arcuate nucleus (3, 9) that are responsible for the control of appetite and energy intake. Interestingly, lack of autophagic activity in POMC



**Figure 4.** *p62-deficient mice exhibit longer escape latencies due to lower activity.* **A.** The probe trial was completed after 5 days of hidden platform training in the Morris water maze. Wild type (WT, n = 10), p62 knockout (KO, n = 11),  $\alpha$ -synuclein Tg (Tg, n = 9) and  $\alpha$ -synuclein mice lacking p62 (Tg/KO, n = 9) were tested at 9 weeks of age. KO mice take longer to reach the platform location. The groups differed significantly [analysis of variance (ANOVA), *F*(3, 39) = 4.53, *P* < 0.01]. **B.** The percentage of time spent in the target quadrant (black) during a 60 s probe trial of the Morris water maze test. KO mice spend less time in the target

quadrant. **C.** The immobility time of the Morris water maze. Longer immobility times are evident in KO mice. **D.** Representative path tracings are shown. Light pink indicates the position of the platform. **E.** A forced swim test was performed at 9–10 weeks of age (n = 9–11 per group) and shows a significant difference in immobility latency, with KO mice lasting longer than 2 s and WT mice remaining mobile for the first 1 minute. **F.** KO mice exhibit higher immobility times for the first 6 minutes. The groups differed significantly [ANOVA, *F*(3, 39) = 2.14, P < 0.05]. \**P* < 0.05.

Tg/KO





**Figure 5.** The effect of p62 deficiency on several kinds of genes. The *mRNA* levels of genes related to proteolysis and oxidative stress in the brains of WT, KO, Tg and TG/KO mice were determined at 9 weeks of age (n = 3 per group). *mRNA* was measured by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) using the right hemisphere of the brain. Data are normalized by the *Cyclophilin A mRNA* level in each sample, and the average and standard deviation was calculated. qRT-PCR reveals that there is no significant difference in the

*Keap1*, glutamate-cysteine ligase catalytic subunit (*Gclc*), NAD(P)H quinone oxidoreductase 1 (*Nqo1*), heme oxygenase-1 (*Ho-1*), *Lamp1*, *Cathepsin D*, *TfEB* and *Rab7l1* levels among the four groups. In contrast, the *mRNA* level of *p62* (*P* < 0.01) and *Nbr1* (*P* < 0.05) are significantly different. The groups differed significantly [analysis of variance, *F*(3, 11) = 226.86, *P* < 0.01 in *p62 mRNA*, *F*(3, 11) = 14.15, *P* < 0.01 in *Nbr1 mRNA*]. The WT values are defined as 100%. \**P* < 0.05, \*\**P* < 0.01.

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The influence of p62 on  $\alpha$ -synuclein in mice

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**Figure 6.** The effect of p62 deficiency on molecules related to proteolysis, oxidative stress and the synapse. **A.** Expression of Keap1 and NBR1 is significantly increased in Tg/KO mice compared with Tg mice. NAD(P)H quinone oxidoreductase 1 (NQO1), LC3, ubiquitin, synaptophysin and SNAP25 levels are not significantly different between the four groups (9 weeks of age, n = 6 per group). **B.** A

quantitative analysis indicates that the Keap1 and NBR1 levels are significantly increased in p62-deficient mice compared with mice with p62. The Tg values are defined as 100%. \*P<0.05, \*\*P<0.01. The groups differed significantly [analysis of variance, F(3, 11) = 7.44, P = 0.011 in Keap1, F(3, 11) = 4.27, P = 0.045 in NBR1].

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Figure 7. The spatial patterns of p62 and NBR1 in the mouse brain. A. Equal amounts of homogenates from the indicated regions were analyzed by immunoblotting (12 weeks of age, n = 2 in wild-type mice). Antibodies against NBR1 (upper) or p62 (bottom) were used to detect endogenous proteins. NBR1 is mainly expressed in the olfactory bulb, temporal and occipital cortices, striatum, thalamus and hypothalamus. The highest expression of p62 is observed in the olfactory bulb, striatum, temporal and occipital cortices, hippocampus, thalamus, hypothalamus and medulla oblongata. β-Actin is used as a loading control. B. Distribution patterns of NBR1 and p62 in the sagittal section of mice brains. NBR1 or p62 levels are normalized by β-actin. The circles represent the size of the expression level. C. NBR1 immunostaining in Tg and Tg/KO mice (9 weeks of age, n = 6 per group). NBR1 immunoreactivity is mainly detected in neurons of the thalamus of both Tg and Tg/KO mice. Note the increased intensity of NBR1 immunoreactivity in Tg/KO mice compared with Tg mice. Bar =  $30 \,\mu$ m.

of p62 target molecules. Accordingly, we revealed that P-syn level is increased in Triton X-100 insoluble fraction of Tg/KO mice compared with Tg mice. Thus, p62 dysfunction observed in autophagy-deficient POMC neurons or p62 KO mice might also affect intercellular environment through disturbance of p62 binding partners or substrates. One of p62 binding partners is known to be dopamine receptor (15). Because dopamine is widely involved in physiological conditions such as mood, cognition and motor control, it is possible that p62 modulates dopamine system, and p62 dysfunction may cause pathogenesis of PD.

Our immunoblot results confirmed that the hypothalamus is one of the regions with the highest p62 expression level. The hypothalamus is known to regulate various physiological functions, particularly the hypothalamus-pituitary-adrenal axis, which coordinates emotional, neuroendocrine and autonomic inputs in response to stress. Regarding behavioral abnormalities, we could not distinguish Tg mice from Tg/KO mice; however, p62-deficient mice exhibited less activity and depression-like behavior in the Morris water maze and forced swim test. This is consistent with previous results (33). It is conceivable that p62 deficiency affects the hypothalamus-pituitary-adrenal axis, leading to behavioral abnormalities in response to stress. The immobility rate of Tg/KO mice was comparable with that of normal control mice. Considering previous reports that mice overexpressing  $\alpha$ -synuclein are hyperactive (8, 30, 44), we speculate that the degree of immobility in Tg/KO mice is recovered because of the hyperactivity of Tg mice. Taken together, p62 plays an important role in modulating multiple physiological responses, including nutritional, oxidative and water stressors.

We screened multiple protein and *mRNA* levels to study the molecular mechanisms associated with the loss of p62 in Tg mice. We found that NBR1 was significantly increased in Tg/KO mice compared with Tg mice at both the *mRNA* and protein levels. p62 and NBR1 contain an N-terminal PB1 domain, an intermediate LC3 binding region, and a C-terminal UBA domain, and they function as cargo adapters for the autophagic degradation of ubiquitinated substrates (10, 12, 29). Intriguingly, our immunoblotting results suggest that these molecules are similarly distributed in distinct regions of the mouse brain. This spatial pattern and functional similarity raise the possibility that NBR1 levels can be up-regulated to compensate for the loss of p62 protein. Therefore, the functional redundancy of NBR1 may mask the anticipated abnormalities of p62-deficient mice.

In conclusion, we have provided evidence that p62 is unnecessary for the formation of inclusions in an animal model that overexpresses  $\alpha$ -synuclein. In addition, p62 deficiency enhanced  $\alpha$ -synuclein pathology based on the number of inclusions and staining intensity of P-syn. In support of this finding, it is likely that p62 indirectly helps sequester abnormal molecules through its own oligomerization (35). Further analyses at the molecular level suggest that NBR1 plays a compensatory role for p62 in the central nervous system. NBR1 and p62 double KO mice would be a useful tool to test this hypothesis.

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# **SUPPORTING INFORMATION**

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

**Video Clip S1.** The Morris water maze test. A wild-type mouse successfully reaches the platform within 30 s on the fourth day of training course.

**Video Clip S2.** The Morris water maze test. A p62-knockout mouse does not take an action within 60 s, and end up failing on the fourth day of training course.





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# Filamentous aggregations of phosphorylated a-synuclein in Schwann cells (Schwann cell cytoplasmic inclusions) in multiple system atrophy

Keiko Nakamura<sup>1,2</sup>, Fumiaki Mori<sup>1\*</sup>, Tomoya Kon<sup>3</sup>, Kunikazu Tanji<sup>1</sup>, Yasuo Miki<sup>1</sup>, Masahiko Tomiyama<sup>3</sup>, Hidekachi Kurotaki<sup>4</sup>, Yasuko Toyoshima<sup>5</sup>, Akiyoshi Kakita<sup>6</sup>, Hitoshi Takahashi<sup>5</sup>, Masahito Yamada<sup>2</sup> and Koichi Wakabayashi<sup>1</sup>

# Abstract

**Background:** The histological hallmark of multiple system atrophy (MSA) is the presence of filamentous aggregations of phosphorylated  $\alpha$ -synuclein in oligodendrocytes, referred to as glial cytoplasmic inclusions (GCIs). Although GCIs can occur widely in the central nervous system, accumulation of phosphorylated  $\alpha$ -synuclein in Schwann cells has not been reported in MSA. We immunohistochemically examined the cranial and spinal nerves, peripheral ganglia and visceral autonomic nervous system of patients with MSA (n = 14) and control subjects (n = 20).

**Results:** In MSA, accumulation of phosphorylated α-synuclein was found in the cytoplasm of Schwann cells. These Schwann cell cytoplasmic inclusions (SCCIs) were also immunopositive for ubiquitin and p62. SCCIs were found in 12 of 14 patients with MSA (85.7 %). They were most frequent in the anterior nerve of the sacral cord and, to a lesser extent, in the cranial nerves (oculomotor, glossopharyngeal-vagus and hypoglossal nerves), and spinal and sympathetic ganglia. SCCIs were rarely found in the visceral organs. Immunoelectron microscopy demonstrated that the SCCIs consisted of abnormal filaments, 15–20 nm in diameter. No such inclusions were found in controls.

**Conclusion:** The present findings indicate that Schwann cells are also involved in the disease process of MSA.

**Keywords:** α-synuclein, Multiple system atrophy, Peripheral nerve, Schwann cell, Schwann cell cytoplasmic inclusion, Ultrastructure

# Introduction

Multiple system atrophy (MSA) is an adult-onset neurodegenerative disorder manifested clinically as a combination of parkinsonism, cerebellar ataxia and autonomic dysfunction. MSA is now divided into two clinical subtypes: MSA with predominant parkinsonian features (MSA-P) and MSA with predominant cerebellar dysfunction (MSA-C) [1]. MSA is characterized pathologically by any combination of coexisting olivopontocerebellar atrophy, striatonigral degeneration and preganglionic

Full list of author information is available at the end of the article

autonomic lesions [2]. The histological hallmark of MSA is widespread glial cytoplasmic inclusions (GCIs) in the central nervous system [3–6]. These GCIs can be visualized by silver staining such as the Gallyas-Braak method [3], and ultrastructurally they consist of granule-associated filaments 20–30 nm in diameter [3, 4, 7]. The major component of GCIs is  $\alpha$ -synuclein [8], which is phosphorylated at Serine 129 [9] and ubiquitinated [10]. Although primary oligodendroglial pathology is the main feature of MSA [11–13], accumulation of phosphorylated  $\alpha$ -synuclein is also consistently found in the neuronal cytoplasm, processes and nuclei [14]. Similar neuronal inclusions are found less frequently in the peripheral sympathetic ganglia [13, 15].



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<sup>\*</sup> Correspondence: neuropal@hirosaki-u.ac.jp

<sup>&</sup>lt;sup>1</sup>Department of Neuropathology, Institute of Brain Science, Hirosaki

University Graduate School of Medicine, 5 Zaifu-cho, Hirosaki 036-8562, Japan

Although immunoreactivity of non-phosphorylated  $\alpha$ -synuclein has been reported in normal and neoplastic Schwann cells in the peripheral nervous system of humans [16], accumulation of phosphorylated  $\alpha$ -synuclein in Schwann cells of patients with MSA has not been described hitherto. Here we immunohistochemically examined the cranial and spinal nerves, peripheral ganglia and visceral autonomic nervous system of patients with MSA using antibodies against phosphorylated  $\alpha$ -synuclein, and report for the first time that Schwann cells in these patients are also affected by filamentous aggregations of phosphorylated  $\alpha$ -synuclein.

### Materials and methods

### Subjects

Thirty-four autopsy cases were included in this study. Fourteen of the patients (age 49–79 years, average = 64.6 years) had a clinical history of MSA, which was confirmed at autopsy by the presence of numerous GCIs (Table 1). All of the MSA cases lacked Lewy body pathology. The clinical and neuropathological features of early MSA (cases 2 and 12) have been reported previously [17, 18]. Twenty patients used as controls (age 40–84 years, average = 70.0 years) were clinically and histopathologically free of neurodegenerative disease. This study was approved by the Institutional Ethics Committee of Hirosaki University Graduate School of Medicine.

### Immunohistochemistry

Immunohistochemical analysis was carried out using formalin-fixed, paraffin-embedded, 4-µm-thick sections from the midbrain, upper pons, medulla oblongata, spinal cord (cervical, thoracic, lumbar and sacral segments), and dorsal root and paravertebral sympathetic ganglia. Oculomotor and trigeminal nerves were examined at the level of the midbrain and upper pons, respectively. Glossopharyngeal and vagus nerves were examined at the level of the dorsal vagal nucleus. Since it was difficult to differentiate glossopharyngeal nerve from vagus nerve on the sections, these two nerves were described as a whole. Hypoglossal nerves were examined at the level of the gracile nucleus. Paraffin sections were also cut from block samples of the esophagus, stomach, small intestine, colon, heart, lung, thyroid, liver, pancreas, kidney, adrenal gland and urinary bladder. The sections were subjected to immunohistochemical processing using the avidin-biotin-peroxidase complex method with diaminobenzidine as the chromogen. The primary antibodies used were mouse monoclonal antibodies against phosphorylated α-synuclein (#64; Wako, Osaka, Japan; 1:5,000), aggregated  $\alpha$ -synuclein (5G4; EMD Millipore, Temecula, CA, USA; 1:1,000) [19] and ubiquitin (1B3; MBL, Nagoya, Japan; 1:2,000), rabbit monoclonal antibody against phosphorylated  $\alpha$ -synuclein (EP1536Y; Abcam, Cambridge, UK; 1:5,000), and rabbit polyclonal antibody against p62 (MBL; 1:1,000). #64 is a monoclonal antibody against a synthetic peptide corresponding to amino acid residues 124–134 of human  $\alpha$ -synuclein with a phosphorylated Serine 129 residue. EP1536Y is also a monoclonal antibody against a synthetic peptide corresponding to residues surrounding phosphorylated Serine 129 of human  $\alpha$ -synuclein.

In addition to routine immunohistochemical techniques, selected sections from the spinal cord of MSA patients were first stained using the modified Gallyas-Braak method [20]. The spinal nerve roots were observed under a ×40 objective lens. After removing the cover glasses from the slides using xylene, the specimens were decolorized in alcohol, then immunostained with antiphosphorylated  $\alpha$ -synuclein (Wako; 1:5,000). The spinal nerve roots were then observed again under a ×40 objective lens.

Semiquantitative assessment of inclusions in Schwann cells was performed in each region by anti-phosphorylated  $\alpha$ -synuclein immunolabeling. The numbers of inclusions were estimated as: –, none; +, 1 to 5 inclusions; ++, >5 inclusions.

### Double immunostaining

To characterize the inclusion-bearing cells, anti-S-100 was used as a marker of Schwann cells [21], anti-tubulin polymerization promoting protein (TPPP)/p25α as a marker of oligodendroglia [22], and anti-phosphorylated neurofilament as a marker of axons [23]. TPPP/p25 $\alpha$  is also known to be a component of GCIs in MSA [24]. Double immunofluorescence analysis was also performed to detect overlapping expression of phosphorylated  $\alpha$ -synuclein and ubiquitin. Paraffin sections from the spinal cord of patients with MSA (n = 3) were processed for double-label immunofluorescence. Deparaffinized sections were blocked with donkey serum and then incubated overnight at 4 °C with a mixture of the monoclonal anti-phosphorylated α-synuclein (Wako; 1:500) and polyclonal anti-S-100 (DAKO, Tokyo, Japan; 1:500), anti-TPPP/p25α (Sigma-Aldrich Japan, Tokyo, Japan; 1:500) or anti-ubiquitin (DAKO; 1:200), or a mixture of the mouse monoclonal anti-phosphorylated neurofilament (SMI31; Cosmo Bio, Tokyo, Japan; 1:500) and rabbit monoclonal anti-phosphorylated a-synuclein (Abcam; 1:500). The sections were then rinsed and incubated for 1 h at 38 °C with anti-rabbit IgG tagged with Alexa Fluor 488 (Invitrogen, Carlsbad, CA, USA; 1:200) and anti-mouse IgG tagged with Alexa Fluor 594 (Invitrogen; 1:200), or anti-rabbit IgG tagged with Alexa Fluor 594 (Invitrogen; 1:200) and anti-mouse IgG tagged with Alexa Fluor 488 (Invitrogen; 1:200). The sections were examined using an Olympus BX63 fluorescence microscope (Olympus, Tokyo, Japan).

Table	1 Summary of	clinical fir	ndings of patients	s with multiple sy	stem atrophy	(MSA)							
Case	Age at	Gender	Disease	Clinical	Pathological	Initial symptoms	Symptoms						
No.	death (years)		duration (years)	diagnosis	diagnosis		Parkinsonian signs	Cerebellar signs	НО	DD	Constipation	Dyshidrosis	Impotence
	49	ш	7	MSA-P	MSA-P	limping	+	1	I	+	+	+	I
2	57	ш	-	spinocerebellar degeneration	MSA-C	ataxia	I	+	I	I	I	I	I
m	58	Z	7	SDS	MSA-C	UD, constipation, impotence	+	+	+	+	+	I	+
4	61	M	7	MSA-P	MSA-P	DD	+	+	I	+	+	I	I
5	61	ш	4.5	MSA-P	MSA-P	tremor	+	+	Ι	+	+	I	I
9	63	ш	Ŷ	MSA-P	MSA-P	gait disturbance	+	I	+	+	I	I	I
7	65	M	7	MSA-C	MSA-C	gait disturbance	+	+	I	+	+	I	I
8	65	M	14	MSA-C	MSA-P	gait disturbance	+	+	+	+	Ι	I	I
6	99	M	6	SDS	MSA-P	DD	+	+	+	+	+	I	I
10	99	M	13	MSA-C	MSA-C	sensory disturbance	+	+	+	+	+	I	I
11	69	M	Ø	SDS	MSA-C	snoring	I	+	+	+	+	I	I
12	71	ш	$\overline{\nabla}$	neurologically normal	MSA-C	none	I	I	I	I	I	I	I
13	75	Σ	ω	progressive supranuclear palsy	MSA-P	gait disturbance	+	+	+	+	I	I	I
14	79	M	4	SDS	MSA-C	unsteady gait	I	+	+	+	Ι	Ι	Ι
OH, orth	iostatic hypotensi	on; UD, urog	enital dysfunction; SI	DS, Shy-Drager syndr	ome; +, present;	, absent							

### Immunoelectron microscopy

The anterior spinal nerve roots from a case of MSA (case 1) were processed for immunoelectron microscopy. Fifty-micrometer-thick vibratome sections were cut from the formalin-fixed tissue. The sections were incubated with a rabbit monoclonal anti-phosphorylated  $\alpha$ -synuclein antibody (Abcam; 1:500), followed by incubation with a biotinylated secondary anti-rabbit IgG (Vector, Burlingame, CA, USA; 1:200) and avidinbiotin-peroxidase complex (Vector; 1:200), and the reaction was developed with diaminobenzidine. The immunolabeled sections were post-fixed in 1 % glutaraldehyde and 1 % osmium tetroxide, dehydrated in ethanol, and then embedded in Poly/Bed 812 resin (Polysciences, Inc., Warrington, PA, USA). Ultrathin sections were cut and viewed with a JEOL1230 electron microscope (JEOL Ltd., Tokyo, Japan).

### Results

### Morphology and immunohistochemical features

Immunostaining with anti-phosphorylated and antiaggregated  $\alpha$ -synuclein antibodies as well as the modified Gallyas-Braak method demonstrated widespread occurrence of GCIs throughout the brain and spinal cord of patients with MSA, but not in control subjects. The immunostaining with two monoclonal anti-phosphorylated  $\alpha$ -synuclein antibodies and a monoclonal anti-aggregated α-synuclein antibody revealed Schwann cell cytoplasmic inclusions (SCCIs) in the cranial and spinal nerves, peripheral ganglia and visceral autonomic nervous system of MSA patients (Fig. 1a-q). They appeared crescent-shaped, coil-like, or cigar-shaped (Fig. 1d-f). The SCCIs enveloped the axons (Fig. 1g) and extended their processes from the cytoplasm to the axons (Fig. 1h, i). Similar inclusions were detected with anti-ubiquitin and anti-p62 antibodies (Fig. 1r, s). The inclusions could not be visualized with hematoxylin and eosin, Klüver-Barrera or Bodian's method. GCIs appeared argyrophilic with the modified Gallyas-Braak method, whereas SCCIs were stained only weakly or partially (Fig. 1 t, u). No such inclusions were found in controls.

To further characterize the inclusion-bearing cells, anti-S-100 was used as a Schwann cell marker, anti-TPPP/ p25 $\alpha$  as an oligodendroglia marker, and phosphorylated neurofilament as an axon marker. Double immunofluores-cence analysis revealed co-localization of phosphorylated  $\alpha$ -synuclein and S-100 (Fig. 2a–c), but not TPPP/p25 $\alpha$  (Fig. 2d–f) or phosphorylated neurofilament (Fig. 2g–i), in the inclusions. Phosphorylated  $\alpha$ -synuclein and ubiquitin were also co-localized in the inclusions (Fig. 2 j–l).

### Immunoelectron microscopy

Pre-embedding immunoelectron microscopy demonstrated phosphorylated  $\alpha$ -synuclein-immunoreactive structures in the cytoplasm of Schwann cells (Fig. 3a). The SCCIs consisted of randomly arranged, loosely packed, granulecoated fibrils, approximately 15–20 nm in diameter (Fig. 3b). Immunodeposition was also detected in the outer and inner loops of the myelinated axons, where fibril formation was not apparent (Fig. 3c).

### Distribution and incidence

The distribution and semiquantitative assessment of SCCIs in patients with MSA are summarized in Table 2. SCCIs were present in the cranial nerves (oculomotor, glossopharyngeal-vagus and hypoglossal nerves) and the spinal nerve roots. In the spinal nerve roots, SCCIs were found in the anterior nerves at the levels of the cervical, thoracic, lumbar and sacral segments, as well as in the posterior nerves in all the segments, except at the cervical level. They were also seen in the dorsal root and sympathetic ganglia and visceral autonomic nervous system.

SCCIs were found in 12 of 14 patients with MSA (85.7 %). They were most frequent in the anterior nerves of the sacral cord (69.2 %) and tended to be more frequent in the anterior than in the posterior nerves at each level. In one case of MSA (case 1), we examined the proximal and distal portions of the sacral nerve roots, and found that SCCIs were more numerous in the proximal than in the distal portion. In the cranial nerves, the inclusions were more frequent in the glossopharyngealvagus nerves (46.2 %) than in the oculomotor (28.6 %) and hypoglossal (9.1 %) nerves. SCCIs were found in 66.7 % and 33.3 % of the dorsal root and sympathetic ganglia, respectively. A small number of SCCIs were also found in the visceral organs in 2 of 14 patients with MSA (14.3 %): the subserosal nerves of the stomach in one patient (case 1) and the adrenal gland and urinary bladder in the other (case 12). There appeared to be no relationship between the frequency of SCCIs and the disease duration or clinical phenotype (MSA-C vs MSA-P) of patients with MSA.

Several neuronal cytoplasmic inclusions were found in the dorsal root ganglia in 2 of 9 MSA patients (cases 5 and 13) (Fig. 1 v, w). No such inclusions were found in the sympathetic ganglia or visceral organs.

### Discussion

In the present study, we have demonstrated for the first time that phosphorylated  $\alpha$ -synuclein accumulates in the cytoplasm of Schwann cells in patients with MSA. These SCCIs were also immunopositive for aggregated  $\alpha$ -synuclein, ubiquitin and p62, a ubiquitin- proteasome system-related protein. Thus, the immunohistochemical profile of SCCIs is similar to that of GCIs [3, 7, 9, 19, 25]. Ultrastructurally, SCCIs were composed of randomly arranged, loosely packed, granule-coated fibrils, approximately 15–20 nm in diameter. Both GCIs and neuronal cytoplasmic inclusions also consisted of granule-coated fibrils, approximately 20–



**Fig. 1** Schwann cell (**a**–**u**) and neuronal (**v**, **w**) cytoplasmic inclusions stained with anti-phosphorylated  $\alpha$ -synuclein (**a**, **b**, **d**–**q**, **u**–**w**), anti-aggregated  $\alpha$ -synuclein (**c**), anti-ubiquitin (**r**), anti-p62 (**s**) and the Gallyas-Braak method (**t**). **a**–**i** Schwann cell cytoplasmic inclusions (SCCIs) (arrowheads) in the anterior spinal nerve roots. SCCIs displaying crescent-shaped (**d**), coil-like (**e**), or cigar-shaped morphology (**f**). SCCIs enwrapping the axons (**g**). SCCIs extending their processes to the axons (**h**, **i**). **j**–**I** SCCIs in the oculomotor (**j**), glossopharyngeal-vagus (**k**) and hypoglossal (**l**) nerves. **m** and **n** SCCIs in the dorsal root (**m**) and sympathetic (**n**) ganglia. **o**–**q** SCCIs in the stomach (**o**), adrenal grand (**p**) and urinary bladder (**q**). **r** and **s** SCCIs showing immunopositivity for ubiquitin (**r**) and p62 (**s**). **t** and **u** Sequential staining of the same sections of the spinal nerve with Gallyas-Braak (**t**) and anti-phosphorylated  $\alpha$ -synuclein (**u**). SCCIs (arrowheads) are only weakly or partially stained with the Gallyas-Braak method. **v** and **w** Neuronal cytoplasmic inclusions in the dorsal root ganglia. Immunostaining with anti-phosphorylated  $\alpha$ -synuclein antibodies (#64 for **a**, **e**, **g**–**q**, **u**–**w**; and EP1536Y for **b**, **d**, **f**). Bars = 50 µm in **a**–**c**; 10 µm in **d**–**w** 



30 nm in diameter [3, 4, 7, 26–28]. These findings indicate that Schwann cells are also involved in the disease process of MSA.

SCCIs were found in 12 of 14 patients with MSA (85.7 %) in the present study. GCIs were consistently found in the brainstem and spinal cord in all of the MSA patients. By contrast, SCCIs were not observed in the cranial or spinal nerves in three patients (cases 2, 12 and 14), two of whom had early MSA [17, 18]. These findings suggest that the occurrence of GCIs precedes that of SCCIs in MSA.

Recently, expression of human  $\alpha$ -synuclein has been reported in Schwann cells ensheathing the nerve fibers of the urinary bladder in a transgenic mouse model of MSA showing oligodendroglial overexpression of human  $\alpha$ -synuclein under the control of the proteolipid protein promoter [29]. Urodynamic analysis revealed a less efficient and unstable urinary bladder in this MSA mouse model. In human MSA, widespread occurrence of GCIs in the central nervous system is a cardinal pathological



feature [3–6]. Moreover, neuronal cytoplasmic and nuclear inclusions have been observed in the inferior olivary and pontine nuclei, substantia nigra, putamen and cerebral cortex in patients with MSA [14, 28]. Filamentous aggregates of  $\alpha$ -synuclein are also found in neurons in the sympathetic ganglia [14, 15]. In the present study, we further demonstrated that accumulation of phosphorylated  $\alpha$ -synuclein occurs in the neuronal cytoplasm in the dorsal root ganglia. Sural nerve biopsy from patients with MSA shows a 23 % reduction of unmyelinated fibers (sensory afferent fibers and postganglionic sympathetic fibers) [30]. Mild degeneration of cardiac sympathetic nerves can occur in MSA [31]. Thus, MSA is a glio-neuronal  $\alpha$ -synucleinopathy involving the central and peripheral nervous systems.

It is noteworthy that SCCIs tend to be more frequent in the peripheral nerves associated with autonomic function, i.e. glossopharyngeal-vagus nerves, and anterior spinal nerves of the thoracic and sacral cord. The vagus nerve is a mixed cranial nerve containing axons of branchiomeric motor neurons, parasympathetic preganglionic fibers, visceral afferent fibers, and somatic sensory afferent fibers. The glossopharyngeal nerve is related closely to the vagus nerve, sharing common medullary

Case No. Cranial nerves					Spinal nerves							DRG	SG	Visceral organs	
					С		Т		L		S				
		V	IX/X	XII	А	Ρ	А	Р	А	Ρ	А	Ρ			
1	NE	-	+	+	NE	NE	+	+	++	-	++	+	NE	NE	+ (stomach)
2	_	_	_	_	_	_	_	_	-	_	-	-	_	_	_
3	_	NE	+	_	+	_	_	_	+	_	+	+	+	_	_
4	_	-	+	NE	NE	NE	+	_	+	-	+	_	NE	NE	_
5	+	NE	_	_	_	-	+	_	+	_	+	-	_	+	_
6	NE	NE	+	-	+	-	-	-	_	-	+	_	+	-	_
7	NE	NE	_	-	+	-	_	_	_	-	+	_	+	_	_
8	++	NE	+	NE	_	-	_	_	-	_	-	+	+	-	_
9	NE	-	+	NE	+	-	+	+	_	-	+	+	NE	NE	_
10	NE	NE	_	-	+	-	+	_	_	-	+	_	+	_	_
11	_	NE	_	-	_	-	_	_	-	+	+	-	_	+	_
12	-	-	-	-	-	-	-	-	-	-	-	-	NE	NE	+ (adrenal, urinary bladder)
13	NE	NE	-	-	-	-	+	-	-	-	-	-	+	+	_
14	NE	_	NE	-	_	-	_	_	-	_	NE	NE	NE	NE	_
Percent positive for inclusions	28.6	0	46.2	9.1	41.7	0	42.9	14.3	28.6	7.1	69.2	30.8	66.7	33.3	14.3

Table 2 Distribution and frequency of Schwann cell cytoplasmic inclusions (SCCIs) in patients with multiple system atrophy

DRG, dorsal root ganglia; SG, sympathetic ganglia; C, cervical; T, thoracic; L, lumbar; S, sacral; A, anterior; P, posterior. Semiquantitive assessment:-, none; +, 1 to 5 SCCIs per area; ++, more than 5 SCCIs per area; NE, not examined

nuclei and having similar functional components [32]. The sympathetic ganglia receive preganglionic fibers from the intermediolateral nucleus of the spinal cord through the anterior roots of all the thoracic and the upper two lumber nerves [32]. The sacral preganglionic parasympathetic fibers exit from the sacral cord and go to the terminal ganglia of the pelvic plexuses, as well as to the myenteric and submucosal plexuses of the descending colon and rectum [32]. The widespread occurrence of SCCIs, at least in part, may play a role for the manifestation of a variety of autonomic symptoms in MSA.

Using the modified Gallyas-Braak method, GCIs were positive whereas SCCIs were stained only weakly or partially. Ultrastructurally, the constituent filaments of SCCIs (approximately 15–20 nm) appeared thinner than those of GCIs (approximately 20–30 nm) [3, 4, 7]. Phosphorylated  $\alpha$ -synuclein-immunoreactive filamentous inclusions are also found in oligodendrocytes and astrocytes in the brains of patients with Parkinson's disease and dementia with Lewy bodies [33–35] and are argyrophilic with the modified Gallyas-Braak method [36], suggesting that the process of  $\alpha$ -synuclein aggregation in glial cells may differ somewhat between the central and peripheral nervous systems.

Cranial nerves are composed of myelinated and unmyelinated fibers in various proportions [37]. The nerve fibers of the anterior spinal nerve roots projecting to the autonomic ganglia are myelinated [38]. Both myelinated and unmyelinated fibers in the peripheral nervous system are enveloped with Schwann cells. Although the number of samples was small, our immunoelectron microscopy examination demonstrated that inclusionbearing Schwann cells, at least in part, ensheath the myelinated fibers. Considering that postganglionic sympathetic nerve fibers are unmyelinated [39] and a small number of SCCIs were observed in the visceral autonomic nervous system in MSA, SCCI formation may also occur in Schwann cells ensheathing the unmyelinated fibers. Moreover, immunodeposition was also found in the outer and inner loops of Schwann cells. In the central nervous system, constituent filaments of GCIs are not evident in the outer or inner loops of oligodendrocytes in MSA [7]. By contrast, tau- and Gallyas-positive filamentous structures are found in the outer and inner loops of oligodendrocytes in progressive supranuclear palsy and corticobasal degeneration [39-41]. These findings suggest that phosphorylated  $\alpha$ -synuclein pathology develops both in the perikarya and distal processes of Schwann cells, whereas the perikarya is chiefly involved in oligodendrocytes in MSA.

It is unclear how aggregated  $\alpha$ -synuclein in the cytoplasm of Schwann cells interacts with the axon, myelin and Schwann cell itself. Both oligodendrocytes and Schwann cells are essential for axonal function and integrity. These enwrapping glia support axonal growth and myelination by transfer of metabolic substrates and secretion of neurotrophic factors [42]. Glial cell linederived neurotrophic factor (GDNF) is one of the neurotrophic factors produced by oligodendrocytes [43] and Schwann cells [44]. The level of GDNF is significantly decreased in the frontal white matter and cerebellum of human MSA patients and in the brain of a MSA mouse model overexpressing human a-synuclein under the control of the myelin basic protein promoter [45]. Intraventricular infusion of GDNF improves behavioral deficits and ameliorates the neurodegenerative pathology in this MSA mouse model [45]. GDNF induces Schwann cell migration and axonal regeneration in the peripheral nervous system [46] and also prevents atrophy of facial motoneurons following axotomy [47]. Liver kinase B1 (LKB1) is also a crucial regulator of the major metabolic pathway in Schwann cells, which are central to axonal stability [48]. Deletion of LKB1 leads to energy depletion, mitochondrial dysfunction, abnormalities of lipid homeostasis and increased lactate release in Schwann cells [48]. The loss of viability in human neuroblastoma cells overexpressing wild-type a-synuclein is associated with reduced activation of intracellular energy sensors, including LKB1 [49]. α-Synuclein-overexpressing rat primary neurons also display lower LKB1 activity [49]. Based on the above findings, it is likely that overexpression of  $\alpha$ -synuclein in Schwann cells impairs the activity of neurotrophic factors, leading to axonal destabilization in peripheral nerves.

The origin of  $\alpha$ -synuclein in SCCIs is uncertain. Immunoreactivity of non-phosphorylated  $\alpha$ -synuclein has been reported in normal and neoplastic Schwann cells in the peripheral nervous system of humans [16]. Therefore, it is possible to consider that overexpression of  $\alpha$ -synuclein in Schwann cells would cause SCCI formation. As another possible mechanism, neuron-to-neuron transmission of  $\alpha$ synuclein fibrils through anterograde axonal transport has been demonstrated in primary cortical mouse neurons in vitro [50]. The fact that SCCIs tended to appear more frequently in the proximal than in the distal spinal nerve roots is appropriate for anterograde transport of  $\alpha$ -synuclein.  $\alpha$ -Synuclein in SCCIs could be derived from neurons. Future studies will be necessary to clarify the origin of  $\alpha$ -synuclein in MSA Schwann cells.

### Conclusion

In conclusion, we have provided for the first time evidence that filamentous aggregation of phosphorylated  $\alpha$ -synuclein occurs in Schwann cells in patients with MSA. Similar inclusions are also observed in the oligodendrocytes and neurons of the central nervous system as well as in neurons of the peripheral ganglia. Both central and peripheral mechanisms may contribute to the neurodegeneration in MSA.

#### **Competing interests**

The authors declare that they have no competing interests.

#### Authors' contributions

KN, FM and KW designed the study and analyzed data. KN, FM, KT, HK, YT, AK, HT and KW performed the pathological observations and evaluations. TK, YM and MT performed the clinical evaluations. FM, MY and KW supervised the whole process of the study. KN, FM and KW wrote the manuscript. All authors read and approved the final manuscript.

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### Author details

<sup>1</sup>Department of Neuropathology, Institute of Brain Science, Hirosaki University Graduate School of Medicine, 5 Zaifu-cho, Hirosaki 036-8562, Japan. <sup>2</sup>Department of Neurology and Neurobiology of Ageing, Kanazawa University Graduate School of Medical Science, Kanazawa, Japan. <sup>3</sup>Department of Neurology, Aomori Prefectural Central Hospital, Aomori, Japan. <sup>4</sup>Department of Pathology, Aomori Prefectural Central Hospital, Aomori, Japan. <sup>5</sup>Department of Pathology, Brain Research Institute, University of Niigata, Niigata, Japan. <sup>6</sup>Department of Pathological Neuroscience, Center for Bioresource-based Researches, Brain Research Institute, University of Niigata, Niigata, Japan.

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# Research paper Sortilin-related receptor CNS expressed 2 (SorCS2) is localized to Bunina bodies in amyotrophic lateral sclerosis



<sup>a</sup> Department of Neuropathology, Institute of Brain Science, Hirosaki University Graduate School of Medicine, Hirosaki, Japan

<sup>b</sup> Department of Pathological Neuroscience, Center for Bioresource-based Researches, Brain Research Institute, University of Niigata, Niigata, Japan

<sup>c</sup> Department of Pathology, Brain Research Institute, University of Niigata, Niigata, Japan

<sup>d</sup> Department of Neurology, Hokkaido University Graduate School of Medicine, Sapporo, Japan

### HIGHLIGHTS

• Amyotrophic lateral sclerosis (ALS) is one of the most devastating neurodegenerative disease.

- ALS-specific Bunina bodies are immunoreactive for SorCS2, a member of vacuolar protein sorting 10 family proteins (VPS10Ps).
- Immunoreativity for VPS10Ps, sortilin and SorLA, is decreased in anterior horn cells of ALS patients.

• VPS10Ps may be involved in the pathomechanisms of ALS.

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### ABSTRACT

Sortilin-related receptor CNS expressed 2 (SorCS2) is one of the vacuolar protein sorting 10 family proteins (VPS10Ps) that have pleiotropic roles in protein trafficking and intracellular and intercellular signaling. Bunina bodies (BBs) are specifically detected in the lower motor neurons in patients with amyotrophic lateral sclerosis (ALS). BBs are immunolabeled with antibodies against cystatin C, transferrin and peripherin and are considered to originate from the endoplasmic reticulum, which is part of the protein sorting pathway. The present study investigated whether VPS10Ps are involved in the formation of BBs in ALS. We immunohistochemically examined the spinal cord from patients with ALS and control subjects using antibodies against VPS10Ps immunolabeled the cytoplasm of anterior horn cells in a fine granular pattern. In ALS, almost all BBs (95.1%) were strongly immunopositive for SorCS2, and immunoreativity for sortilin and SorLA was decreased in anterior horn cells. These findings suggest that VPS10Ps may be involved in the disease process of ALS.

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### 1. Introduction

Sortilin-related receptor CNS expressed 2 (SorCS2) is a type I transmembrane glycoprotein receptor that belongs to the mammalian vacuolar protein sorting 10 protein (VPS10P) family [6,19]. VPS10Ps include sortilin, sorting protein-related receptor with A-type repeats (SorLA), SorCS1, SorCS2, and SorCS3 proteins. All SorCS proteins are expressed predominantly in the brain, especially

E-mail address: neuropal@hirosaki-u.ac.jp (F. Mori).

http://dx.doi.org/10.1016/j.neulet.2015.09.030 0304-3940/© 2015 Elsevier Ireland Ltd. All rights reserved. during development [6,8,19]. Interactions have been documented between VPS10Ps and retromer coat complex (VPS35 and VPS26), a key component of the intracellular trafficking apparatus that sorts cargo from the early endosome to the trans-Golgi network [9]. In recent years, it has been shown that a number of neurodegenerative diseases including Alzheimer's disease (AD), Parkinson's disease and frontotemporal lobar degeneration (FTLD) are characterized by the misprocessing and missorting of intracellular proteins (amyloid precursor protein, tau,  $\alpha$ -synuclein, TDP-43, FUS) within endosomal–lysosomal pathways. Dysfunction within these pathways is proposed to be a major contributing factor to disease progression [9].

Bunina bodies (BBs) are small round eosinophilic inclusions,  $1-5\,\mu m$  in diameter, observed in the lower motor neurons of







<sup>\*</sup> Corresponding author at: Department of Neuropathology, Institute of Brain Science, Hirosaki University Graduate School of Medicine, 5 Zaifu-cho, Hirosaki 036-8562, Japan. Fax: +81 172 39 5132.

patients with amyotrophic lateral sclerosis (ALS), one of the most prevalent TDP-43 proteinopathies. BBs are immunolabeled with antibodies against cystatin C [16], transferrin [10] and peripherin [11]. BBs are considered to originate from the endoplasmic reticulum [21,23], which serves many general functions, including the folding of protein molecules in sacs called cisternae and the transport of synthesized proteins in vesicles to the Golgi apparatus. Our previous immunoelectron microscopy observations indicating that cystatin C is localized to the vesicular membranous structures of BBs [13] prompted us to examine whether VPS10Ps are involved in the formation of BBs. Therefore, we immunohistochemically investigated the expression of VPS10Ps in spinal cord samples from patients with ALS and control subjects. Here we report that SorCS2 immunoreactivity is detectable in BBs in anterior horn cells (AHCs) and that immunoreativity for sortilin and SorLA is decreased in AHCs of ALS patients in comparison to controls.



**Fig. 1.** Light micrographs of the anterior horn of the lumbar cord of normal controls (a,c,e,g,i) and patients with amyotrophic lateral sclerosis (ALS) (b,d,f,h,j), showing immunoreactivity for sortilin (a,b), sorting protein-related receptor with A-type repeats (SorLA) (c,d), sortilin-related receptor CNS expressed 1 (SorCS1) (e,f), SorCS2 (g,h), and SorCS3 (i,j). In ALS, immunoreactivity for sortilin and SorLA is decreased in anterior horn cells (b,d). Bars = 50 µm.



**Fig. 2.** Semiquantification of immunoreactivity for sortilin (a), SorLA (b), SorCS1 (c), SorCS2 (d), and SorCS3 (e) of anterior horn cells in normal controls and ALS patients. The proportions of neurons with intense/moderate (++, black column), weak (+, gray column) or no immunostaining (-, white column) relative to the total number of neurons in the anterior horn are shown. Values are expressed as mean  $\pm$  standard error of the mean. Correlations at *p* < 0.05 were considered significant.

### 2. Materials and methods

### 2.1. Subjects

Twelve patients with sporadic ALS (aged 53–77 years, average 65.8 years) were studied. The diagnosis was confirmed both clinically and histopathologically. BBs were found in the AHCs in 8 of the 12 cases, and TDP-43-positive inclusions were evident in all the cases. We also examined five neurologically normal individuals (aged 53–84 years, average 67.8 years). For routine histological examination, 4-µm-thick, formalin-fixed, paraffin-embedded sections from multiple cortical and subcortical regions were stained with hematoxylin and eosin (H&E) or by the Klüver–Barrera method. We also examined the sections immunohistochemically using anti-TDP-43 antibody (10782-1-AP; ProteinTec Group, Inc., Chicago, IL, USA; 1:4000). This study was approved by the Institutional Ethics Committee of Hirosaki University Graduate School of Medicine.

### 2.2. Immunohistochemistry

Sheep polyclonal antibody against SorCS2 (AF4238; R&D Systems, Inc., Minneapolis, MN, USA; 1:40) and rabbit polyclonal antibodies against sortilin (HPA006889; Atlas Antibodies AB, Stockholm, Sweden; 1:50), SorLA (ab16642; Abcam, Cambridge, UK; 1:50), SorCS1 (23002-1-A1; ProteinTec Group, Inc.; 1:50) and SorCS3 (HPA049097; Atlas Antibodies AB; 1:500) were used in this study.

We identified BBs using H&E-stained sections. BBs were defined as small eosinophilic perikaryal inclusions, 1-5 µm in diameter, which were sometimes clustered. Serial sections (4 µm thick) were cut from paraffin blocks of the 4th lumbar segment in all cases and were first stained with H&E. Digital images of the anterior horn on both sides were captured by a virtual slide system (VS110-S1; Olympus, Tokyo, Japan). AHCs were defined as Nissl body-containing cells in Rexed VIII and IX, whose somal diameters were greater than  $37 \,\mu m$  [12]. AHCs with or without BBs were observed under a ×40 objective lens, measured by a virtual slide system ( $\times$ 600 on a display) and numbered on enlarged prints ( $\times$ 80). When the same neuronal cell body was recognized on several contiguous sections, the same number was marked on the neuron. This approach ensured we avoided missing tiny BBs. After removing the cover glass from the slides in xylene, the specimens were decolorized in alcohol, subjected to heat retrieval using an autoclave for 10 min in 10 mM citrate buffer (pH 6.0), and immunostained with sheep antibody against SorCS2 (1:40) using a Vectastain ABC kit (Vector, Burlingame, CA, USA). Diaminobenzidine was used as the chromogen. The sections were counterstained with hematoxylin. Digital images of the anterior horn on both sides were captured by the virtual slide system again. The SorCS2 immunoreactivity of BBs was confirmed under a ×40 objective lens in each neuron on a display.

Selected sections were double-immunolabeled with sheep polyclonal anti-SorCS2 (1:20) and rabbit polyclonal anti-cystatin C (A0541; DakoCytomation, Glostrup, Denmark; 1:500) antibodies. The secondary antibodies were Alexa Fluor 488 donkey anti-sheep IgG (A-11015; Invitrogen, Carlsbad, CA, USA; 1:200) and Alexa Fluor 594 donkey anti-rabbit IgG (A-21207; Invitrogen; 1:200). The sections were examined with an Olympus Provis fluorescence microscope (Olympus, Tokyo, Japan).

#### 2.3. Semi-quantitative analysis

The numbers of neurons immunoreactive for sortilin, SorLA, SorCS1, SorCS2 and SorCS3 in control subjects and patients with ALS were assessed using a semi-quantitative rating scale: –, unstained; +, weakly stained; ++, moderately or intensely stained. The staining intensity was first graded as – or ++, and any intensity that was neither – nor ++ was defined as +. Three authors (FM, YM, KW), experienced neuropathologists, rated immunostained sections of ALS and control cases under blinded fashion. The proportions of sortilin, SorLA, SorCS1, SorCS2 and SorCS3-positive neurons relative to the total number of neurons were calculated in each case.



**Fig. 3.** Contiguous sections stained with anti-SorCS2 antibody (a,b) and hematoxylin and eosin (c,d). Higher-magnification view of the area indicated by the asterisks in a and c showing Bunina bodies (arrowheads) in the lumbar anterior horn of a patient with ALS (b,d). Bars in a and c = 50  $\mu$ m. Bars in b and d = 10  $\mu$ m.

#### 2.4. Statistical analysis

Calculations were performed using Statcel software (OMS Publishing, Tokorozawa, Japan). Statistical comparison was performed with two-way factorial ANOVA. Values were expressed as mean  $\pm$  standard error of the mean. Correlations at p < 0.05 were considered significant. Good interrater reliability was achieved by three observers using the method, because the data from each rater showed the same significant result.

### 3. Results

In normal controls, antibodies against sortilin, SorLA and SorCS3 moderately immunolabeled the cytoplasm of AHCs in a diffuse granular pattern (Fig. 1a,c,i). With anti-SorCS1 and anti-SorCS2 antibodies, the cytoplasm of AHCs was barely immunolabeled, or was unstained (Fig. 1e,g). In ALS, immunoreactivity for sortilin and SorLA was decreased in the majority of AHCs (Fig. 1b,d). There was no apparent difference in staining intensity of the neuronal cytoplasm immunolabeled with anti-SorCS1, -SorCS2 or -SorCS3 between ALS patients and controls (Fig. 1f,h,j).

Semi-quantitative analysis showed that 20.2% of AHCs were moderately or intensely immunolabeled with anti-sortilin antibody and that 60.5% were weakly immunolabeled in normal controls (Fig. 2a, left). In ALS, 31.2% of AHCs showed weak immunoreactivity and 68.8% were unstained (Fig. 2a, right). Similarly, 27.0% of AHCs were moderately or intensely immunolabeled with anti-SorLA antibody and 57.7% were weakly immunolabeled in normal controls (Fig. 2b, left). In ALS patients, 15.6% of AHCs showed weak immunoreactivity and the rest were unstained (Fig. 2b, right). The differences in the staining intensity of AHCs immunolabeled with anti-sortilin or anti-SorLA between ALS patients and controls were statistically significant (p < 0.01). There were no significant differences in immunoreactivity for SorCS1, SorCS2 and SorCS3 between ALS patients and controls (Fig. 2c-e).

Although the cytoplasm of AHCs showed no or only weak immunoreactivity for SorCS2, BBs were intensely immunolabeled with anti-SorCS2 antibody (Fig. 3). Sequential staining of the same sections with H&E and anti-SorCS2 antibody revealed that almost all BBs were immunopositive for SorCS2 (90.9–100%, average 95.1%). No SorCS2-positive inclusions were observed in 4 cases of ALS, in which no BBs had been detected. No significant



**Fig. 4.** Double-labeling immunofluorescence demonstrating co-localization of SorCS2 and cystatin C in Bunina bodies in spinal anterior horn cells of a patient with ALS. SorCS2 appears green (a) and cystatin C appears red (b). Overlap of SorCS2 and cystatin C appears yellow (c). Bars = 10  $\mu$ m.

immunoreactivity was detectable in sections from controls and ALS cases treated with phosphate-buffered saline solution or by preincubating the antibody (AF4238) with the corresponding synthetic protein (4238-SR; R&D Systems, Inc.) (data not shown).

Double-label immunofluorescence revealed co-localization of SorCS2 and cystatin C in BBs (Fig. 4). No significant immunoreactivity was detectable in negative reaction control sections incubated without the primary antibodies.

### 4. Discussion

A number of genome-wide association studies and biochemical studies have identified members of the VPS10Ps (sortilin, SorLA, SorCS1, SorCS2 and SorCS3) that regulate endosomal sorting as risk factors for neurodegenerative diseases [9]. In the present study, we demonstrated that immunoreativity for sortilin and SorLA was significantly decreased in AHCs from patients with ALS and that BBs were immunoreactive for SorCS2, indicating that sortilin, SorLA and SorCS2 are linked to the pathomechanism of ALS.

Sortilin functions as a trafficking receptor for both Trk receptors and brain-derived neurotrophic factor through the secretory pathway. Progranulin is targeted to the endosomal–lysosomal pathway through a sortilin-dependent mechanism [9]. Haploinsufficiency of progranulin is a common genetic cause of FTLD with TDP-43 aggregates. Progranulin levels were sharply increased upon TDP-43 reduction and splicing of sortilin was altered [17]. TDP-43 is a major constituent of inclusions in motor and non-motor neurons in ALS and FTLD [1,14,22]. Decreased immunoreativity for sortilin in AHCs of ALS might be associated with altered splicing of sortilin.

SorLA was the first member of the family of VPS10 proteins to be genetically linked with late-onset AD [20], and SorLA mutations have now also been suggested as causes of familial AD [18]. Subsequent *in vitro* and *in vivo* studies have shown that SorLA is indeed required for endosome to TGN trafficking of amyloid precursor protein [4,24]. Disruption of the VPS26 binding motif within the SorLA cytoplasmic tail results in increased localization of amyloid precursor protein to endosomal compartments and increased amyloidogenic processing of amyloid precursor protein to produce  $\beta$ -amyloid [4].  $\beta$ -Amyloid has been reported to trigger ALS-associated TDP-43 pathology in AD models [7]. These findings suggest that decreased immunoreativity for SorLA in AHCs of ALS might contribute to the pathomechanism of ALS.

We further demonstrated that BBs are immunoreactive for SorCS2, a member of the VPS10Ps which play various roles in membrane trafficking [9]. Our findings indicate that SorCS2 is another immunohistochemical marker for BBs in addition to cystatin C [16], which is a member of a super family of protease inhibitors, transferrin, which is an iron-binding plasma protein [10], and peripherin, which is a type III intermediate filament protein [11]. These three proteins show moderate to strong positive reactions in the cytoplasm in almost all neurons without BBs, suggesting that they might be incorporated into BBs. On the other hand, SorCS2 protein appears to be less detectable in the cytoplasm of AHCs without BBs, suggesting a different role of SorCS2 in the formation of BBs. SorCS2 is a member of the VPS10Ps, which have pleiotropic roles in protein trafficking and intra- and intercellular signaling in neuronal and non-neuronal cells [9]. Ultrastructurally, BBs are considered to originate from the endoplasmic reticulum [21,23], and cystatin C is localized to the vesicular structures of BBs [13]. These findings indicate that deposition of SorCS2 in BBs results from dysfunction of protein trafficking and intra- and intercellular signaling, leading to incorporation or mislocalization of cystatin C [16], transferrin [10] and peripherin [11] into BBs.

Sortilin is a sorting receptor that directs target proteins, such as growth factors, signaling receptors, and enzymes, to their destined location in secretory or endocytic compartments of cells [2]. However, sortilin is not unique, and just one of five structurally related VPS10Ps including SorCS2 [25]. Emerging evidence suggests that VPS10Ps functionally cooperate, adding another level of complexity to the molecular network in which sortilin acts [15]. SorCS2 has been identified as a receptor that binds to pro-nerve growth factor, thereby mediating acute collapse of growth cones of hippocampal neurons [3]. SorCS2 has also been identified as a proneurotrophin receptor, mediating both trophic and apoptotic signals in conjunction with p75NTR [5]. These findings indicate that SorCS2 is involved in regressive changes of neuronal cells. In the present study, we have demonstrated that BBs, a specific hallmark of ALS, are strongly immunopositive for SorCS2. These findings indicate that, in ALS, SorCS2-positive BBs represent dysfunctional sorting of certain cellular proteins to their destined location in AHCs.

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# **Surgical Neurology International**

James I. Ausman, MD, PhD University of California, Los Angeles, CA, USA

# Case Report

# Cortico-cortical activity between the primary and supplementary motor cortex: An intraoperative near-infrared spectroscopy study

Masafumi Fukuda, Tetsuro Takao, Tetsuya Hiraishi, Hiroshi Aoki, Ryosuke Ogura, Yosuke Sato, Yukihiko Fujii

Department of Neurosurgery, Brain Research Institute, University of Niigata, Niigata-City, Japan

E-mail: \*Masafumi Fukuda - mfuku529@bri.niigata-u.ac.jp; Tetsuro Takao - takao@bri.niigata-u.ac.jp; Tetsuya Hiraishi - tetsuya\_hiraishi@me.com; Hiroshi Aoki - aoki1123@bri.niigata-u.ac.jp; Ryosuke Ogura - oguryou@bri.niigata-u.ac.jp; Yosuke Sato - yosuke0420@hotmail.com; Yukihiko Fujii - yfujii@bri.niigata-u.ac.jp \*Corresponding author

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### Abstract

**Background:** The supplementary motor area (SMA) makes multiple reciprocal connections to many areas of the cerebral cortices, such as the primary motor cortex (PMC), anterior cingulate cortex, and various regions in the parietal somatosensory cortex. In patients with SMA seizures, epileptic discharges from the SMA rapidly propagate to the PMC. We sought to determine whether near-infrared spectroscopy (NIRS) is able to intraoperatively display hemodynamic changes in epileptic network activities between the SMA and the PMC.

**Case Descriptions:** In a 60-year-old male with SMA seizures, we intraoperatively delivered a 500 Hz, 5-train stimulation to the medial cortical surface and measured the resulting hemodynamic changes in the PMC by calculating the oxyhemoglobin (HbO2) and deoxyhemoglobin (HbR) concentration changes during stimulation. No hemodynamic changes in the lateral cortex were observed during stimulation of the medial surface corresponding to the foot motor areas. In contrast, both HbO2 and HbR increased in the lateral cortex corresponding to the hand motor areas when the seizure onset zone was stimulated. In the premotor cortex and the lateral cortex corresponding to the trunk motor areas, hemodynamic changes showed a pattern of increased HbO2 with decreased HbR.

**Conclusions:** This is the first reported study using intraoperative NIRS to characterize the epileptic network activities between the SMA and PMC. Our intraoperative NIRS procedure may thus be useful in monitoring the activities of cortico-cortical neural pathways such as the language system.

**Key Words:** Cortico-cortical activity, epilepsy, hemodynamic change, near-infrared spectroscopy, primary motor cortex, supplementary motor area



# **INTRODUCTION**

Supplementary motor area (SMA) seizures are short in duration and characterized by abrupt, bilateral, tonic

posturing of the extremities, and vocalization without loss of consciousness.<sup>[6]</sup> Epileptic discharges from the SMA can rapidly propagate through the primary motor cortex (PMC), anterior cingulate cortex (ACC),

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and various parietal somatosensory areas, which form multiple reciprocal connections to the SMA.<sup>[2,4]</sup> Better understanding of the mechanisms of SMA propagation can be achieved through improved methods to probe cortical network activities associated with epileptic discharges from the SMA. For example, an ictal single photon emission computed tomography study indicated that seizure-associated hyperperfusion areas did not localize within the SMA, but rather spread to adjacent cortical regions ipsilateral to epileptic foci, such as the ACC and PMC.<sup>[1]</sup> Recently, we demonstrated using simultaneous transcranial near-infrared spectroscopy (NIRS) and electrocorticography (ECoG) recordings in a patient with SMA seizures that increased cerebral blood flow was observed from the epileptic discharges in the ipsilateral SMA and spread to the ipsilateral premotor cortex and PMC as well as the contralateral hemisphere.<sup>[7]</sup>

In a patient with SMA seizure, we used a novel 4-probe device attached to the brain surface to conduct intraoperative NIRS from four probes to show increased blood flow in the PMC elicited by stimulation to the SMA. This is the first report to determine that intraoperative NIRS can reveal the cortico-cortical activities between the SMA and PMC. Moreover, by placing probes on the brain surface, we were able to obtain greater resolution than with transcranial NIRS methods. Our technique will thus allow improved mapping of cortico-cortical network activities intraoperatively.

# **CASE REPORT**

A 60-year-old male presented with seizures a few months before admission to our hospital. His seizures were characterized by tonic posturing in the left extremities and occurred 3-4 times monthly. T2-weighted magnetic resonance (MR) imaging revealed a high intensity lesion in the medial surface of the right frontal lobe. The lesion was not enhanced by Gd on the Tl-weighted MR images, and was suspected to be a low-grade glioma. In order to confirm the relationship between the lesion and the PMC corresponding to the lower extremities, subdural grid electrodes were placed to cover the lateral and medial surfaces adjacent to the PMC. Video-ECoG monitoring demonstrated seizure onset at the right medial surface corresponding to the SMA. The seizure activities rapidly propagated from the SMA to the lateral cortex, including the PMC.

Cortical electrical stimulation was performed for functional cortical mapping. A repetitive square wave with electrical currents of alternating polarity, a pulse width of 0.2 ms, and a frequency of 50 Hz were delivered for 5 s (Nihon Koden, Corporation, Japan). Two neighboring electrodes, with an intensity of 2–5 mA, were stimulated in a bipolar manner. Positive motor and sensory areas were identified by positive motor response (i.e. muscle

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twitch) and subjective sensory sensation, respectively. To define the precise location of each electrode on the surface of the brain, subdural electrodes extracted from computed tomography (CT) images were co-registered to three-dimensional volume-rendered MR images (3.0 T) using image-analysis software (Zed-View, LEXI, Inc., Japan). The results of this functional cortical mapping are depicted in Figure 1. Cortical stimulation to the anterior and posterior areas of the seizure onset zone induced habitual seizures.

Before a partial resection of the lesion for pathology, intraoperative NIRS recording was performed upon stimulation of the placed subdural electrodes apparatus of the medial cortical surface. Constant current stimuli, consisting of five rectangular pulses with 2-ms interstimulus intervals, were generated and recorded with a Neuropac (Nihon Koden, Corporation, Japan). The cathode was positioned at Fz. Motor-evoked potentials were recorded from the abductor pollicis brevis and abductor halluces brevis muscles through paired stainless-steel needle electrodes inserted subdermally. The band-pass filter was set to a range of 5–3000 Hz. The applied stimuli were adjusted to the supra-threshold intensity.

For NIRS monitoring, we developed a novel device comprising of four recording probes spaced 1.5 cm apart and equipped with fixable spatula retractors at the tip of each probe to enable attachment to the brain surface [Figure 2a and b]. The four probes were set to cover the lateral cortex, including the PMC [Figure 2c]. NIRS was carried out with a 695/830 nm spectrometer equipped to our novel monitoring device (ETG-7100; HITACHI Medical, Japan). Emitting light intensity was adjusted to 1 mW (approximately one-fourth



Figure 1: Results of video-electrocorticography (ECoG) monitoring and functional cortical mapping. 3D brain surface images showing recording electrodes (pale blue) and the brain tumor (green). The seizure onset zone (square) was confirmed to reside in the medial surface. The sites in which stimulation induced habitual seizures (closed square) were noted anterior and posterior to the seizure onset zone. Before partial tumor resection, both the foot motor area and seizure onset zone were stimulated for intraoperative NIRS study (stars). UE: Upper extremities, LE: Lower extremities, CS: Central sulcus

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of scalp NIRS). The oxyhemoglobin (HbO2) and deoxyhemoglobin (HbR) concentration changes corresponding to medial surface stimulation in each session were calculated for each segment, which consisted of a 30 s intervals comprising of a prestimulation block (10 s), a stimulation block, and a poststimulation block (20 s). Each session was repeated five times. The present study was approved by the Institutional Review Board Committee at the Niigata University School of Medicine, and informed consent was obtained from the patients (IRB#1559).

In the results, no hemodynamic changes were observed during stimulation of the medial surface corresponding to the foot motor areas at the intensity of 20 mA [Figure 3A]. In contrast, both HbO2 and HbR increased in the lateral cortex corresponding to the hand motor areas when the seizure onset zone was stimulated at an intensity of 16 mA [b and c in Figure 3B]. In the lateral cortex corresponding to the trunk motor areas and the premotor cortex, hemodynamic changes showed a pattern of increased HbO2 with decreased HbR [a and d in Figure 3B].

# DISCUSSION

To our knowledge, this is the first report using intraoperative NIRS during cortical stimulation to demonstrate cortico-cortical activity between the SMA and PMC. We observed that stimulation of the foot motor area elicited no detectable hemodynamic responses in the lateral cortex, whereas stimulation to the seizure onset zone elicited hemodynamic responses at all four probes despite the stimulation intensity decreasing from 20 to 16 mA. These results likely reflected the epileptic network activities between the SMA and PMC.



Figure 2: (a) Our novel device for the intracranial setting of four NIRS probes. The inter-probe distance was 1.5 cm. (b) Four probes were equipped to the NIRS devices. (c) During surgery, the novel device and its four probes were wrapped by a sterilized cover. The device was fixed by spatula retractors at the tip of each probe for attachment to the brain surface. The probes were placed to cover the lateral cortex including the primary motor cortex

It should be noted that we used high frequency stimulation (5-trains, 500 Hz) in the present study, because we were concerned that 50 Hz-stimulation to the seizure onset zone during surgery and functional mapping might induce seizures. Previously, we characterized hemodynamic connectivity in the language system in a patient with temporal lobe epilepsy.<sup>[8]</sup> In this study, 50 Hz-stimulation in the left superior temporal gyrus via subdural electrode contacts gave rise to hemodynamic increases in the inferior frontal gyrus, indicating a strong connection to the stimulation site. In a cortico-cortical evoked potential study,<sup>[5]</sup> stimulation of the foot motor cortex elicited responses in the pre- and postcentral gyrus. Thus, 50 Hz-stimulation of the foot motor areas, rather than 500 Hz employed in our present experiments, might induce hemodynamic changes in the lateral cortex as well.

In this study, high frequency stimulation caused the hemodynamic changes characterized by increase in both HbO2 and HbR in the lateral cortex corresponding to the hand motor areas. This phenomenon was also observed in a study of the language system of the frontal and temporal cortex using simultaneous NIRS and ECoG recordings during cortical stimulation.<sup>[8]</sup> In contrast, in the areas corresponding to the trunk motor areas and premotor cortex, hemodynamic changes showed a pattern of increased HbO2 with decreased HbR as is typical of task-evoked hemodynamic changes. In a previous NIRS study carried out simultaneously with cortical stimulation via subdural electrodes,<sup>[3]</sup> 50 Hz-stimulation produced significant increases in both HbO2 and HbR at the stimulation site and surrounding areas, while 5 Hz-stimulation produced a localized increase in HbO2 and a decrease in HbR. An increase in HbO2 with an



Figure 3: Time course changes of HbO2 and HbR in the lateral cortex. The left picture shows the relationship between the sites of each of the four probes (circle) and subdural electrodes. Recording sites of NIRS were a, b, c, and d. CS: Central sulcus. (A) No hemodynamic changes were noted in any sites, when the foot motor area was stimulated at the intensity of 20 mA. (B) Both HbO2 and HbR increased in the hand motor areas when the seizure onset zone was stimulated at an intensity of 16 mA (b and c). In the trunk motor areas (a) and the premotor cortex (d), hemodynamic changes showed a pattern of increased HbO2 with decreased HbR

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associated increase in HbR is thus indicative of higher levels of neural activity and indicates that oxygen consumption exceeds oxygen delivery via the blood supply.

These findings also suggest that stronger stimulations to the brain surface may likely induce increases in both HbO2 and HbR not only in stimulation sites but also in remote areas that make strong neuronal connections to the stimulation sites. This implies that areas with robust connections areas are prone to seizure spread, even if remote, and that their connectivity will be reflected in the NIRS data. For instance, in the present study, hand motor cortex exhibited increases in both HbO2 and HbR upon stimulation of the seizure onset zone. Oxygen consumption exceeded the rate of oxygen delivery, because the two areas had robust connections that lay within one of the epileptic networks. However, in the trunk motor and premotor cortex, which were conceivably not located within main epileptic networks and weakly connected to the seizure onset zone, the rate of oxygen consumption did not exceed the rate of delivery. Further studies are needed to clarify and extend the implications and relevance of such hemodynamic changes in resolving epileptic networks and more weakly connected surrounding areas.

In this study, we employed intraoperative NIRS from four probes to demonstrate that abnormal connectivity between regions of cortex characterizes epileptic network activities. Our results highlight the capability of intraoperative NIRS to provide us with useful information about the dynamics of cortico-cortical activity at high resolution and without artefacts due to scalp blood flow. This approach can thus expand on previous studies using NIRS scalp recordings, such as our description of hemodynamic connectivity between the superior temporal and inferior frontal cortex in the language system. The additional ability to make simultaneous electrical recording would enhance the level of information obtained through this approach. Recently, a thin flexible probe, with a probe head of  $5.6 \times 10 \text{ mm}$ and the total thickness of 0.7 mm, was developed for simultaneous recording of NIRS and ECoG,<sup>[9]</sup> by integrating near-infrared light-emitting diodes and photodiodes for NIRS measurement placed on the brain surface and ECoG electrodes. In their initial experimental study using this novel device, these researchers reported hemodynamic changes associated with both focal brain cooling and hyperventilation. If such small devices

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were made widely available and applied in patients undergoing neurosurgery, it could be used to further our understanding of cortico-cortical projections and expand on intraoperative NRIS studies.<sup>[8]</sup>

### **CONCLUSIONS**

In a patient with SMA seizures, we employed intraoperative NIRS to demonstrate hemodynamic changes between the SMA, corresponding to the seizure onset zone, and the lateral PMC, corresponding to the seizure propagation areas. This is the first report to report that NIRS can reveal cortico-cortical activities from the brain surface intraoperatively. In the future, intraoperative NIRS will be useful in monitoring cortico-cortical activities such as in the language system and other cortical processes.

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# Limitations of Neuroendoscopic Treatment for Pediatric Hydrocephalus and Considerations from Future Perspectives

Kenichi NISHIYAMA,<sup>1</sup> Junichi YOSHIMURA,<sup>1</sup> and Yukihiko FUJII<sup>1</sup>

<sup>1</sup>Brain Research Institute, University of Niigata, Niigata, Niigata

# Abstract

Neuroendoscopy has become common in the field of pediatric neurosurgery. As an alternative procedure to cerebrospinal fluid shunt, endoscopic third ventriculostomy has been the routine surgical treatment for obstructive hydrocephalus. However, the indication is still debatable in infantile periods. The predictors of late failure and how to manage are still unknown. Recently, the remarkable results of endoscopic choroid plexus coagulation in combination with third ventriculostomy, reported from experiences in Africa, present puzzling complexity. The current data on the role of neuroendoscopic surgery for pediatric hydrocephalus is reported with discussion of its limitations and future perspectives, in this review.

Key words: hydrocephalus, neuroeodoscopy, cerebrospinal fluid, pediatric, shunt

# Introduction

The cerebrospinal fluid (CSF) shunt has long been the classic treatment for pediatric hydrocephalus. It can resolve nearly all forms of hydrocephalus, regardless of the etiology. It is a simple procedure that can aid in having a relatively normal life. However, its failure rate is significant. The reported rate of shunt malfunction in the first year of placement is approximately 30%, and about 10% per year thereafter. The risk of infection is between 5% and 10%. In addition, the high rates of shunt complication such as slit ventricle syndrome are unacceptable by current standards. Children with shunts are dependent on surgical maintenance throughout their lives.<sup>1-8)</sup> Therefore, the advent of neuroendoscopy was received with enthusiasm. Endoscopic third ventriculostomy (ETV) has been a routine surgical practice for the past two decades and provides an alternative to the CSF shunt. It is a straightforward procedure for diversion of the CSF and does not require placing devices in children's bodies. The utility and safety of ETV have been proven for obstructive hydrocephalus that occur secondary to aqueductal stenosis. However, for other indications, it is necessary to examine the anatomy and etiology, as well as the patient's age. During follow-up after

ETV, late failure can occur and may lead to rapid deterioration. The aim of this report is to review the current data on the use of neuroendoscopy in children and to discuss the limitations and future perspectives regarding this procedure.

# Patient Selection and ETV in Infants

ETV has two main purposes: to restore CSF communication between the ventricle and subarachnoid space and to reduce transmantle pulsatile stress by increasing compliance of the ventricular wall.<sup>9)</sup> Since the 1990s, patients with aqueductal stenosis have been considered ideal candidate for ETV.<sup>10,11)</sup> ETV quietly developed into a common procedure, without any prospective randomized trials comparing its efficacy to that of the CSF shunt. In spite of strict patient selection, the overall ETV success rate does not exceed 80%.<sup>12-16)</sup> However, as neuroendoscopic technology has evolved and pediatric neurosurgeons have gained experience with the procedure, the indications for ETV have broadened. For example, patients with hydrocephalus who had a previously failed shunt have become ETV candidates. The success rate of ETV for shunt malfunction is notable, around 80%.<sup>17–20)</sup> In recent years, the use of ETV to treat many pathological conditions has been debated. These include myelomeningocele, Chiari type 1 malformation, Dandy-Walker malformation, and previous meningitis or hemorrhage.14,19,21,22)

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In infants, the benefits conferred by ETV may be considerable due to the high complication rate of CSF shunts. However, there are two concerns with the use of ETV in infants. The first is safety of the procedure, including the possibility of damage to the ventricular and cisternal structures during surgery, CSF leakage, or infection soon after surgery and late closure of the stoma with rapid deterioration. The second concern is that an infant successfully treated with ETV may be transformed from active hydrocephalus to an arrested type. These infants often have larger ventricles than children treated with CSF shunts. No study has attempted to correlate the larger ventricle size with any measurements of psychomotor development. According to reports from a multitude of international studies, the shunt independence rate ranges from 25% to 89%.<sup>23-28)</sup> Two-thirds of the reports suggested that success rate is dependent on age of the infant at the time of ETV.<sup>29-34)</sup> To increase ETV success rates, greater accuracy of appropriate patients would be advised. However, because of the high rate of shunt failure and complication, ETV is sometimes preferred as a first-line treatment. Further, the range of what is defined as failure in this age group is very wide. A multicenter prospective randomized study on infants up to 2 years of age with no flow at the level of the aqueduct, named the International Infant Hydrocephalus Study (IIHS) is now under way. This study focuses primarily on the neurodevelopmental outcomes associated with different treatment paradigms at 5 years, and includes a comprehensive assessment of relevant risks and benefits.<sup>35)</sup>

# Failed ETV and Its Management

ETV failure is a possible event. Although most failures from ETV occur in the early period, within a few days to 2 weeks following the procedure, late failure after many months may lead to rapid deterioration and even sudden death. A rapid increase in intracranial pressure caused by late obstruction of the stoma is typically regarded as the mechanism of failure.<sup>36-42)</sup> Early failure is attributed to the incorrect surgical technique or different criteria in the selection of patients. However, the predictors of late failure are still unknown. Therefore, patients who have undergone successful ETV should be followed on an ongoing basis. Neurosurgeons should encourage patients and their parents to return as soon as possible if any adverse symptoms develop, because these may have severe consequences.<sup>43)</sup> Setting the CSF reservoir concurrently with ETV is one option for the treatment of emergencies, even though it means implantation of a foreign material. In Japan, a follow-up magnetic resonance imaging (MRI) examination

including sagittal  $T_2$ -weighted images, cine-MRI, or constructive interference in steady state (CISS) is often scheduled to detect the CSF flow across the stoma. However, there is currently no evidence regarding whether the patients with no flow on MRI following ETV may be at a greater risk to develop clinical symptoms. For these patients, a repeat ETV may be performed immediately rather than close observation. In the literature, repeat ETV has a good success rate.<sup>32,44–49)</sup> Therefore, this is one option for patients with a failed ETV, and it provides a means of even avoiding the CSF shunt.

# ETV vs. Aqueductoplasty

Endoscopic aqueductoplasty (EAP) is a means to restore the physiological CSF dynamics. This procedure provides an alternative to ETV, because it avoids the risk of severe arterial bleeding. EAP has been performed in cases with membranous or short segmental occlusion of the sylvian aqueduct. However, the long-term results of EAP have not been as successful as one would expect.<sup>50-54)</sup> EAP has been shown to fail frequently. Schroeder et al. reported a re-closure rate of 50%, and proposed that one contributing factor to re-closure could be lower aqueductal CSF flow through the stoma than that following ETV.<sup>55,56)</sup> In addition, aqueductoplasty is generally considered a riskier procedure due to the higher risk of injuring midbrain structures. It may lead to neurological deficits such as oculomotor or trochlear nerve palsy, Parinaud's syndrome, and periaqueductal syndrome. Therefore, ETV, which has higher long-term success rates and less risk, would be a better alternative for membranous, short segmental, and even tumor-related occlusion of the aqueduct.<sup>55,57</sup> However, the condition of isolated fourth ventricle (IFV) is an exception. Almost all patients with IFV have a medical history of hydrocephalus within first year of life, mostly post-infectious or post-hemorrhagic. Further, following shunt placement, they often experience complicated overdrainage with aqueductal stenosis. Aqueductoplasty could be a means of establishing CSF communication to the formerly isolated ventricular compartment; however, a stent is mandatory to keep this pathway open. EAP with a stent could be one choice in the endoscopic treatment of IFV.<sup>50,53,55,57,58)</sup>

# ETV vs. CSF Shunt in Children

As described above, ETV has been widely applied for pediatric hydrocephalus as an alternative to the CSF shunt mainly in an attempt to avoid foreign body implantation and to better simulate physiological

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CSF dynamics. However, the results of CSF shunt have rarely been compared to ETV. According to the pediatric study by de Ribaupierre et al., the failure rate of ETV was 26% and that of ventriculoperitoneal (VP) shunt was 42% at 5 years follow-up. In a review of the literature, the same trend was seen in other studies.<sup>59)</sup> Kulkarni et al. reported that the relative risk of ETV failure is initially higher than that for the CSF shunt. However, the risk became progressively lower at approximately 3 months following the procedure, and was approximately half the risk of shunt failure at 2 years.<sup>60)</sup> The decrease in ventricle size is usually smaller and happens more slowly after ETV compared to shunt. Pediatric neurosurgeons are sometimes concerned about the relationship between these neuroimaging changes and neuropsychological outcomes. Hirsch reviewed that the postoperative intelligence quotient (IQ) was not significantly different in 70 patients with shunts vs. 44 who underwent percutaneous third ventriculostomy.61) Other reports also have found similar results.<sup>62,63)</sup> Recently, Kulkarni et al. reported that at 1 year following surgery, the quality of life and IQ scores were not significantly different between an ETV and a VP shunt group.<sup>64,65)</sup>

# **Challenging Procedure of ETV + CPC**

There has been a resurgence in the technique of choroid plexus coagulation (CPC) in combination with ETV ever since this procedure was performed for African children with hydrocephalus of various etiologies in 2005.66-73) Warf and colleagues highlighted that hydrocephalus with shunt dependency is inadvisable in developing countries because of limited access to medical centers in the event of shunt malfunction. In 2005, Warf and colleagues reported the results of a combined ETV + CPC trial performed mainly in infant with post-infectious hydrocephalus and in those with myelomeningocele. A total of 266 patients underwent ETV + CPC, whereas ETV alone was performed in 284 patients. The results demonstrated that the ETV + CPC increased the success rate in infants from 47% to 66%.<sup>66)</sup> In 2008, they reported the long-term results of ETV + CPC in hydrocephalus with myelomeningocele. The intention-to-treat analysis showed a shunt independence rate of 76% in 338 infants, which was higher than the results of ETV alone reported in the literature.<sup>22,32,67)</sup> Further analyses regarding ETV + CPC performed in patients with encephalocele, Dandy-Walker complex, and congenital aqueductal stenosis have been conducted using their database.<sup>70–72)</sup> The success rates achieved were between 70% and 80%. However, all pediatric neurosurgeons should

be cautious about the meaning of "success." In these reports, success usually referred to controlling raised intracranial pressure and avoiding an extra-cranial CSF shunt. Cognitive function was not the main outcome index. In addition, all these results have been reported from Africa. In the coming years, the challenge will be to see whether these results from Africa can be extrapolated to developed countries.74) The main advantage of ETV + CPC was avoidance of a shunt in patients who were difficult to follow-up. In Africa, geographic and socioeconomic constraints reasons for poor follow-up. The low possibility of cognitive salvage or the high rate of shunt complications, such as holoprosencephaly or hydroanencephaly, may be further reasons in developed nations. Technically, Warf and colleagues used a steerable endoscope via a single burr hole, sometimes in combination with septostomy, and coagulated the bilateral choroid plexus from the foramen of Monro to the entrance of the temporal horn.<sup>66,73)</sup> This procedure appeared to be safe in some cases of severely dilated ventricles. For Japanese neuroendoscopic surgeons, the flexible endoscope is more familiar than it is in other European nations. Nevertheless, almost all pediatric neurosurgeons still believe that the use of ETV + CPC in pediatric hydrocephalus needs further evaluation.

### Conclusion

It is difficult to determine the best strategy for CSF diversion in pediatric hydrocephalus. ETV is one of the alternative procedures to the CSF shunt in conditions of obstructive hydrocephalus such as aqueductal stenosis. However, the preferred indications in infants have not been fully determined. The mechanisms and predictors of late ETV failure that might cause rapid deterioration are not still known. Recently, other clinical dilemmas, such as the use of ETV + CPC have arisen. Definition of the optimal indications, perfection of endoscopic techniques, developing strategies for follow-up, and considering socioeconomic constraints are required from pediatric neurosurgeons when selecting a surgical strategy in pediatric hydrocephalus.

# **Conflicts of Interest Disclosure**

The authors declare that they have no conflicts of interest.

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e-mail: nishiken@d4.dion.ne.jp

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Address reprint requests to: Kenichi Nishiyama, MD, PhD, Brain Research Institute, University of Niigata, 1-757 Asahimachi, Chuo-ku, Niigata, Niigata 951-8585, Japan.

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# BRAIN A JOURNAL OF NEUROLOGY

# Multiple therapeutic effects of progranulin on experimental acute ischaemic stroke

Masato Kanazawa,<sup>1,\*</sup> Kunio Kawamura,<sup>1,\*</sup> Tetsuya Takahashi,<sup>1</sup> Minami Miura,<sup>1</sup> Yoshinori Tanaka,<sup>2</sup> Misaki Koyama,<sup>1</sup> Masafumi Toriyabe,<sup>1</sup> Hironaka Igarashi,<sup>3</sup> Tsutomu Nakada,<sup>3</sup> Masugi Nishihara,<sup>2</sup> Masatoyo Nishizawa<sup>1</sup> and Takayoshi Shimohata<sup>1</sup>

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\*These authors contributed equally to this work.

In the central nervous system, progranulin, a glycoprotein growth factor, plays a crucial role in maintaining physiological functions, and progranulin gene mutations cause TAR DNA-binding protein-43-positive frontotemporal lobar degeneration. Although several studies have reported that progranulin plays a protective role against ischaemic brain injury, little is known about temporal changes in the expression level, cellular localization, and glycosylation status of progranulin after acute focal cerebral ischaemia. In addition, the precise mechanisms by which progranulin exerts protective effects on ischaemic brain injury remains unknown. Furthermore, the therapeutic potential of progranulin against acute focal cerebral ischaemia, including combination treatment with tissue plasminogen activator, remains to be elucidated. In the present study, we aimed to determine temporal changes in the expression and localization of progranulin after ischaemia as well as the therapeutic effects of progranulin on ischaemic brain injury using in vitro and in vivo models. First, we demonstrated a dynamic change in progranulin expression in ischaemic Sprague-Dawley rats, including increased levels of progranulin expression in microglia within the ischaemic core, and increased levels of progranulin expression in viable neurons as well as induction of progranulin expression in endothelial cells within the ischaemic penumbra. We also demonstrated that the fully glycosylated mature secretory isoform of progranulin (~88 kDa) decreased, whereas the glycosylated immature isoform of progranulin (58-68 kDa) markedly increased at 24 h and 72 h after reperfusion. In vitro experiments using primary cells from C57BL/6 mice revealed that the glycosylated immature isoform was secreted only from the microglia. Second, we demonstrated that progranulin could protect against acute focal cerebral ischaemia by a variety of mechanisms including attenuation of blood-brain barrier disruption, neuroinflammation suppression, and neuroprotection. We found that progranulin could regulate vascular permeability via vascular endothelial growth factor, suppress neuroinflammation after ischaemia via anti-inflammatory interleukin 10 in the microglia, and render neuroprotection in part by inhibition of cytoplasmic redistribution of TAR DNA-binding protein-43 as demonstrated in progranulin knockout mice (C57BL/6 background). Finally, we demonstrated the therapeutic potential of progranulin against acute focal cerebral ischaemia using a rat autologous thrombo-embolic model with delayed tissue plasminogen activator treatment. Intravenously administered recombinant progranulin reduced cerebral infarct and oedema, suppressed haemorrhagic transformation, and improved motor outcomes (P = 0.007, 0.038, 0.007 and 0.004, respectively). In conclusion, progranulin may be a novel therapeutic target that provides vascular protection, antineuroinflammation, and neuroprotection related in part to vascular endothelial growth factor, interleukin 10, and TAR DNAbinding protein-43, respectively.

- 1 Department of Neurology, Brain Research Institute, Niigata University, Niigata, Japan
- 2 Department of Veterinary Physiology, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Tokyo, Japan
- 3 Department of Centre for Integrated Human Brain Science, Brain Research Institute, Niigata University, Niigata, Japan
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Correspondence to: Dr Takayoshi Shimohata, Department of Neurology, Brain Research Institute, Niigata University, 1-757 Asahimachi-dori, Chuoku, Niigata, Niigata 951-8585, Japan E-mail: t-shimo@bri.niigata-u.ac.jp

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**Abbreviations:** FTLD-TDP = TAR DNA binding protein positive frontotemporal lobar degeneration; OGD = oxygen glucose deprivation; TDP-43 = TAR DNA-binding protein 43; TGF- $\beta$  = transforming growth factor beta; TNF- $\alpha$  = tumor necrosis factor alpha; tPA = tissue plasminogen activator

# Introduction

Progranulin (PGRN) is a secreted N-linked glycoprotein growth factor implicated in tissue regeneration, wound repair, and inflammation (He *et al.*, 2003*a*). Several *in vitro* studies have revealed that PGRN is classified into two isoforms according to its glycosylation status, the glycosylated immature isoform (58–68 kDa) and the fully glycosylated mature secretory isoform (~88 kDa) (Shankaran *et al.*, 2008; Naphade *et al.*, 2010; Alquézar *et al.*, 2014).

In the CNS, PGRN is considered to play a crucial role in maintaining physiological functions and in the survival of neurons for the following reasons: first, primary cortical neurons derived from *Pgrn* knockout mice showed reduced neuronal survival (Kleinberger *et al.*, 2010); second, PGRN has been shown to exhibit neurotrophic activity *in vitro* (Van Damme *et al.*, 2008) and *in vivo* (Laird *et al.*, 2010); and third, mutations in the *Pgrn* gene caused ubiquitin- and TAR DNA binding protein-positive frontotemporal lobar degeneration (FTLD-TDP), a progressive neurodegenerative disorder with an autosomal dominant inheritance pattern (Baker *et al.*, 2006; Cruts *et al.*, 2006).

Several studies raise the possibility that PGRN might be involved in ischaemic brain injury. First, PGRN might play a vascular protective role against focal cerebral ischaemia via suppression of blood-brain barrier disruption. Recent studies have demonstrated that intraventricular administration of recombinant PGRN could suppress cerebral oedema in a mouse transient focal cerebral ischaemia (suture model) (Egashira *et al.*, 2013), and that *Pgrn* knockout mice may prompt post-ischaemic blood-brain barrier disruption (Jackman *et al.*, 2013).

Second, PGRN has been shown to be involved in neuroinflammation after ischaemic brain injury. Previous studies have demonstrated that PGRN was induced in activated microglia after spinal cord injury (Naphade *et al.*, 2010) and traumatic brain injury (Tanaka *et al.*, 2013), indicating that the induction of PGRN reflects microglial responses. Similarly, in ischaemic brain injury, microglia have been shown to mediate expansion of the infarct via inflammation in the acute phase (Mabuchi *et al.*, 2000; Yenari *et al.*, 2010), while it might also contribute to endogenous anti-inflammatory protection in the subacute phase (Faustino *et al.*, 2011). In this context, recent studies suggested that PGRN might suppress secretion of pro-inflammatory cytokines and recruitment of neutrophils associated with focal cerebral ischaemia (Tao *et al.*, 2012; Egashira *et al.*, 2013). However, a recent study showed that PGRN was not involved in neuroinflammation related to acute focal cerebral ischaemia (Jackman *et al.*, 2013). Therefore, the role of PGRN in neuroinflammation after ischaemic brain injury is controversial.

Third, PGRN might exhibit protective effects on neuronal cells after acute focal cerebral ischaemia based on analyses using transgenic mice expressing PGRN (Tao *et al.*, 2012) and *Pgrn* knockout mice (Jackman *et al.*, 2013), although the mechanism of neuroprotection remains unknown. We previously demonstrated that ischaemic neuronal injury might be caused in part by cleavage and cytoplasmic redistribution of TARDBP (previously known as TDP-43), a key protein in FTLD-TDP and amyotrophic lateral sclerosis (Kanazawa *et al.*, 2011*b*). Because PGRN can suppress the cleavage of TARDBP via inhibiting caspase 3 (Zhang *et al.*, 2007; Kanazawa *et al.*, 2011*b*), it raises the possibility that PGRN may prevent ischaemic neuronal injury via preservation of TARDBP functions.

Based on the abovementioned findings, we speculated that PGRN could exert a positive therapeutic effect via regulation of blood-brain barrier function, suppression of inflammation, and neuroprotection against acute focal cerebral ischaemia. Here, we investigated temporal changes in PGRN after focal cerebral ischaemia, including its cellular expression, glycosylation status, and sources of secretion. We also examined the role of PGRN in neuroinflammation after ischaemic brain injury and the mechanisms of neurovascular protective effects of PGRN. Moreover, we used a rat autologous thrombo-embolic model (Okubo *et al.*, 2007), which shows cerebral oedema and haemorrhagic transformation when tissue plasminogen activator (tPA) is administered beyond the therapeutic time window (Kanazawa *et al.*, 2011*a*; Kawamura *et al.*, 2014) and demonstrated for the first time that intravenous administration of recombinant PGRN showed therapeutic effects on the volumes of cerebral infarct and oedema, haemorrhagic transformation, and prognosis.

# Materials and methods

This study was carried out in strict accordance with the recommendations from the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Niigata University Administrative Panel on Laboratory Animal Care. All surgeries were performed under inhalation and intraperitoneal anaesthesia and according to the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines (Kilkenny *et al.*, 2010).

## Focal cerebral ischaemia

Transient acute focal cerebral ischaemia was induced using the intraluminal filament in male Sprague-Dawley rats weighing 300-370 g and wild-type (C57BL/6) and Pgrn knockout mice (C57BL/6 background) weighing 22-28g (Kanazawa et al., 2011b). Briefly, anaesthesia was administered via inhalation of 1.5% halothane in a mixture of 70% nitrous oxide and 30% oxygen for rats and with intraperitoneal injection 10 mg/kg xylazine and 300 mg/kg chloral hydrate for mice. A nylon monofilament, with a diameter of 0.148 mm for rats and 0.074 mm for mice, was used for suturing. The tip of the nylon monofilament was rounded with heat. An 11-mm distal suture segment was coated with 0.350 mm diameter silicon for rats, and a 4-mm distal suture segment was coated with 0.185 mm diameter silicon for mice. Middle cerebral artery occlusion was performed by insertion of the suture via an external carotid artery into the internal carotid artery. After 90 min of ischaemia, the suture was withdrawn to restore blood flow. Body core temperature monitored with a rectal probe was maintained at  $37.0 \pm 0.5^{\circ}$ C, using a heating pad. To exclude the effects of anomalies of circle of Willis on the ischaemic size and oedema in Pgrn knockout mice, we investigated patterns of circle of Willis in both wild-type and Pgrn knockout mice using transcardial perfusion with carbon black suspended in saline  $(n \ge 7)$  (Supplementary Fig. 1; Özdemir *et al.*, 1999). The sample size was calculated before performing the experiments. We calculated the sample size needed to detect the difference in the amount of cerebral haemorrhage or cerebral oedema volume between the tPA with the recombinant PGRN group and tPA with the control group with 80% power ( $\alpha = 0.05$ ; one-sided analysis). Mice and rats were randomly assigned to various experimental groups, and analyses were performed by an investigator blinded to genotype and treatment.

### Immunoblotting

The rats that survived for 10 min, 4 h, 10 h, 18 h, 24 h, and 72 h after reperfusion were euthanized with an overdose of halothane, followed by intracardiac perfusion with cold normal saline. We defined ischaemic penumbra as the region that was rescued by reperfusion, and the ischaemic core as the

region that was not rescued by reperfusion as described previously (Memezawa *et al.*, 1992; Kanazawa *et al.*, 2011*b*). The cortical tissues corresponding to the ischaemic core or penumbra were dissected for immunoblotting as described (Kanazawa *et al.*, 2011*b*). Cortical tissues from the shamoperated rats were also dissected as controls. For whole-cell extracts, proteins from brain tissues were extracted as described previously (Shimohata *et al.*, 2007). The proteins (50 µg) were subjected to tris-glycine sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

For the whole-cell extracts *in vitro*, cells were harvested in cold cell lysis buffer (Cell Signaling Technology). Conditioned media were collected and concentrated  $(30 \times)$  using Amicon Ultra-0.5 tubes (Millipore). Proteins  $(5 \ \mu g)$  in the samples were separated by tris-glycine SDS-PAGE and were probed with primary antibodies (Supplementary Table 1), followed by a secondary horseradish peroxidase-conjugated antibody (1:10 000). Signals were detected by an enhanced chemiluminescence (Millipore) and semiquantified by densitometry (n = 5). The membranes were stripped and probed with an anti- $\beta$ -actin or an anti-transferrin antibody to confirm even loading of proteins.

# Immunofluorescence staining and confocal microscopy

Rats and mice euthanized with an overdose inhalation of halothane or intraperitoneal injection of xylazine chloral hydrate were sacrificed by transcardial perfusion with normal saline followed by perfusion with cold 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS; pH 7.4) at 24 h and 72 h after reperfusion. We stained free-floating sections as described previously (Kanazawa *et al.*, 2011*b*) and mounted with Vectashield DAPI (Vector Laboratories). Information of the primary antibodies is described in Supplementary Table 1. The sections were examined under a confocal laser-scanning microscope (LSM510 META; Carl Zeiss). The results were confirmed in three independent samples.

### Immunohistochemistry

Deeply anaesthetized rats and mice were perfused intracardially with cold 4% paraformaldehyde at 24 h and 72 h after reperfusion. The brains were removed and embedded in paraffin wax. Serial sections (4- $\mu$ m thick) were cut from the paraffin blocks and stained using antibodies (Supplementary Table 1). The immunoreactive products were visualized using the ABC Vectastain kit (Vector Laboratories) and diaminobenzidine as the chromogen. The results were confirmed in three independent samples.

To measure immunoreactivity, we used ImageJ software 1.37r (National Institutes of Health, Bethesda, MD, USA) as described elsewhere (Krishnamurthy *et al.*, 2009). Briefly, images of the stained sections were visualized, examined blindly, and seven randomly chosen non-overlapping highpower fields (×400) covering the ischaemic cortex were analysed for both wild-type and *Pgrn* knockout mice (n = 21). For each image field, the total number of pixels was quantified on a grey scale (0–255). The levels of immunoreactivities were expressed as the average density (mean pixel value in an image field ± SEM) for each experimental group.

#### Effects of progranulin on stroke

# Measurement of the volume of the cerebral infarct and oedema

At 24 h after reperfusion, the rats and mice were given highly concentrated halothane and deeply anaesthetized. After transcardial perfusion with saline, the brains were extracted. The brains were cut into 3-mm slices and stained with 2% 2, 3, 5-triphenyltetrazolium chloride solution (#264310; BD, Franklin Lakes). After staining, the slices were photographed with a scanner (CanoScan LiDE 50; Canon Inc), and cerebral infarct volume and cerebral oedema volume were measured with ImageJ software 1.37r (National Institutes of Health, Bethesda, MD, USA) according to the method described by Swanson *et al.* (1990). These values were expressed as a proportion of the cerebral hemisphere occupied. The analyses were performed by an investigator blinded to the genotype and treatment.

## **Neurological evaluations**

Neurological evaluations were conducted at 24 h after reperfusion with a 6-point neurological scale (Zausinger *et al.*, 2000). Specifically, grade 5 indicated no neurological findings, grade 4 indicated an inability to move forward with the foot on the affected side, grade 3 indicated weak resistance to a force that was applied from the side on a level surface, grade 2 indicated turning to the affected side when pulled from behind on a level surface, grade 1 indicated spontaneously turning to the affected side, and grade 0 indicated an inability to move spontaneously or death.

## Pgrn knockout mice

*Pgrn* knockout mice were produced by breeding heterozygous pairs in the Graduate School of Agricultural and Life Sciences, at the University of Tokyo, and genotyped using previously described protocols (Kayasuga *et al.*, 2007). Additionally, *Pgrn* knockout mice purchased from RIKEN BioResource Centre (Tsukuba, Japan) were bred and maintained in the Department of Comparative and Experimental Medicine, Brain Research Institute, Niigata University. We used only homozygous pairs to obtain *Pgrn* knockout pups. The mice were maintained under controlled light (lights on, 05:00–19:00), temperature ( $23 \pm 1^{\circ}$ C), and humidity ( $55 \pm 10\%$ ) and given free access to food and water.

# C6 cell culture and analysis of glycosylation

Rat C6 cells were grown in Opti-MEM<sup>®</sup> (Gibco/Invitrogen) with 10% foetal bovine serum (FBS) and 1% penicillinstreptomycin. Cells were grown in Opti-MEM<sup>®</sup> without FBS 24 h before the experiments. Cells were harvested in cell lysis buffer (Cell Signaling Technology) containing 1% Triton<sup>TM</sup> X-100 and inhibitors for proteases and phosphatases (Sigma-Aldrich). After centrifugation at 14 000 rpm for 5 min (4°C), the supernatants were collected. Additionally, conditioned media were also obtained for immunoblotting. To analyse gly-cosylation of PGRN, cell lysates and media were digested at 37°C with peptide-*N*-glycosidase F (PNGase F; New England Biolabs) according to manufacturer's instructions, in triplicate.

## **Primary cell cultures**

Primary murine neuronal cells were prepared from the neocortices of embryonic Day 17 C57Bl/6 mouse embryos (Takeuchi *et al.*, 2005). Briefly, cortical fragments were dissociated into single cells in a dissociation solution (Sumitomo Bakelite) and resuspended in nerve culture medium (Sumitomo Bakelite). Neurons were plated onto poly-D-lysine-coated (P7405, Sigma-Aldrich) six-well dishes ( $10 \times 10^4$  cells per well). The purity of these neuronal cultures was 95% as determined by NeuN-specific immunostaining (Takeuchi *et al.*, 2005).

Primary murine microglia and astrocytes were obtained as previously described (Milner *et al.*, 2008). Primary mixed glial cultures were established from the forebrains of postnatal C57Bl/6 mice by dissociating isolated cerebral cortices in papain and then growing the resulting cell suspension for 10 days in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS. After 10 days, the flasks were shaken for 15 min to remove the loosely attached microglia. The purity of these microglial cultures was 99% as determined by Mac-1 immunoreactivity in flow cytometry (Milner *et al.*, 2008). For astrocytes, the flasks were then shaken overnight to remove the microglia and oligodendrocyte precursors. The remaining monolayer was determined as >95% astrocytes by glial fibrillary acidic protein (GFAP) immunoreactivity (Milner *et al.*, 2008).

To investigate the effect of microglia and astrocytes on secretion of vascular endothelial growth factor (VEGF) from astrocyte after oxygen glucose deprivation (OGD), we cultured astrocytes with microglial conditioned media. Briefly, after we performed OGD using primary murine microglia, we collected the microglial low-glucose conditioned media. Then, we removed the astrocytic high-glucose conditioned media and washed thoroughly with PBS. We finally exchanged the PBS for the collected microglial low-glucose conditioned media. After overnight incubation under OGD condition, we measured the level of VEGF in the conditioned media using VEGF Quantikine® ELISA Kit (RRV00, R&D Systems), according to the manufacturer's instructions (n = 6-7). IL10 levels in the conditioned media from microglia were also determined by a sandwich ELISA using the IL10 Quantikine® ELISA Kit (M1000B, R&D Systems), according to the manufacturer's instructions (n = 5-6).

# Oxygen-glucose deprivation

The standardized conditions for OGD have been described in detail elsewhere (Milner *et al.*, 2008). Briefly, before induction of OGD, serum-containing media was removed from the cell cultures by washing twice thoroughly with PBS before adding serum-free high glucose medium (4.5 mg/l, DMEM containing 4 mM L-glutamine, penicillin, and streptomycin, supplemented with N1 medium) or low-glucose medium (1.0 mg/l, supplemented with DMEM). The cultures containing low-glucose medium were placed in a hypoxia chamber (Billups-Rothenburg), which was flushed with a mixture of 95% N<sub>2</sub> and 5% CO<sub>2</sub> for 1 h, and then closed for 18 h or 24 h. Oxygen levels decreased to 0.1–0.4% at 4 h and was maintained throughout the experiment (del Zoppo *et al.*, 2012).

To determine the effects of PGRN under OGD, we added full-length recombinant mouse PGRN produced from HEK293 cells (AG-40A-0080, AdipoGen) into conditioned media. The recombinant PGRN is N-glycosylated, as determined by a PNGase F assay (manufacturer's datasheet). Because a FLAGtag was attached to the C-terminus of the recombinant PGRN protein, localization of exogenous recombinant PGRN protein was determined by immunohistochemical staining with an anti-FLAG antibody (Supplementary Table 1).

The survival of cells in culture exposed to OGD was determined by lactate dehydrogenase (LDH) release into the culture supernatant using a kit assay in six-well format (Roche Diagnostics), according to the manufacturer's instructions. The relative assessments of cytotoxicity were normalized by comparison with 100% cell damage (n = 5). The survival of neuronal cells in the culture exposed to OGD was determined using the propidium iodide method in which 33 µg/ml propidium iodide solution (Sigma) was added to each well, followed by incubation for 5 min (Osada *et al.*, 2011).

## **Cell counting protocols**

To determine the frequency of propidium iodide-positive neuronal cells after OGD conditions, we examined seven randomly chosen non-overlapping high-power fields (×630) of primary neuronal cells incubated under OGD conditions for 24 h (n = 21) (Kanazawa *et al.*, 2011*b*). To determine the frequency of microtubule-associated protein 2 (MAP2)-positive neurons with cytoplasmic redistribution of TARDBP, seven randomly chosen non-overlapping high-power fields (×630) of primary neuronal cells under normoxic or OGD conditions during 24 h were examined (n = 7) (Kanazawa *et al.*, 2011*b*). We also determined the appearance frequencies of microglia and neutrophils in the ischaemic tissue in wild-type and *Pgrn* knockout mice by examining seven randomly chosen non-overlapping high-power fields (×400) (n = 21). These analyses were conducted blinded to the sample information.

# Reverse transcription polymerase chain reaction

Primary microglia were collected as pellets after centrifugation at 13000 rpm for 10 min. Total RNA was isolated from the samples using NucleoSpin® RNA XS (U0902A, Takara Bio), and the first-strand cDNA was synthesized with ReverTra Ace (FSQ-101, TOYOBO). PCR reactions were performed with PrimeSTAR® Max DNA Polymerase (Takara Bio) and the following primers: TNF-a (forward, 5'- AAGAGGCACT CCCCCAAAAG -3'; reverse, 5'- GCTACAGGCTTGTCACT CGAA -3'), TGF-β (forward, 5'- TGGAGCAACATGTGGA ACTC -3'; reverse, 5'- CAGCAGCCGGTTACCAAG -3'), IL-10 (forward, 5'- CTAACCGACTCCTTAATGCAG -3'; reverse, 5'- TTAAAATCACTCTTCACCT -3'), and actin (forward, 5'- CATCCGTAAAGACCTCTATGCCAAC -3'; reverse, 5'- ATGGAGCCACCGATCCACA -3'). The PCR reactions were carried out as follows: 35 cycles for amplification at 98°C for 10s, 55°C for 5s, and 72°C for 5s. The genes of interest were normalized to actin (n = 5-7).

# **Quantitative real-time PCR**

Primary microglia were collected as pellets after centrifugation at 13 000 rpm for 10 min. Total RNA was isolated from samples using Nucleospin RNA XS (U0902A, Takara Bio), and first-strand cDNA was synthesized from total RNA by

ReverTra Ace (TOYOBO). Quantitative RT-PCR was performed with SYBR<sup>®</sup> Green Premix ExTag on a TP-850 Real-Time PCR Detection system (Takara Bio). We designed pairs for IL10 as follows: (forward, 5'primer ATTTGAATTCCCTGGGTGAGAAG -3'; 5'reverse. CACAGGGGAGAAATCGATGACA -3'). PCR conditions were 30s at 95°C for initial denaturing, followed by 40 cycles of 5s at 95°C and 30s at 60°C. Expression levels of the target gene relative to housekeeping enzyme (Takara Bio) were determined using the  $\Delta\Delta$ CT method (*n* = 3–4).

# Thrombolytic treatment with tissue plasminogen activator and PGRN

Cerebral ischaemia was created using a model of focal embolic ischaemia, in which the animals had their middle cerebral artery occluded by autologous thrombi (Okubo et al., 2007; Kanazawa et al., 2011a). tPA was administered intravenously in the form of alteplase (Mitsubishi Tanabe Pharma Co.) at a dose of 10 mg/kg per animal at 4 h after cerebral ischaemia, because we previously demonstrated that this condition caused cerebral oedema, haemorrhagic transformation, and severe prognosis (Kanazawa et al., 2011a; Kawamura et al., 2014). We demonstrated that cerebral cortical blood flow decreased by 40% from baseline after ischaemia and tPA treatment 4 h after ischaemia improved it by >70% from baseline in this thrombo-emobolic model (Kanazawa *et al.*, 2011*a*). Immediately before tPA administration, 100 µg of FLAGtagged recombinant PGRN or control protein (IgG; R5G10-048, OEM Concepts) was administered as a bolus through a catheter in the inguinal vein (n = 6-7). The volumes of the cerebral infarct and oedema, cerebral haemorrhage, neurological evaluations, and mortality ratio were examined. Cerebral infarct and oedema and cerebral haemorrhage measurements were performed in a randomized and blinded manner.

# **Statistical analyses**

All data were presented as mean  $\pm$  SEM. Differences in the parameters were analysed using one-way or two-way ANOVA, followed by Bonferroni's *post hoc* test or unpaired *t*-test. Statistical analyses were performed using SPSS (ver.21; SPSS Inc.). Differences in the frequencies were assessed with Fisher's exact test or Mann-Whitney's U-test. All tests were considered statistically significant at a *P*-value < 0.05.

# Results

# Post-ischaemic changes of PGRN expression in neuronal cells, microglia and endothelial cells in rat cerebral cortex

To investigate the expression and localization of PGRN in the non-ischaemic cortex of Sprague-Dawley rats, we performed immunofluorescence staining using an anti-PGRN antibody. At first we tested specificity of the anti-PGRN antibody by immunoblotting and immunohistochemistry. The cell lysates and brain tissue from *Pgrn* knockout mice did not show immunoreactivity against the antibody (Supplementary Fig. 1). Confocal microscopic studies revealed that the immunoreactivity for PGRN was detected only in cortical neurons (Fig. 1). PGRN was detected in a punctate pattern within neuronal cytoplasm, and double immunostaining revealed that PGRN was colocalized with marker proteins for the endoplasmic reticulum, Golgi apparatus, and lysosome [ERp57 (now known as PDIA3), Golgi-58k, and LAMP1, respectively]. We did not identify microglia or vessels that expressed PGRN.

We next investigated the effect of acute focal ischaemia on the expression and localization of PGRN using ischaemic Sprague-Dawley rats. Immunohistochemical analyses revealed that PGRN was not observed in the neurons from the ischaemic core (Fig. 2A), but observed in surviving neurons from the ischaemic penumbra (Fig. 2A). The expression pattern of PGRN in the surviving neurons from the ischaemic penumbra is diffused and strong, which is different from that of the non-ischaemic neurons (Fig. 2A). PGRN-positive microglia were increased at 24 h and markedly increased at 72 h after reperfusion, especially on the border of the ischaemic core and penumbra on the ischaemic core side (Fig. 2B). In addition, PGRN expression was observed in the von Willebrand factor-positive endothelial cells in the ischaemic penumbra at 24 h after reperfusion (Fig. 2C).

# Temporal changes in PGRN expression and its glycosylated status in rat cerebral cortex after acute focal cerebral ischaemia

To investigate the effects of acute focal ischaemia on the expression and glycosylation status of PGRN, we next performed immunoblotting using whole-cell extracts of the ischaemic cortex of Sprague-Dawley rats. In the ischaemic core, the level of ~88 kDa PGRN gradually decreased after reperfusion, although it recovered at 72 h after reperfusion (Fig. 3A and B). On the other hand, the level of 58–68 kDa PGRN did not change until 18 h, but increased at 24 h and markedly increased at 72 h after reperfusion (Fig. 3A and B). In the ischaemic penumbra, the ~88-kDa PGRN decreased slightly, but not significantly, after reperfusion, while the 58–68 kDa PGRN moderately increased (Fig. 3A and B).

To confirm whether these two bands indicated secretory isoforms of PGRN, an *in vitro* study was performed. Cell lysates and conditioned media from rat C6 cells were subjected to immunoblotting, as this tumor cell line produces PGRN during growth (Liau *et al.*, 2000). We identified a low molecular-weight band (58–68 kDa) in the cell lysates and a high molecular-weight band (~88 kDa) in the conditioned media (Fig. 3C). In addition, after digestion with PNGase F, which cleaves all N-linked oligosaccharides, the mobility of both bands from rat C6 cells changed to 58 kDa. A similar result was observed from an experiment using rat non-ischaemic brains (Fig. 3D). Therefore, it is considered that the high molecular-weight band (~88 kDa) was the secretory form of the fully glycosylated isoform of PGRN and that the low molecular-weight band (58–68 kDa) was the pre-secretory form of the glycosylated immature isoform of PGRN (Fig. 3D).

# Production and secretion of the two isoforms of PGRN after OGD

To determine the potential cellular sources of the glycosylated immature isoform of 58-68 kDa PGRN, which markedly increased after reperfusion in ischaemic rats, immunoblot analyses of whole-cell extracts and conditioned media from primary murine neurons or microglia were performed. After 18 h incubation under OGD, both neurons and microglia were not dead as determined by the lack of change in LDH release from the cells (Fig. 4A). Under normoxic conditions, 58-68 kDa PGRN was observed in the neuronal cell extracts similar to C6 cell (Fig. 4B), and the ~88-kDa PGRN was observed in both neuronal and microglial conditioned media (Fig. 4C). We found that, after 18h incubation under OGD, neurons produced ~88 kDa and 58-68 PGRN and secreted ~88 kDa PGRN, and the microglia produced ~88 kDa PGRN, and secreted both ~88 kDa and 58-68 kDa PGRN (Fig. 4C). Therefore, the glycosylated immature isoform of 58-68 kDa PGRN was secreted only from microglia.

# Volumes of cerebral infarct and oedema, and outcome after ischaemia in *Pgrn* knockout mice

To investigate the roles of PGRN in acute focal cerebral ischaemia, we compared volumes of cerebral infarct and oedema between Pgrn knockout mice and wild-type mice, and evaluated the outcome by motor scale after middle cerebral artery occlusion. Before the comparison, we evaluated vascular anomaly of the circle of Willis (Özdemir et al., 1999) of Pgrn knockout mice, and found no significant difference in vascular structure between wild-type and Pgrn knockout mice (Supplementary Fig. 2). There was also no significant difference in infarct size at 24 h after reperfusion between the two groups (P = 0.12) (Fig. 5A). However, cerebral oedema in the Pgrn knockout mice was larger than that in the wild-type mice at 24 h after reperfusion (P = 0.048) (Fig. 5B). In addition, motor scale at 24 h after reperfusion in *Pgrn* knockout mice was worse than that in the wild-type mice (P = 0.02) (Fig. 5C). There were no significant differences in cerebral oedema and motor scale at 72 h after reperfusion between the two groups (P = 0.95 and 0.75, respectively).



**Figure 1** Analyses of PGRN expression in neuronal cells and microglia from rat non-ischaemic cerebral cortex. Microtubuleassociated protein 2 (MAP2), endoplasmic reticulum stress protein 57 (ERp57, now known as PDIA3), Golgi-58k, Iysosomal associated membrane protein 1 (LAMP-1), CD68/ED1 (green) / PGRN (red) / 4',6'-diamidino-2-phenylindole (DAPI; blue) triple labelling in the non-ischaemic cortices examined by confocal microscopy. MAP2, ERp57, Golgi-58k, LAMP1, and CD68/ED1 are markers for neuronal cells, endoplasmic reticulum, Golgi apparatus, Iysosome, and microglia, respectively. Scale bars = 10 µm.



Figure 2 Post-ischaemic changes in PGRN expression in neuronal cells, microglia and endothelial cells in the rat cerebral cortex. (A) The absence of PGRN expression in the

# Recruitment of neutrophils and microglia after ischaemia in *Pgrn* knockout mice

Because a recent study reported that recombinant PGRN treatment suppressed neutrophil recruitment into ischaemic mouse brain (Egashira *et al.*, 2013), we investigated whether cerebral oedema in *Pgrn* knockout mice is associated with neutrophil recruitment. Immunohistochemistry using an anti-myeloperoxidase antibody revealed that there were no significant differences in the numbers of myeloper-oxidase-positive neutrophils between the ischaemic wild-type and *Pgrn* knockout mice at 24 h and 72 h after reperfusion (Supplementary Fig. 3). We also found that there were no significant differences in the numbers of IBA1 (now known as AIF1)-positive microglia in the border of the ischaemic core and penumbra between the wild-type and *Pgrn* knockout mice at 24 h and 72 h after reperfusion (Supplementary Fig. 4).

# **VEGF** expression in *Pgrn* knockout mice after ischaemia

We next investigated the possibility that cerebral oedema in Pgrn knockout mice was caused by VEGF expression, because VEGF is a potent angiogenic factor that regulates vascular permeability after focal cerebral ischaemia (Zhang et al., 2002). Additionally, a recent study, in which PDGF (platelet-derived growth factor) was used as an experimental agent to induce blood-brain barrier leakage, did not determine the VEGF protein level after ischaemia (Jackman et al., 2013). Although VEGF expression was undetectable in brains of sham-operated wild-type and Pgrn knockout mice, VEGF expression was observed in the peri-infarct area from brains of ischaemic wild-type and Pgrn knockout mice 24 and 72 h after reperfusion. At 72 h after reperfusion, the expression was much more prominent in Pgrn knockout mice than in wild-type mice (P < 0.001) (Fig. 5D). VEGF expression was observed

#### Figure 2 Continued

ischaemic core and increased PGRN expression in the ischaemic penumbra at 24 h after reperfusion. MAP2, LAMP1 (green) / PGRN (red) / DAP1 (blue) triple labelling in the ischaemic core and penumbra examined by confocal microscopy. Scale bars = 10  $\mu$ m. (**B**) Markedly increased PGRN-positive microglia at 72 h after reperfusion. CD68/ED1 (green) / PGRN (red) / DAP1 (blue) triple labelling in the sham-operated or ischaemic cortex (ischaemic core) examined by confocal microscopy. Red scale bar = 10  $\mu$ m. White scale bars = 20  $\mu$ m. (**C**) PGRN expression in endothelial cells and astrocytic end-feet at 24 h after reperfusion. von Willebrand factor (vWF), glial fibrillary acidic protein (GFAP, green) / PGRN (red) / DAPI (blue) triple labelling in the sham-operated or ischaemic penumbra examined by confocal microscopy. von Willebrand factor is a marker for endothelial cells, and GFAP is a marker for astrocytes. Scale bars = 10  $\mu$ m.



Figure 3 Temporal changes in PGRN expression in the rat cerebral cortex after focal cerebral ischaemia. (A) Representative PGRN protein bands of the rat cerebral cortex from the ischaemic core (*left*) and ischaemic penumbra (*right*). Two isoforms (~88 kDa and 58–68 kDa) were observed.  $\beta$ -actin confirmed equal loading of proteins. (B) Optical densitometry analyses for ~88 kDa (*left*) and 58–68 kDa isoforms (*right*) of PGRN. \**P* < 0.05, \*\**P* < 0.01, ##*P* < 0.01 versus sham. Data represent relative optical densities of ischaemic brain samples compared to those of sham-operated samples. (C) Difference in glycosylation status in PGRN between cell lysates and conditioned media of rat C6 cells analysed by immunoblotting. Note that PGRN shows two isoforms (~88 kDa and 58–68 kDa), both of which change to 58 kDa after digestion with peptide-*N*-glycosidase F (PNGase F). All findings were confirmed in triplicate. (D) Difference in glycosylation status in PGRN shows two isoforms (~88 kDa and 58–68 kDa), both of which change to 58 kDa, both of which change to 58 kDa after digestion with PNGase F. All findings were confirmed in triplicate.

mainly in astrocytes and endothelial cells (but not in microglia) within ischaemic mice (Supplementary Fig. 5).

To confirm the increased level of VEGF in ischaemic *Pgrn* knockout mice, we performed an *in vitro* study. We

first compared VEGF secretion after OGD from microglia and astrocytes between wild-type and *Pgrn* knockout mice (Fig. 5E). The levels of VEGF from microglia of *Pgrn* knockout mice were higher than those of wild-type mice



Figure 4 Effects of OGD on PGRN secretion from murine primary cultured neuronal and microglial cells. (A) Cytotoxicity evaluated by lactate dehydrogenase assay between normoxia and OGD in neuronal cells and microglia (n = 5). (**B** and C) Representative PGRN protein bands in immunoblotting from whole-cell extracts (B) and conditioned media (C) of murine primary cultured cells (neurons and microglia) subjected to normoxia (norm) or OGD. After 18 h incubation under OGD condition, 58- $68\,k\text{Da}$  and  ${\sim}88\,k\text{Da}$  PGRN in the neuronal cell extracts as well as  $\sim$ 88 kDa PGRN in the neuronal conditioned media increased compared to those under normoxic condition (P = 0.001 and 0.011, respectively). The  $\sim$ 88 kDa PGRN in the microglial conditioned media and the 58-68 kDa PGRN in the microglial cell extracts also increased compared to those under normoxic conditions (P < 0.001 and 0.0098, respectively). Additionally, the 58-68 kDa PGRN in the microglial conditioned media markedly increased (P < 0.001).  $\beta$ -actin and transferrin confirmed equal loading of proteins. Optical densitometry analyses for PGRN are also shown. Data represent relative optical densities of each sample compared to microglial samples from OGD (n = 5). \*P < 0.05. \*\*P < 0.01.

(P < 0.001), although the levels of VEGF from astrocytes were not different between wild-type and Pgrn knockout mice (P = 1.00). To investigate the effect of microglia on VEGF secretion from astrocytes, microglial condition media of wild-type and Pgrn knockout mice were added to the astrocytes of wild-type and Pgrn knockout mice, respectively. Interestingly, the levels of VEGF from the astrocytes with microglial conditioned media of knockout mice was higher than that of wild-type under OGD (P < 0.001). In addition, the levels of VEGF from astrocytes with microglial conditioned media of knockout mice was higher than that from microglia alone (P = 0.013) or astrocytes alone (P = 0.014) of Pgrn knockout mice (Fig. 5E). These results suggest that lack of PGRN or unknown humoral factors from ischaemic microglia of Pgrn knockout mice might synergically promote VEGF secretion from the astrocytes.

# The effects of VEGF expression by recombinant PGRN treatment after ischaemia in vitro and in vivo

To investigate the effect of recombinant PGRN on VEGF expression in vitro, we compared VEGF levels in astrocytes with microglial conditioned media for wild-type mice treated with vehicle controls and recombinant PGRN. The level of VEGF in astrocytes cultured in microglial conditioned media was decreased by recombinant PGRN (10 µg/ml) compared to vehicle treatment  $(23.0 \pm 4.8\%)$  reduction; P = 0.046, n = 3-6). We previously reported that delayed tPA treatment 4h after ischaemia promoted expression of VEGF in vivo (Kanazawa et al., 2011a). We investigated the effect of recombinant PGRN on VEGF expression using this thrombo-embolic model. For this purpose we administrated 100 µg recombinant PGRN or vehicle along with tPA treatment 4h after ischaemia. The co-administration of recombinant PGRN and tPA decreased the expression of VEGF compared to co-administration of vehicle and tPA (P = 0.046) (Supplementary Fig. 6).

# Comparison of cytokine response of microglia between wild-type and *Pgrn* knockout mice after OGD

To investigate the effect of PGRN on neuroinflammation after acute ischaemia, we compared mRNA levels of several cytokines in microglia from wild-type and *Pgrn* knockout mice, under normoxic and OGD conditions, because the balance between pro-inflammatory and anti-inflammatory microglial phenotypes is considered to influence expansion of cerebral infarct (Hu *et al.*, 2012). We found that there were no differences in the mRNA levels of pro-inflammatory cytokines (TNF- $\alpha$  and TGF- $\beta$ ) between wild-type and *Pgrn* knockout mice, while the mRNA level of anti-inflammatory cytokine IL10 in the *Pgrn* knockout microglia was lower than that in the wild-type microglia after OGD



Figure 5 Comparison of ischaemic outcomes and VEGF expression between wild-type and *Pgrn* knockout mice. These panels show volumes of the cerebral infarct (**A**) and cerebral oedema (**B**) at 24 h and 72 h after reperfusion (all,  $n \ge 9$ ), and outcome evaluated by a 6-point neurological scale score (**C**) at 24 h after reperfusion [wild-type (WT) n = 29, knockout (KO) n = 22]. Volumes of cerebral infarct and oedema are expressed as proportions on the ischaemic side of the cerebral hemisphere. \*P < 0.05, \*\*P < 0.01. (**D**) VEGF expression in the shamoperated and ischaemic brains (at 24 h and 72 h after reperfusion) from the wild-type and *Pgrn* knockout mice. The results were confirmed in triplicate. A secondary-only antibody control confirms that extracellular stainings of VEGF after ischaemia were not non-specific (data not shown). At 72 h after reperfusion, the expression of VEGF was higher in ischaemic brains of *Pgrn* knockout mice than in those of wild-type mice (\*\*P < 0.001) (n = 21). Scale bars = 200  $\mu$ m. (**E**) The levels of secretory VEGF from microglia, astrocytes, and astrocytes with microglial conditioned media of wild-type and *Pgrn* knockout mice after OGD (n = 6-7). \*P = 0.01, \*\*P < 0.01.



**Figure 6** Comparison of cytokine response of microglia between wild-type and *Pgrn* knockout mice after OGD. (A) Representative cytokine mRNA bands from primary microglial cells of wild-type (WT) and *Pgrn* knockout (KO) under normoxic and OGD conditions. (B) Optical densitometry analyses for inflammatory cytokines such as TNF- $\alpha$  and transforming growth factor- $\beta$  (TGF- $\beta$ ), and antiinflammatory cytokines such as IL-10 (n = 5-7). Data represent relative optical densities of respective samples compared to samples from wildtype mice under normoxic conditions by one-way ANOVA. (C) Data represent relative *II10* mRNA levels compared to samples from wild-type mice under normoxic conditions (n = 3-4). \*P < 0.05, \*\*P < 0.01.

(P = 0.037) (Fig. 6A and B). We also found that *Il10* mRNA levels in the *Pgrn* knockout microglia were lower than that in the wild-type microglia after OGD by real-time PCR (P = 0.001) (Fig. 6C). The level of *Il10* after 18 h incubation under OGD from microglial conditioned media of *Pgrn* knockout mice ( $10.3 \pm 3.1$  pg/ml) was lower than that from microglial conditioned media of wild-type mice ( $37.4 \pm 12.2$  pg/ml) (P = 0.040).

We also investigated the expression of TNF- $\alpha$ , TGF- $\beta$ , and IL10 in brains from ischaemic wild-type and *Pgrn* knockout mice 24 and 72 h after reperfusion. Although the expressions of TNF- $\alpha$  and TGF- $\beta$  in the ischaemic brains were not different between wild-type and *Pgrn* knockout mice (P = 0.262 and P = 0.228, respectively) (Supplementary Fig. 7A and B), IL10 expression was lower in the ischaemic brains of *Pgrn* knockout mice than in those of wild-type mice (P < 0.001) (Supplementary Fig. 7C). IL10 was expressed mainly in the extracellular parenchyma.

# Effects of PGRN on neuronal cell death and cytoplasmic redistribution of TARDBP

We investigated the neuroprotective effect of PGRN on murine primary neuronal cells under OGD condition. Twenty-four hours incubation under OGD promoted neuronal cell death as evaluated by a lactate dehydrogenase assay (Fig. 7A), and the percentage of propidium iodidepositive neuronal cells after 24-h incubation under OGD was  $8.4 \pm 3.7\%$  (*n* = 21) (Supplementary Fig. 8). The attached neuronal cells that showed cytoplasmic redistribution of TARDBP under the OGD conditions were confirmed to be living as they were propidium iodide-negative (Fig. 7B). However, we found that the recombinant PGRN (5µg/ml) suppressed the neuronal cell death compared to the control (P = 0.045)(Fig. 7C). Immunocytochemistry confirmed that these surviving neurons incorporated the exogenously administered



**Figure 7** Suppression of neuronal cell death and cytoplasmic redistribution of TARDBP (TDP-43) under OGD conditions by recombinant PGRN. (A) Cytotoxicity of neuronal cells between normoxic and OGD conditions at 18 h and 24 h (n = 5). \*\*P < 0.01. (**B**) Confocal microscopic observation of TDP-43 (now known as TARDBP, green)/propidium iodide (PI) (red)/DAPI (blue)-triple labelling in neuronal cells under OGD conditions. Magnification is double in the small inset. Cytoplasmic redistribution of TDP-43 was observed in surviving neurons under the OGD conditions. These neuronal cells were propidium iodide-negative. Scale bar = 50 µm. (**C**) Recombinant PGRN (rPGRN) alleviated cytotoxicity at 24 h under OGD conditions (n = 6). \*P < 0.05. (**D**) MAP2 (green)/FLAG (red) / DAPI (blue) triple labeling in neurons between normoxic and OGD conditions by confocal microscopy. FLAG-tagged recombinant PGRN was co-localized in surviving neurons under OGD conditions (arrow). Scale bars = 20 µm. (**E**) MAP2 (green)/TDP-43 (red)/DAPI (blue) triple labelling in neurons between normoxic and OGD conditions. Scale bars = 20 µm. (**F**) The frequencies cytoplasmic redistribution of TARDBP in MAP2-positive neuronal cells between normoxic and OGD conditions. recombinant PGRN blocked cytoplasmic redistribution of TARDBP at 24 h under OGD conditions. recombinant PGRN blocked cytoplasmic redistribution of TARDBP at 24 h under OGD conditions. \*P < 0.05, \*P < 0.05 (b) hocked cytoplasmic redistribution of TARDBP at 24 h under OGD conditions. \*P < 0.05, \*P < 0.05 (b) hocked cytoplasmic redistribution of TARDBP in MAP2-positive neuronal cells between normoxic and OGD conditions. recombinant PGRN blocked cytoplasmic redistribution of TARDBP at 24 h under OGD conditions. \*P < 0.05, \*P < 0.05 (b) versus normoxic condition (n = 7).





FLAG-tagged recombinant PGRN (Fig. 7D). We also found that the neuronal cells, which incorporated the exogenous FLAG-tagged recombinant PGRN, showed a decrease in the cytoplasmic redistribution of TARDBP (Fig. 7E): the frequency of cytoplasmic redistribution of TARDBP in the neurons treated with recombinant PGRN under OGD was lower than that treated with the vehicle control ( $48.0 \pm 35.4\%$  versus  $86.9 \pm 10.4\%$ ; P = 0.010) (Fig. 7F).

# Therapeutic effects of recombinant PGRN against delayed tPA treatment for acute focal cerebral ischaemia

Finally, we investigated therapeutic effects of recombinant PGRN using a rat autologous thrombo-embolic model (Okubo *et al.*, 2007), because this model shows cerebral

oedema and haemorrhagic transformation when tPA is administered beyond the therapeutic time window (4 h) (Kanazawa *et al.*, 2011*a*; Kawamura *et al.*, 2014). The group treated with tPA and intravenous administration of recombinant PGRN at 4 h after reperfusion showed therapeutic effects on the cerebral infarct volume (Fig. 8A), oedema volume (Fig. 8B), haemorrhage (Fig. 8C), motor scale (Fig. 8D), and mortality ratio (Fig. 8E) compared to the group treated with tPA and control (P = 0.007, 0.038,0.007, 0.004 and 0.026, respectively).

# Discussion

We demonstrated for the first time the dynamic changes in PGRN *in vivo* in the neurons, microglia, and endothelial cells after ischaemia, including decreased level of PGRN

expression in neurons within the ischaemic core, increased level of PGRN expression in the surviving neurons, as well as induction of PGRN expression in microglia and endothelial cells in the ischaemic penumbra. Immunoblot analyses demonstrated that the fully glycosylated mature secretory isoform (~88 kDa) decreased, while the glycosylated immature isoform (58-68 kDa) markedly increased after reperfusion. In addition, in vitro experiments revealed that the glycosylated mature isoform was secreted from neurons and microglia, and the glycosylated immature isoform was secreted only from microglia. Consistent with this finding, immunohistochemical analyses demonstrated a marked increase in PGRN-positive microglia at 72 h after reperfusion in ischaemic rats. These findings suggest that the marked increase in the glycosylated immature isoform of 58-68 kDa PGRN after reperfusion was caused by microglia.

We next demonstrated that PGRN has multiple therapeutic effects against ischaemic brain injury. First, we found that PGRN could attenuate blood-brain barrier disruption after acute focal cerebral ischaemia. We demonstrated for the first time that PGRN is expressed in the endothelial cells in the ischaemic penumbra. A previous report has shown that PGRN is induced in the capillary endothelium of wound granulation tissue and promotes the mitosis and migration of adult dermal microvascular cells (He et al., 2003b), although PGRN is not expressed in the healthy endothelium (Daniel et al., 2000). We speculated that the expression of PGRN in endothelial cells may be involved in the vascular protection or repair against ischaemic injury. Indeed, cerebral oedema volume in Pgrn knockout mice was larger than that in wild-type mice after focal cerebral ischaemia. Regarding the mechanism by which PGRN regulates vascular permeability, we considered that cerebral oedema is not caused by recruitment of neutrophils and microglia based on the findings from immunostainings using wild-type and Pgrn knockout mice. A very recent study demonstrated the involvement of the PDGF receptor pathway using Pgrn knockout mice (Jackman et al., 2013). We proposed another possibility that PGRN might regulate vascular permeability via VEGF pathway, because we observed more prominent expression of VEGF in Pgrn knockout mice than in wild-type mice after focal cerebral ischaemia. In addition, Pgrn knockout microglia itself secreted VEGF and the conditioned media from Pgrn knockout microglia synergically stimulated VEGF secretion from Pgrn knockout astrocytes after OGD. Although we could not identify the specific molecules that stimulate VEGF expression, a recent study demonstrated that PGRN might inhibit cerebral oedema via NF-kB (Egashira et al., 2013), which activates VEGF transcription (Yoshida et al., 1998). PGRN may regulate vascular permeability by inhibiting microglial production of molecules that activate NF-kB-VEGF signalling pathway.

In breast cancer, high levels of PGRN result in increased VEGF production (Tangkeangsirisin *et al.*, 2004), while in this study of ischaemia, depletion of PGRN increased

VEGF expression. Furthermore, we showed clear increases in PGRN expression in vessels of the penumbra (Fig. 2C), which is similar to what has been reported in peripheral wounds (He *et al.*, 2003*b*). Because elevated levels of PGRN have a significant biological effect on vessel growth that might be independent of VEGF (Toh *et al.*, 2013), some of the effects of *Pgrn* knockout on penumbral blood vessels might be mediated directly by PGRN loss rather than via increased VEGF expression (Jackman *et al.*, 2013). Further studies are needed to clarify the mechanism underlying PGRN-mediated vascular permeability.

Second, PGRN may suppress neuroinflammation after acute focal cerebral ischaemia via the anti-inflammatory cytokine, IL10. We demonstrated that the number of PGRN-positive microglia (CD68/ED1-positive cells) increased in the ischaemic core of Sprague-Dawley rats, and that the primary cultured-microglia from Pgrn knockout mice showed a decrease in the levels of IL10 under OGD, although levels of inflammatory cytokines such as TNF- $\alpha$  and TGF- $\beta$  were not changed. Consistent with this finding, previous studies reported that the level of IL10 in glial cells from PGRN-overexpressing transgenic mice was higher than that from wild-type mice (Tao et al., 2012), and that the macrophage/microglia from Pgrn knockout mice released less IL10 than from wildtype mice when exposed to bacterial lipopolysaccharide (Yin et al., 2010). However, findings on pro-inflammatory cytokines are controversial and inconclusive. It has been demonstrated that levels of other pro-inflammatory cytokines (IL1B, IL6 and TNF- $\alpha$ ) in glial cells from PGRNoverexpressing transgenic mice were lower than those from wild-type mice (Tao et al., 2012), and that they were increased in the macrophage/microglia from Pgrn knockout mice compared to wild-type mice when mice exposed to bacterial lipopolysaccharide (Yin et al., 2010). In contrast, a very recent study demonstrated that mRNA levels of *Il1b*, *Il6*, TNF- $\alpha$  (now known as *Tnf*), and TGF- $\beta$ (now known as Tgfb1) (Il10 not examined) after ischaemic brain injury were not associated with Pgrn knockout condition as seen in our results (Jackman et al., 2013). We consider, based on our findings, that PGRN may suppress neuroinflammation via IL10 not by inhibiting pro-inflammatory cytokines.

Third, we confirmed that PGRN might play a neuroprotective role against acute focal cerebral ischaemia *in vitro* and *in vivo*. Neuronal PGRN decreased in the ischaemic core where neuronal death occurred, and increased in the ischaemic penumbra where neuronal survival occurred after acute focal cerebral ischaemia. *In vitro* studies demonstrated that the neuronal cells and microglia produced and secreted PGRN after ischaemia, and that exogenous recombinant PGRN could suppress neuronal cell death under OGD. Although PGRN has been shown to have neurotrophic effects, the exact mechanism remains unknown (Van Damme *et al.*, 2008; Laird *et al.*, 2010). We consider that the neurotrophic effects of PGRN might be explained in part by the inhibition of abnormal cytoplasmic redistribution of nuclear TARDBP (Zhang *et al.*, 2007, 2009). Stated another way, the decreased level of PGRN might cause loss of function of TARDBP in neurons, resulting in neuronal damage.

Finally, we demonstrated that intravenously administered recombinant PGRN reduced volumes of cerebral infarct and oedema, suppressed haemorrhagic transformation, and improved motor outcome with delayed tPA treatment resulting in haemorrhagic transformation that is similar to human stroke, in a rat autologous embolic model (Kanazawa *et al.*, 2011*a*; Kawamura *et al.*, 2014). A previous study reported that intraventricular administration of recombinant PGRN reduced cerebral infarct and oedema in a mouse focal suture ischaemic model (Egashira *et al.*, 2013), although this model and injection route poorly reflected the clinical practice. Our findings indicate the possibility that recombinant PGRN could be used as a novel neurovascular protective drug with anti-inflammatory effect after delayed tPA treatment.

Because of the importance of protein glycosylation in mediating a wide range of biological processes, characterization of glycan structures is necessary to understand the structure-function relationship (Songsrirote *et al.*, 2010). Therefore, we chose the full-length glycosylated recombinant PGRN protein. We could not evaluate the impact of glycosylated status of the PGRN on neuronal cells because it is technically hard to use deglycosylated PGRN after PNGase assay because of reagent toxicities for cells. Further studies are needed to determine the impact of glycosylation status for recombinant PGRN on this effect.

In conclusion, we demonstrated dynamic changes of expression and localization of PGRN after acute focal cerebral ischaemia. PGRN may be a novel therapeutic target that provides vascular protection, anti-neuroinflammation, and neuroprotection related in part to VEGF, IL10 and TARDBP, respectively.

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# Supplementary material

Supplementary material is available at Brain online.

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# Clinicopathological Features in Anterior Visual Pathway in Neuromyelitis Optica

Mariko Hokari, MD,<sup>1</sup> Akiko Yokoseki, MD, PhD,<sup>1</sup> Musashi Arakawa, MD, PhD,<sup>1</sup>

Etsuji Saji, MD, PhD,<sup>1</sup> Kaori Yanagawa, MD, PhD,<sup>1</sup> Fumihiro Yanagimura, MD,<sup>1</sup>

Yasuko Toyoshima, MD, PhD,<sup>2</sup> Kouichirou Okamoto, MD, PhD,<sup>3</sup>

Satoshi Ueki, MD, PhD,<sup>4</sup> Tetsuhisa Hatase, MD, PhD,<sup>5</sup> Riuko Ohashi, MD, PhD,<sup>6</sup>

Takeo Fukuchi, MD, PhD,<sup>5</sup> Kohei Akazawa, PhD,<sup>7</sup> Mitsunori Yamada, MD, PhD,<sup>8</sup>

Akiyoshi Kakita, MD, PhD,<sup>2</sup> Hitoshi Takahashi, MD, PhD,<sup>2</sup>

Masatoyo Nishizawa, MD, PhD,<sup>1</sup> and Izumi Kawachi, MD, PhD<sup>1</sup>

**Objective:** Neuromyelitis optica spectrum disorder (NMOsd) is an autoimmune disorder of the central nervous system characterized by aquaporin-4 (AQP4) autoantibodies. The aim of this study was to elucidate the characteristics of involvement of the anterior visual pathway (AVP) and neurodegeneration via glia–neuron interaction in NMOsd. **Methods:** Thirty Japanese patients with serologically verified NMOsd were assessed with a neuro-ophthalmological study. Using 27 tissue blocks from 13 other cases of NMOsd, we performed neuropathological analysis of glial and neuroaxonal involvement in the AVP.

**Results:** The AVP involvement in NMOsd was characterized by the following, compared to multiple sclerosis: (1) longitudinally extensive optic neuritis (ON); (2) more severe visual impairment and worse prognosis for ON; (3) unique AQP4 dynamics, including loss of AQP4 immunoreactivity on astrocytes with complement activation in ON lesions, loss of AQP4 immunoreactivity on Müller cells with no deposition of complement in the retinas, and densely packed AQP4 immunoreactivity on astrocytes in gliosis of secondary anterograde/retrograde degeneration in the optic nerves and retinal nerve fiber layer (RNFL); and (4) more severe neurodegeneration, including axonal accumulation of degenerative mitochondria and transient receptor potential melastatin 4 channel with complement-dependent astrocyte pathology in ON lesions, mild loss of horizontal cells, and RNFL thinning and loss of ganglion cells with abundance of AQP4<sup>+</sup> astrocytes, indicating secondary retrograde degeneration after ON.

**Interpretation:** Severe and widespread neuroaxonal damage and unique dynamics of astrocytes/Müller cells with alterations of AQP4 were prominent in the AVP and may be associated with poor visual function and prognosis in NMOsd.

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 $\mathbf{N}$  euromyelitis optica (NMO) spectrum disorder (NMOsd)<sup>1</sup> and multiple sclerosis (MS) are the two main autoimmune, inflammatory, and demyelinating syndromes of the central nervous system (CNS). The serum NMO immunoglobulin G autoantibody targets

the water channel aquaporin-4 (AQP4) and is a specific marker of NMOsd.<sup>2,3</sup> The anterior visual pathway (AVP), which includes the retinas and the pathway from the optic nerves to the lateral geniculate nuclei, is a frequent site of injury, as shown by the presence of optic

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Address correspondence to Dr Kawachi, Department of Neurology, Brain Research Institute, Niigata University, 1-757 Asahimachi, Chuo-ku, Niigata 951-8585, Japan. E-mail: ikawachi@bri.niigata-u.ac.jp

From the <sup>1</sup>Department of Neurology, Brain Research Institute, Niigata University, Niigata; <sup>2</sup>Department of Pathology, Brain Research Institute, Niigata University, Niigata; <sup>3</sup>Department of Neurosurgery, Brain Research Institute, Niigata University, Niigata; <sup>4</sup>Center for Integrated Human Brain Science, Brain Research Institute, Niigata University, Niigata; <sup>5</sup>Department of Ophthalmology, Niigata University, Niigata; <sup>6</sup>Department of Pathology, Niigata University Medical and Dental Hospital, Niigata; <sup>7</sup>Department of Medical Informatics, Niigata University Medical and Dental Hospital, Niigata; and <sup>8</sup>Department of Brain Disease Research, Shinshu University School of Medicine, Matsumoto, Nagano, Japan.

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neuritis (ON) during the course of NMOsd. Compared to MS, ON in NMOsd is characterized as follows: (1) visual loss is generally more severe in NMOsd<sup>4</sup>; (2) the occurrence of bilateral simultaneous ON is more suggestive of NMOsd<sup>5</sup>; and (3) optic coherence tomography (OCT) assessment in NMOsd demonstrates a thinner retinal nerve fiber layer (RNFL), suggesting more wide-spread axonal injury.<sup>6,7</sup>

In the optic nerves, chiasm, and tracts, expression of AQP4 is generally enriched in astrocytic endfoot membrane domains that face capillaries and the pia in the CNS.8 In the retinas, a striking feature of AQP4 expression is its polarized distribution in 2 types of retinal glia: (1) Müller cells, which are a specialized form of radial glia spanning the width of the retina from the inner limiting membrane to the outer limiting membrane, with the cell soma lying within the inner nuclear layer (INL); and (2) retinal astrocytes, which are mostly localized in the RNFL, which is composed predominantly of unmyelinated axons of retinal ganglion cells. However, little is known about the details of the pathology in the AVP, including the dynamics of AQP4 expression in Müller cells and astrocytes in NMOsd. Here we report that more severe and widespread neuroaxonal damage and unique dynamics of AQP4 in astrocytes/ Müller cells was prominent in the AVP and may be associated with visual function and prognosis in NMOsd by using neuro-ophthalmological, radiological, and pathological assessments.

# **Patients and Methods**

## Neuro-ophthalmological and Neuroradiological Assessments

We retrospectively reviewed the medical records of 79 Japanese patients (67 women, 12 men; 30 patients with NMOsd, 49 patients with MS) between 2000 and 2013 at the MS/NMO clinic in the Department of Neurology at Niigata University Hospital. Patients were included in the study according to these ocular criteria: (1) intraocular pressure <20mmHg; (2) absence of current or previous history of other ocular diseases, including glaucoma, retinal detachment, early age-related macular degeneration, other macular degeneration, and retinal vascular diseases; and (3) adequate clinical data on follow-up (Supplementary Table 1). AQP4 autoantibodies were measured in the sera of all patients using the method described in our previous reports.<sup>9,10</sup> We stringently defined the definite form of NMOsd as fulfilling all items of the 2006 NMO criteria,1 and limited NMOsd to either: (1) ON and seropositivity for AQP4 autoantibodies but without spinal cord involvement or (2) myelitis with seropositivity for AQP4 autoantibodies but without optic nerve involvement. As a disease control, MS was defined as clinically definite MS according to the International Panel criteria for MS,11 after excluding definite and limited NMOsd.

In NMOsd and MS patients, ON-affected eyes were defined as eyes with a previous clinical episode of ON or abnormalities in the AVP on magnetic resonance imaging (MRI) findings. ONunaffected eyes were defined as eyes with no history of ON, no abnormalities in the AVP on MRI findings, and no abnormalities in the visual field (VF).

Visual function testing was performed using visual acuity (VA; Landolt broken ring chart), VF (automatic perimetry; Humphrey; Carl Zeiss Meditec, Jena, Germany), and visualevoked potential (Neuropack X1; Nihon Kohden, Tokyo, Japan). OCT was performed on both eyes using Fourier/spectral-domain (F/SD)-OCT with an RTVue-100 instrument (Optovue, Fremont, CA) with software version A4.0.

To detect ON lesions with MRI, coronal-oblique fat-saturated dual echo fast spin-echo images with 3mm-thick slices, coronal-oblique 3-dimensional (3D) fast imaging employing steady-state acquisition with 0.8- to 1.0mm-thick slices, and coronal-oblique fat-saturated T1-weighted spin-echo images with 3mm-thick slices before and after administration of 0.1mmol/kg intravenous gadolinium were acquired using a 1.5T scanner (GE Medical Systems, Milwaukee, WI; Philips Achieve, Philips Medical Systems, Best, the Netherlands). The 3D double inversion recovery (DIR) images (repetition time = 5,500 milliseconds, echo time = 350 milliseconds, inversion time 1 (TI1) = 2,350 milliseconds; TI2 = 350 milliseconds, number of excitations = 2, echo train length = 208, measured voxel size =  $1.30 \times 1.31 \times 1.30$ mm) were acquired using a 1.5T scanner.

The present study was approved by the institutional review board of the Niigata University School of Medicine, Niigata, Japan. Written informed consent was obtained from all patients or their guardians.

## Neuropathological Assessments

This study was conducted on retina and optic nerve tissues from 13 patients with NMOsd (3 retinas, 13 optic nerves), 7 patients with MS (1 retina, 7 optic nerves), and 8 patients with other diseases (as controls; 4 retinas, 4 optic nerves; Supplementary Table 2). These individuals were different from those who underwent neuro-ophthalmological and neuroradiological assessments. One to 4 blocks of retinas and optic nerves were assessed per autopsy case. Four-micrometer-thick, paraffinembedded sections were obtained and stained with hematoxylin and eosin, Klüver-Barrera staining, and Bodian silver impregnation. Selected sections were immunostained with polyclonal or monoclonal antibodies (Supplementary Table 3).9,12,13 All NMOsd and MS lesions were classified according to lesion activity.<sup>14</sup> The area of Wallerian degeneration (Wld) was defined as an extensive loss of myelin and axons and profound gliosis with densely packed glial fibrillary acidic protein (GFAP) and AQP4 immunoreactivity (IR). For quantitative assessment, the content of mitochondria or channel proteins was determined as the volume occupied in axons using X-Y and X-Z confocal images, or as a percentage of the axonal volumes. Serial optical sections at intervals of  $0.49\mu m$  in the Z dimension were captured by using a LSM510 or LSM710 laser

TABLE 1. Clinical Characteristics of Neuro-ophthalmological Assessment		
Characteristic	NMOsd Patients, 25 Eyes, 28 Attacks	MS Patients, 22 Eyes, 25 Attacks
Episodes of anterior visual pathway involvement, No.ª	$1.1 \pm 0.4$	$1.2 \pm 0.4$
Spherical equivalent, diopters <sup>a</sup>	$-3.1 \pm 2.1$	$-2.1 \pm 2.5$
Optic nerve head involvement at ON attacks, No. (%)	10/28 (36)	4/25 (16)
Visual acuity analysis		
Visual acuity, logMAR, at ON attacks, median [range]	1.52 [0.73–3.00] <sup>b</sup>	0.30 [0–1.35] <sup>b</sup>
Severe visual loss at ON attacks, No. (%) <sup>c</sup>	20/28 (71) <sup>b</sup>	9/25 (36) <sup>b</sup>
Severe visual loss at 6 months after ON attacks, No. (%) <sup>c</sup>	9/28 (32) <sup>b</sup>	0/25 (0) <sup>b</sup>
Chronic progressive visual disturbance, No. (%) <sup>d</sup>	7/28 (25)	2/25 (8)
Visual field defects at ON attacks		
MD, dB <sup>a</sup>	$-16.4 \pm 9.1^{b}$	$-8.4 \pm 6.7^{b}$
PSD, dB <sup>a</sup>	$11.0 \pm 3.8^{b}$	$6.0 \pm 3.8^{b}$
CPSD, dB <sup>a</sup>	$10.6 \pm 4.6^{b}$	$4.9 \pm 2.7^{b}$
I. Neurologic abnormalities, No. (%)	7/28 (25)	1/25 (4)
I.1. Vertical step	0 (0)	1 (4)
I.2. Quadrant	0 (0)	0 (0)
I.3. Partial hemianopia	0 (0)	0 (0)
I.4. Hemianopia	3 (11)	0 (0)
I.5. Three quadrant	4 (14) <sup>b</sup>	$0 (0)^{b}$
II.1. Nerve fiber bundle abnormalities, No. (%)	5/28 (18)	3/25 (12)
II.1.a. Temporal wedge	0 (0)	0 (0)
II.1.b. Enlarged blind spot	0 (0)	1 (4)
II.1.c. Nasal step	1 (4)	0 (0)
II.1.d. Paracentral	1 (4)	0 (0)
II.1.e. Partial arcuate	0 (0)	0 (0)
II.1.f. Arcuate	0 (0)	2 (8)
II.1.g. Altitudinal	3 (11)	0 (0)
II.2. Diffuse abnormalities, No. (%)	0/28 (0)	6/25 (24)
II.2.a. Multiple foci	0 (0)	2 (8)
II.2.b. Widespread	0 (0)	4 (16)
II.3. Central abnormalities, No. (%)	4/28 (14) <sup>b</sup>	13/25 (52) <sup>b</sup>
II.3.a. Centrocecal	2 (7)	0 (0)
II.3.b. Central	2 (7) <sup>b</sup>	13 (52) <sup>b</sup>
II.4. Total loss of vision, No. (%)	12/28 (43) <sup>b</sup>	1/25 (4) <sup>b</sup>

## TABLE 1: Continued

Characteristic	NMOsd Patients, 25 Eyes, 28 Attacks	MS Patients, 22 Eyes, 25 Attacks
Artifactual abnormalities, No. (%)	0/28 (0)	1/25 (4)
Normal, No. (%)	0/28 (0)	0/25 (0)
VEP analysis at ON attacks		
VEP abnormalities, No. (%)	12/13 (92)	15/16 (94)
<sup>a</sup> Mean $\pm$ standard deviation.		

<sup>b</sup>Statistically significant difference between the linked values (p < 0.05).

<sup>c</sup>Severe visual loss was defined as visual acuity worse than 20/200 (1.0 logMAR).

<sup>d</sup>Chronic visual disturbance was defined as progressive abnormalities of visual acuity (+0.3 logMAR or more) and/or visual field for >2 months.

CPSD = corrected pattern standard deviation; MD = mean deviation; MS = multiple sclerosis; NMOsd = neuromyelitis optica spectrum disorder; ON = optic neuritis; PSD = pattern standard deviation; VEP = visual evoked potential.

confocal microscope (Carl Zeiss, Gottingen, Germany), and Imaris software 7.6.4 (Bitplane, Zurich, Switzerland) was used to generate the 3D reconstructed images.<sup>15</sup> Sections with long ( $\geq 20\mu$ m) axonal segments were identified based on an established morphological study and after minimizing identification of false-positive segments lacking mitochondrial elements because of the naturally discontinuous distribution of mitochondrial elements and the variability in the intermitochondrial distance within axons. To obtain these data, we quantified axonal morphology and the volume of mitochondria or channel proteins in 262 images (×100 objective) acquired from 2 to 6 fields of each area in 20 tissue blocks of the optic nerves from 8 NMOsd cases, 4 MS cases, and 5 controls.

### Statistical Analyses

Data analyses were performed using GraphPad Prism 5 software (GraphPad Software, San Diego, CA), IBM SPSS 19 (IBM, Chicago, IL), and Stata 11 software (StataCorp, College Station, TX). Comparisons of medians and proportions between NMOsd and MS groups were performed using the Mann–Whitney *U* test or the Fisher exact test. Statistical tests among  $\geq$ 3 subgroups were performed using analysis of variance, the Kruskal–Wallis *H* test, or the chi-square test. Multiple comparisons among subgroups were performed using the Bonferroni or Bonferroni– Dunn tests. Generalized estimating equation (GEE) models, accounting for the history of ON, age, and the effects of withinpatient and intereye correlations, were used to determine the

TABLE 2. Clinical Characteristics of Neuroradiological Assessment: Contrast-Enhanced Magnetic Resonance Imaging Analysis of the Optic Nerves, Chiasma, and Tracts at the Time of ON Attack

Characteristic	NMOsd Patients, 21 Eyes, 28 Attacks	MS Patients, 18 Eyes, 21 Attacks
Optic perineuritis, No. (%)	13/28 (46)	6/21 (29)
Simultaneous bilateral involvement, No. (%)	5/28 (18)	2/21 (10)
Distribution of abnormal enhancement during ON attacks, No. (%)		
Nerve head segment involvement	0/28 (0)	0/21 (0)
Orbital segment involvement	24/28 (86) <sup>a</sup>	10/21 (48) <sup>a</sup>
Canalicular segment involvement	21/28 (75)	13/21 (62)
Intracranial segment involvement	9/28 (32)	6/21 (29)
Optic chiasm involvement	1/28 (4)	2/21 (10)
Optic tracts involvement	1/28 (4)	1/21 (5)
<sup>a</sup> Statistically significant difference between the linked values ( $p < 0.05$ ). MS = multiple sclerosis; NMOsd = neuromyelitis optica spectrum disorder; ON = optic neuritis.		

Distribution of Abnormal Intensity Lesion	NMOsd Patients, 13 Eyes, 36 Attacks	MS Patients, 18 Eyes, 41 Attacks
Nerve head segment involvement, No. (%)	0/13 (0)	0/18 (0)
Orbital segment involvement, No. (%)	13/13 (100)	17/18 (94)
Canalicular segment involvement, No. (%)	12/13 (92)	11/18 (61)
Intracranial segment involvement, No. (%)	10/13 (77) <sup>a</sup>	5/18 (28) <sup>a</sup>
Optic chiasm involvement, No. (%)	9/13 (69) <sup>a</sup>	3/18 (17) <sup>a</sup>
Optic tracts involvement, No. (%)	6/13 (46)	2/18 (11)
<sup>a</sup> Statistically significant difference between the linked values ( $p < 0.05$ ). MS = multiple sclerosis; NMOsd = neuromyelitis optica spectrum disorder.		

TABLE 3. Clinical Characteristics of Neuroradiological Assessment: Magnetic Resonance Imaging Double Inversion Recovery Analysis of the Optic Nerves, Chiasma, and Tracts in Remission

relationship with clinical parameters, including neuroophthalmological and neuroradiological assessments (Tables 1–3; Fig 1). For all GEE analyses, age was controlled in the models, because this variable is an important factor in visual function and RNFL thickness.<sup>16</sup> Cumulative probabilities of the followup visual performance as assessed with time to recovery of VA with 3 lines change from the nadir of ON attacks or to recovery of VA to 20/25 (0.1 logMAR) were estimated by the Kaplan– Meier method, and the significance test among curves of cumulative probabilities and survival rates was performed by the logrank test. All statistical analyses were considered significant at *p* values of <0.05.

# Results

# Neuro-ophthalmological Features of NMOsd at the Time of ON Attacks

Of the 158 eyes that met the inclusion criteria in the series, 31 of 60 eyes (20 of 30 patients, 67%, 56 attacks) with NMOsd and 41 of 98 eyes (26 of 49 patients, 53%, 57 attacks) with MS were diagnosed as having AVP involvement.

For the neuro-ophthalmological assessments at the time of ON attacks, eyes were excluded<sup>17</sup> if the affected eyes had a prior ON attack and vision did not return to 20/20 (0 logMAR), mean deviation (MD) < -2.00dB at a prior ON attack, or if optic disk pallor, a known baseline-corrected VA < 20/40 (>0.3 logMAR), or VF loss due to any other ophthalmic disorder was present, and then we evaluated each attack of ON-affected eyes with NMOsd (25 eyes, 28 attacks) and MS (22 eyes, 25 attacks; see Table 1). Among ON-affected eyes with NMOsd and MS, the mean (standard deviation [SD]) number of episodes of AVP involvement in eyes with NMOsd and MS was 1.1 (0.4) and 1.2 (0.4), respectively. In the VA analysis, the median VA (logMAR) at the time of ON attacks was significantly impared. The frequency of eyes with

severe visual loss, which was defined as VA worse than 20/ 200 (1.0 logMAR), at onset was significantly higher in eyes with NMOsd compared with those with MS. In the VF analysis, the VF defects indicated by the average VF-MD, pattern SD, and corrected pattern SD were significantly impaired in eyes with NMOsd compared with those with MS. VF morphological analysis using the Optic Neuritis Treatment Trial classification<sup>18</sup> demonstrated that "neurologic abnormalities (I)" and "total loss of vision (II.4)" were prominent in ON-affected eyes with NMOsd, whereas "central abnormalities (II.3)" were evident in those with MS. "Altitudinal abnormalities (II.1.g)" were present in 3 of 28 (11%) attacks with NMOsd, but 0 of 25 (0%) attacks with MS.

Intriguingly, 7 of 28 (25%) attacks in NMOsd and 2 of 25 (8%) attacks in MS were associated with chronic progressive visual deterioration, which was defined as progressive abnormalities in VA (+0.3 logMAR or more) and/or VF for >2 months.

# Neuroradiological Features on Enhanced MRI in NMOsd at the Time of ON Attack

For the neuroradiological assessments at the time of ON attacks, we evaluated the AVP using enhanced T1-weighted images with fat suppression. Of 34 patients (17 patients with NMOsd; 17 patients with MS) who underwent MRI examination at ON attack, abnormal enhancement was present in 16 of 17 (94%) patients (21 eyes, 28 attacks) with NMOsd, and in 16 of 17 (94%) patients (18 eyes, 21 attacks) with MS (see Table 2; Fig 1). MRI findings demonstrated that the length of abnormally enhanced lesions in NMOsd patients was significantly longer than those in MS patients, suggesting that longitudinally extensive lesions were prominent in the optic nerves in NMOsd. During attacks, radiologic



FIGURE 1: Radiological profiles of optic neuritis (ON) at the time of attacks in neuromyelitis optica (NMO) spectrum disorder (NMOsd) and multiple sclerosis (MS). Anterior visual pathways (AVP) during ON in NMOsd and MS were examined by using axial and coronal views of enhanced T1-weighted magnetic resonance images with fat suppression. (A) They were divided into 6 segments: head, orbital, canalicular, and intracranial segments of the optic nerves, the optic chiasm, and the optic tracts. Abnormal enhancement on fat-suppressed magnetic resonance imaging (MRI; arrows) was present in patients with NMOsd and MS. (B) The length of abnormally enhanced lesions in the AVP in NMOsd at the time of ON attacks (n = 28 events) was significantly longer than in MS attacks (n = 21 events). The pattern of abnormal enhancement in the AVP at the time of ON attacks was generally homogeneous in most NMOsd and MS patients (A). However, the pattern of optic nerve sheath enhancement, which is referred to as a "tram track sign" on axial views and a "doughnut sign" on coronal views, was also present in 46% of attacks in NMOsd patients and 29% of attacks in MS patients (A). This was defined as optic perineuritis (OPN).<sup>19</sup> The pathologic evidence (see Fig 4) indicated that OPN was associated with more widespread meningeal and perineural inflammation around the optic nerves. (C, D) Three-dimensional (3D) double inversion recovery (DIR) MRI detected accumulation of archival ON lesions in NMOsd and MS. DIR MRI simultaneously suppresses the signals from white matter and cerebrospinal fluid (CSF). A substantial increase in cortical demyelinating lesions in patients with MS was found compared to more conventional sequences, including fluid-attenuated inversion recovery (FLAIR) imaging.<sup>49</sup> Therefore, not only cortical demyelinating lesions but also ON lesions, which usually contact the CSF, were detected with high sensitivity by using DIR imaging (C, arrows) compared to FLAIR imaging in ON lesions in NMOsd and MS. The distribution of abnormal intensity on 3D-DIR images showed accumulation of archival lesions through the disease course, based on longitudinally evaluated MRI data. A representative case is shown in D. A 38-year-old woman experienced 3 attacks of acute ON and 6 attacks of myelitis (M) during the 12 years after disease onset. The distribution of abnormal intensity on 3D-DIR images 12 years after disease onset (\*4, red, blue, and yellow arrowheads) equaled the addition of each abnormally enhanced lesion in 3 attacks of ON (+1, red arrowheads, 6 years after onset; ★2, blue arrowheads, 7 years after onset; ★3, yellow arrowheads, 11 years after onset; D). Gd T1WI = gadolinium-enhanced T1-weighted image; Lt. = left eye; Rt. = right eye.

	NMOsd Patients		MS Patients	
Characteristic	Unaffected Eyes, 14 Eyes	Affected Eyes, 8 Eyes	Unaffected Eyes, 18 Eyes	Affected Eyes, 8 Eyes
Mean RNFL thickness				
Mean RNFL, $\mu m^a$	$95.9 \pm 9.4$	$63.9 \pm 10.2$	$96.5 \pm 9.7$	$78.9 \pm 9.5$
Within normal limits, No. (%)	11/14 (79)	0/8 (0)	11/18 (61)	1/8 (13)
Borderline, No. (%)	1/14 (7)	0/8 (0)	5/18 (28)	0/8 (0)
Outside normal limits, No. (%)	2/14 (14)	8/8 (100)	2/18 (11)	7/8 (88)
16-sector RNFL analysis				
Average number of sectors with borderline or outside normal limits <sup>a</sup>	2.2 ± 2.5	13.3 ± 4.1	2.1 ± 2.3	9.9 ± 4.9
Mean GCC thickness				
Mean GCC, $\mu m^a$	87.3 ± 12.5	$60.4 \pm 11.0$	$89.2 \pm 5.8$	$70.1 \pm 7.9$
Within normal limits, No. (%)	9/14 (64)	0/8(0)	13/18 (72)	0/8 (0)
Borderline, No. (%)	0/14 (0)	0/8 (0)	2/18 (11)	1/8 (13)
Outside normal limits, No. (%)	5/14 (36)	8/8 (100)	3/18 (17)	7/8 (88)
<sup>a</sup> Mean $\pm$ standard deviation.				

### TABLE 4. Clinical Characteristics of OCT Assessment: OCT Analysis of the Retinas in Remission

GCC = ganglion cell complex; MS = multiple sclerosis; NMOsd = neuromyelitis optica spectrum disorder; OCT = optical coherence tomography; RNFL = retinal nerve fiber layer.

characteristic features of optic perineuritis (OPN)<sup>19</sup> were marked in NMOsd patients (13 of 28 [46%] attacks) compared to MS patients (6 of 21 [29%] attacks), but the difference was not significant. Some patients with NMOsd and MS retained abnormal enhancement with the OPN pattern for >4 weeks.<sup>20</sup> Five of 28 (18%) attacks in NMOsd and 2 of 21 (10%) attacks in MS simultaneously had bilateral optic nerve involvement.

### Neuroradiological Features on 3D-DIR MRI in NMOsd in Remission

Based on longitudinally evaluated MRI data, the distribution of abnormal intensity on 3D-DIR images showed accumulation of archival lesions throughout the disease course (see Fig 1C, D). In remission, 3D-DIR MRI assessments were performed in 14 patients (28 eyes) with NMOsd and 28 patients (56 eyes) with MS. Thirteen of 28 (46%) eyes with NMOsd and 18 of 56 (32%) eyes with MS had abnormal intensities in the AVP. The numbers of segments with optic chiasma and intracranial involvement were significantly higher in NMOsd patients compared to MS patients (see Table 3).

### OCT Analysis of Retinas with NMOsd in Remission

The cohort that underwent OCT assessment included 11 patients with NMOsd (4 with the definite form and 7 with the limited form; 22 eyes; 14 ON-unaffected eyes and 8 ON-affected eyes) and 13 patients with MS (26 eyes; 18 ON-unaffected eyes and 8 ON-affected eyes; Tables 4 and 5; Fig 2). In NMOsd and MS patients, the RNFL and ganglion cell complex (GCC) were significantly thinner in ON-affected eyes than in ONunaffected eyes. For the ON-affected eyes, RNFL was significantly thinner in NMOsd patients than in MS patients.

When damage was classified as "borderline" and "outside normal limits" for the RNFL or GCC thickness, the frequency of ON-unaffected eyes with RNFL thinning was 21% in NMOsd patients and 39% in MS patients, and the frequency of ON-unaffected eyes with GCC thinning was 36% in NMOsd patients and 28% in MS patients (see Table 4). In patients with the limited form of NMOsd with myelitis but no history of ON, the frequency of ON-unaffected eyes with RNFL thinning was 30%, and the frequency of ONunaffected eyes with GCC thinning was 40% (see Table 5). These data suggest that RNFL and GCC loss was evident in ON-affected and ON-unaffected eyes, and not only secondary retrograde changes after ON, but also in vivo primary neuroaxonal pathology, regardless of ON history, were present in retinas in NMOsd and MS.

TABLE 5. Clinical Characteristics of OCT Assessment:OCT Analysis of the Retinas in Limited Formof NMOsd with Myelitis Patients in Remission		
Characteristic	Limited Form of NMOsd with Myelitis Patients, Unaffected Eyes, 10 Eyes	
Mean RNFL thickness		
Mean RNFL, $\mu m^a$	$94.0 \pm 10.4$	
Within normal limits, No. (%)	7/10 (70)	
Borderline, No. (%)	1/10 (10)	
Outside normal limits, No. (%)	2/10 (20)	
16-sector RNFL analysis		
Average number of sectors with borderline or outside normal limits <sup>a</sup>	2.5 ± 2.8	
Mean GCC thickness		
Mean GCC, $\mu m^a$	87.3 ± 11.2	
Within normal limits, No. (%)	6/10 (60)	
Borderline, No. (%)	0/10 (0)	
Outside normal limits, No. (%)	4/10 (40)	
<sup>a</sup> Mean $\pm$ standard deviation. GCC = ganglion cell complex; NMOsd = neuromyelitis optica spectrum disorder; OCT = optical coherence tomog- raphy: RNFL = retinal nerve fiber layer.		

# Follow-up Visual Performance in NMOsd

We evaluated the follow-up visual performance from the nadir of ON attacks in NMOsd and MS (Fig 3, Table 1). The frequency of eyes with severe visual loss, which was defined as VA worse than 20/200 (1.0 logMAR), at 6 months after ON onset was significantly higher in eyes with NMOsd compared with those with MS (see Table 1). Kaplan-Meier analysis of time to recovery of VA with 3 lines change from the nadir of ON attacks or to recovery of VA to 20/25 (0.1 logMAR) was performed. Patients with NMOsd developed a significantly worse outcome regarding visual performance over time than those with MS when we evaluated all cumulative attacks of ON in patients with NMOsd and MS or all first attacks of ON in each patient with NMOsd and MS (data not shown). Mean follow-up times between the ON onset and assessment were 11.5 months in NMOsd patients (25 attacks) and 9.2 months in MS patients (13 attacks) or 11.5 months in NMOsd patients (25 attacks)

and 9.4 months in MS patients (14 attacks). All patients with NMOsd and MS in the analysis were treated with methylprednisolone pulse treatment (1g per day) for 3 days and/or high-dose oral prednisolone treatment (1.0mg/kg per day) followed by prednisolone tapered to maintenance level. No patients in this study were treated with plasma exchange.

## Astrocytopathy with Increased Meningeal Inflammation in Plaques of the Optic Nerves, Chiasm, and Tracts in NMOsd

Clinically, 85% (11 of 13) of NMOsd cases and 86% (6 of 7) of MS cases had a previous history of ON. Pathologically, all cases in NMOsd and MS had several plaques in the AVP (Fig 4). The pathology in the AVP in NMOsd cases was evaluated in 22 lesion areas, 13 areas of periplaque white matter (PPWM), 11 areas of normal-appearing white matter (NAWM), and 10 areas of Wld. Similarly, in cases with MS, we evaluated 14 lesion areas, 11 areas of PPWM, and 11 areas of NAWM. All plaques in the optic nerves in NMOsd cases showed hallmarks of NMO pathology, including pattern-specific loss of AQP4 IR with thickened hyalinized blood vessels.9,21 Moreover, thickening of the perioptic meninges with inflammatory infiltration including CD45RO<sup>+</sup> T cells and Iba-1<sup>+</sup> cells was prominent in the meninges adjacent to the plaques in NMOsd cases. The meningeal infiltrates also extended into the pial septa and parenchyma of the optic nerves (see Fig 4). Therefore, lesions in the AVP in NMOsd were pathologically defined as ON and/or OPN with meningeal inflammation, consistent with radiological findings (see Fig 1A).<sup>19</sup>

### Severe axonal damage with abnormal mitochondrial dynamics and aberrant expression of the TRPM4 cation channel in the optic nerves, chiasm, and tracts in NMOsd

Axonal pathology was qualitatively and quantitatively assessed with Bodian silver impregnation, and immunohistochemistry for phosphorylated neurofilaments (SMI-31) and amyloid precursor protein. Axonal damage, including axonal loss, transections, swellings, and distorted axons, and axonal spheroids were evident in plaques and PPWM, and had accumulated toward the plaque center of the AVP in NMOsd and MS (Figs 5-7). The axonal damage was the most prominent in plaques and areas of Wld in NMOsd. The density of SMI-31<sup>+</sup> axons was significantly reduced, and the percentages of swollen axons and axonal spheroids were significantly increased in plaques, PPWM, and Wld in NMOsd, and plaques, PPWM, and NAWM in MS compared to controls. Swollen axons and axonal spheroids with no significant reduction in axonal density were also present, even in the NAWM in NMOsd.



FIGURE 2: Optical coherence tomography (OCT) analysis in patients with neuromyelitis optica (NMO) spectrum disorder (NMOsd) and multiple sclerosis (MS). (A, B) The data shown are representative of optic neuritis (ON)-affected eyes with retinal nerve fiber layer (RNFL) thinning (A) and ON-unaffected eyes with RNFL thinning (B) in NMOsd and MS patients. In the 16sector RNFL analysis, sectors with a classification of "within normal limits" (ie, sectors for which the probability of no damage  $\geq$  5%) are indicated in green, sectors with a "borderline" classification (probability < 5 but  $\geq$  1%) are indicated in yellow, and sectors with a classification of "outside normal limits" (probability < 1%) are indicated in red. The 16-sector RNFL analysis (A) revealed that sectors with a classification of "outside normal limits" (red) and "borderline" (yellow) were present in the ONaffected eyes of NMOsd and MS patients. (C–E) The average thicknesses of the RNFL (C) and ganglion cell complex (GCC; E) were significantly decreased in the ON-affected eyes compared with the ON-unaffected eyes in patients with NMOsd and MS. The 16-sector RNFL analysis (D) revealed the average number of normal sectors with significantly decreased thickness in the ON-affected eyes compared with the ON-unaffected eyes in patients with NMOsd and MS. For the ON-affected eyes, RNFL was significantly thinner in NMOsd patients than in MS patients (C). Intriguingly, the 16-sector RNFL analysis (B) revealed that sectors with a classification of "outside normal limits" (red) and "borderline" (yellow) were present in the ON-unaffected eyes of NMOsd and MS patients. When damage was classified as "borderline" and "outside normal limits" for RNFL or GCC thickness, the frequency of ON-unaffected eyes with RNFL thinning was 21% in the NMOsd patients and 39% in the MS patients (see Table 4). The frequency of ON-unaffected eyes with GCC thinning was 36% in the NMOsd patients and 28% in the MS patients (see Table 4). These data suggest that RNFL and GCC loss is present and is independent of ON in NMOsd and MS. The peripapillary RNFL results were obtained based on Fourier/spectrum domain OCT using an RTVue-100 instrument in patients with NMOsd and MS. GCC scanning using the OCT system encompasses the following 3 layers in the retina: (1) the RNFL, which is composed of ganglion cell axons; (2) the ganglion cell layer, which is composed of ganglion cell bodies; and (3) the inner plexiform layer, which is composed of interlaced dendrites of ganglion cells and axons of bipolar cells with dendrites of amacrine cells (see Fig 8F). \*p < 0.05, \*\*\*p < 0.001. IN = inferonasal; IT = inferotemporal; Lt = left eye; NL = nasal lower; NU = nasal upper; Rt = right eye; SN = superonasal; ST = superotemporal; TL = temporal lower; TU = temporal upper.

Mitochondrial dynamics is an important contributor to damage and loss of axons in several neurodegenerative disorders.<sup>22</sup> We thus assessed the dynamics of voltage-dependent anion channel (VDAC)/porin-labeled mitochondria located in SMI-31<sup>+</sup> axons in NMOsd compared to mitochondria in MS and controls (see Fig



FIGURE 3: Follow-up visual performance in neuromyelitis optica (NMO) spectrum disorder (NMOsd) and multiple sclerosis (MS). We performed Kaplan–Meier analysis of time to (A) recovery of visual acuity (VA) with 3 lines change and (B) recovery of VA to 20/25 (0.1 logMAR) from the nadir of optic neuritis (ON) attacks in NMOsd and MS patients. Eyes were excluded if deterioration of VA with <3 lines change was present at presentation (A) or VA was >20/25 (<0.1 logMAR) at presentation (B). Patients with NMOsd developed significantly worse visual performance outcomes over time than those with MS, when we evaluated not only all accumulative attacks of ON in patients with NMOsd and MS, but also all first attacks of ON in each patient with NMOsd and MS (data not shown).

6). First, morphological analysis of VDAC-1<sup>+</sup> mitochondria showed that shortened, fragmented, or swollen mitochondria, indicating extensive fission of mitochondria and mitochondrial degeneration, accumulated within axons in all areas including plaques, PPWM, NAWM, and Wld in NMOsd and MS compared to controls. Abnormal morphological changes were more evident in axons in NMOsd compared to those in MS, and were more prominent in swollen axons compared to nonswollen axons in NMOsd and MS. Second, compared to controls, quantitative analysis of the mitochondrial volume in axons demonstrated that the VDAC-1<sup>+</sup> mitochondrial content in SMI-31<sup>+</sup> axons was significantly increased in swollen axons of PPWM and NAWM and in areas of Wld in NMOsd, but not in these areas in MS. The VDAC-1<sup>+</sup> mitochondrial content in the axons of active plaques (17.1%, 6.6; mean, SD) was increased compared to inactive plaques (6.1%, 3.4) in NMOsd. These qualitative and quantitative data suggest that the accumulation of mitochondria with abnormal morphology in axons was more extensive in NMOsd compared to MS and controls and was dependent on morphological changes in axons (swollen axons) and on the disease stage (active stage) from the lesion center to even NAWM in NMOsd (see Figs 5-7).

Consistent with the dynamics of degenerative mitochondria in axons, the axon-specific mitochondria docking protein, syntaphilin,<sup>23</sup> was also significantly increased in axons in plaques, PPWM, NAWM, and Wld in NMOsd and MS, suggesting immobile mitochondria or decreased mitochondrial movement within axons (see Fig 6C).

Next, to investigate the pathophysiological mechanisms of axonal damage in NMOsd, we assessed the transient receptor potential melastatin 4 (TRPM4). This protein crucially contributes to axonal damage, and pharmacological inhibition of TRPM4 using the antidiabetic drug glibenclamide reduces axonal degeneration in an animal model of MS, experimental autoimmune encephalomyelitis (EAE).<sup>24</sup> Quantification of axonal TRPM4 expression revealed significantly more TRPM4 IR in areas of plaques and Wld and in the swollen axons of PPWM and NAWM in NMOsd and MS compared to controls, in which we detected only a few TRPM4<sup>+</sup> axons (see Figs 6B and 7D, E). TRPM4<sup>+</sup> axons were more evident in swollen axons than in nonswollen axons in NMOsd and MS. Therefore, aberrant axonal accumulation of mitochondria with abnormal morphology and decreased movement, and abnormal axonal expression of TRPM4 extended over wider areas in NMOsd compared to MS.

### Neuroaxonal Damage with Müller Cell Pathology in the Retina in NMOsd

The retina is anatomically isolated and represents a unique unmyelinated model in which to study neurodegeneration and inflammation, as well as the distal effects of demyelination of optic nerves in inflammatory and demyelinating syndromes of the CNS. All retina samples from NMOsd autopsy cases showed severe RNFL thinning with an abundance of GFAP<sup>+</sup> and AQP4<sup>+</sup> astrocytes and neuronal loss of ganglion cells in the retina, suggesting the presence of secondary retinal retrograde degeneration after ON (Fig 8). The calcium-binding

A. Pattern-specific loss of AQP4 in the optic chiasma



FIGURE 4: Characterization of typical pathological findings of neuromyelitis optica (NMO) spectrum disorder (NMOsd) in optic nerves including pattern-specific loss of aquaporin-4 (AQP4) immunoreactivity (IR) in inflammatory lesions. (A, B) All patients with NMOsd in this study had typical pathological findings of NMOsd in optic nerves (*arrowheads*), including pattern-specific loss of AQP4 and glial fibrillary acidic protein (GFAP) IR and immunoglobulin deposits colocalizing with a product of complement activation, C9neo, in a vasculocentric pattern around thickened hyalinized vessels in inflammatory demyelinating lesions. Moreover, Iba-1<sup>+</sup> microglia, CD45RO<sup>+</sup> T cells, and CD20<sup>+</sup> B cells were abundant in inflammatory demyelinating lesions. In particular, thickening of the perioptic meninges with inflammatory infiltration including CD45RO<sup>+</sup> T cells and Iba-1<sup>+</sup> cells was prominent in the meninges adjacent to the plaques in NMOsd patients. (C) The meningeal infiltrates also extended into the pial septa and parenchyma of the optic nerves. HE = hematoxylin and eosin; KB = Klüver–Barrera; MBP = myelin basic protein.



FIGURE 5: Severe axonal damage in the optic nerves in neuromyelitis optica (NMO) spectrum disorder (NMOsd). Axonal pathology of the optic nerves in NMOsd and multiple sclerosis (MS) was qualitatively assessed with Bodian silver impregnation, and immunohistochemistry for phosphorylated neurofilaments (SMI-31) and amyloid precursor protein (APP). Axonal damage, including axonal loss, transections, swellings, and distorted axons, and axonal spheroids were evident in plaques and periplaque white matter (PPWM) and had accumulated toward each plaque center in the optic nerves in NMOsd and MS compared to control. Areas of Wallerian degeneration (Wld), which were defined by an extensive loss of myelin and axons, and profound gliosis with densely packed glial fibrillary acidic protein (GFAP) and aquaporin-4 (AQP4) immunoreactivity (IR), were present in NMOsd. Axonal damage, including axonal loss, transections, swellings, and distorted axons, and axonal spheroids were most prominent in plaques and areas of Wld in NMOsd and MS. All NMO and MS lesions were classified according to lesion activity, as previously described.<sup>14</sup> In brief, active plaques were characterized by macrophages with minor myelin products (cyclic nucleotide phosphodiesterase) or myelin basic protein (MBP) and profound inflammation, and inactive plaques were characterized by macrophages without minor or major myelin products within their cytoplasm. PPWM was defined as normal myelin, microglial activation, and few inflammatory infiltrates adjacent to the plaque. Additionally, PPWM in NMOsd included loss of AQP4 IR adjacent to the plaque.<sup>14</sup> Normal-appearing white matter (NAWM) was defined as myelin integrity and at least 1cm away from a plaque's edge.<sup>50</sup> Additionally, NAWM in NMOsd included preservation of AQP4 IR. \*Plaque. KB = Klüver–Barrera; neg = negative.

protein calbindin is a known marker of cone photoreceptors, horizontal cells, and subpopulations of bipolar cells and amacrine cells in the human retina. In the retina in NMOsd cases, we observed mild loss of calbindin<sup>+</sup> horizontal cells in the INL and unique dynamics of Müller cells including scattered loss of AQP4 IR in Müller cells



NMO FIGURE 6: Abnormal axonal mitochondrial dynamics and aberrant axonal expression of transient receptor potential melastatin 4 (TRPM4) cation channel in the optic nerves in neuromyelitis optica (NMO) spectrum disorder (NMOsd). (A) We assessed the dynamics of mitochondria labeled with voltage-dependent anion channel (VDAC)/porin (green) located in SMI-31 (blue)<sup>+</sup> axons in NMOsd compared to controls. Myelin basic protein (MBP; red)<sup>+</sup> myelinated and MBP<sup>-</sup> demyelinated axons were identified based on the lesion classification. Morphological analysis of VDAC-1<sup>+</sup> mitochondria showed shortened, fragmented, or swollen mitochondria, indicating extensive fission of mitochondria and mitochondrial degeneration. These mitochondria accumulated within axons in all areas including plaques, periplaque white matter (PPWM), normal-appearing white matter (NAWM), and Wallerian degeneration (Wld) in NMOsd and multiple sclerosis (MS) compared to controls (arrowheads in A). (B) We then assessed the dynamics of the TRPM4 cation channel (green) in SMI-31 (red)<sup>+</sup> axons in NMOsd and MS compared to controls. TRPM4<sup>+</sup> axons were more evident in areas of plaques and Wld and in the swollen axons of PPWM and NAWM in NMOsd and MS compared to controls, in which we detected only a few TRPM4<sup>+</sup> axons. (C) Consistent with the dynamics of degenerative mitochondria in axons (A), the axon-specific mitochondrial docking protein, syntaphilin (red), was also increased within SMI-31<sup>+</sup> axons (blue) in plaques, PPWM, NAWM, and Wld in NMOsd and MS, suggesting immobile mitochondria or decreased mitochondrial movement within axons. Therefore, aberrant accumulation of mitochondria with abnormal morphology and with decreased movement in axons was evident in the optic nerves in NMOsd and MS. Scale bars =  $50\mu$ m.

and mild to moderate loss of glutamine synthetase  $(GS)^+$  Müller cell soma in the INL. Iba-1<sup>+</sup> microglia were present in the inner retina in NMOsd cases; how-

ever,  $CD45RO^+$  T cells,  $CD20^+$  B cells, and perivascular deposition of complement, C9neo, were not observed in all retina samples from NMOsd cases (data not

shown). The retinas of MS cases showed severe RNFL thinning with an abundance of GFAP<sup>+</sup> and AQP4<sup>+</sup> astrocytes and neuronal loss of ganglion cells in the ganglion cell layer and calbindin<sup>+</sup> horizontal cells in the INL. However, retinas from MS cases showed no changes in AQP4 or GS IR in Müller cells. Reported evidence supports the involvement of AQP4 in retinal inflammatory responses,<sup>25</sup> retinal swelling,<sup>25</sup> and light-induced retinal neurodegeneration.<sup>26</sup> Moreover, optic nerve

transection in animal models results in RNFL thinning and ganglion cell loss but not in atrophy or degeneration of deep retinal layers including the INL.<sup>27</sup> If this is the case in the eyes of NMO patients, the Müller cells with unique dynamics observed in NMO patients may be related to primary retinal degeneration. These data suggest that the characteristics of the retinal pathology observed in NMOsd were strikingly distinct not only from the characteristics of the retinas in MS but also



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from myelitis or ON in NMOsd (see Fig 4), and the retina of NMOsd cases was characterized by primary as well as secondary neuronal and axonal loss with unique dynamics of Müller cells.

### Discussion

The optic nerves are the major site of involvement in NMOsd and have several distinguishing structures. First, the long, thin cylindrical dimension of the optic nerves with the cul-de-sac anatomy of the subarachnoid space may cause unique cerebrospinal fluid (CSF) dynamics, including restricted diffusion of proinflammatory elements (eg, AQP4 antibodies), retained inflammation, and limited clearance of myelin and axonal debris, followed by enhanced NMOsd lesion formation.<sup>28,29</sup> We revealed prominent pathological meningeal thickening with inflammation (see Fig 4) and radiologically abnormal enhancement of the OPN pattern in NMOsd (see Fig 1). Inflammatory processes may contribute to the closing of the arachnoid apertures that drain CSF into the meningeal lymphatics, thus adding to CSF compartmentalization.<sup>29</sup> Second, the absence of classical bloodbrain barrier (BBB) properties with nonspecific permeability of microvessels in the prelaminar region in the optic nerve head<sup>30</sup> may cause transmigration of AQP4 antibodies. Third, the unique structure of pial septa that partition each optic nerve fiber bundle from the adjoining glial limitans (a network of AQP4<sup>+</sup> astrocytic foot processes) may form an immunological barrier that prevents immune cells from entering the CNS parenchyma. Penetration of this barrier by encephalitogenic T lymphocyte requires the activity of matrix metalloproteinases,

which are produced by myeloid cells in an EAE model.<sup>31</sup> Thus, the presence of CD45RO<sup>+</sup> T cells and Iba-1<sup>+</sup> macrophages in the pial septa in NMOsd (see Fig 4) may increase the chance of breaking the barrier and promote effector responses for AQP4 on astrocytes. Finally, abundant large orthogonal arrays of particles (OAPs) in astrocytic endfeet of the optic nerves enhance binding of AQP4 antibodies and complement-dependent astrocyte damage.<sup>28</sup> These unique structures of the optic nerves may promote typical NMOsd lesions that show a pattern-specific loss of AQP4 with complement activation.

Importantly, we demonstrated that severe and widespread neurodegeneration with aberrant accumulation of degenerative mitochondria and TRPM4 channels was evident in the optic nerves in NMOsd. In the pathogenesis of MS, key elements that drive neurodegeneration and that can result in permanent clinical deficits<sup>32</sup> include accumulation of axonal mitochondrial damage, ion channel dysfunction including TRPM4, and involvement of Wld in axons of the CNS as follows.<sup>24,33,34</sup> First, axons are extremely susceptible to mitochondrial dysfunction, because the geometry of the neuron-axon unit presents a substantial challenge for efficient distribution of mitochondria and adenosine triphosphate (ATP) production within axons, which can reach 1m or more in length in humans.<sup>33</sup> "Fusion and fission" and an "increased number" of mitochondria play critical roles in maintaining functional mitochondria in the presence of environmental stress.<sup>22</sup> These dynamics of mitochondria fine-tune fundamental cellular processes such as calcium homeostasis and the generation of ATP and reactive

FIGURE 7: Quantitative analysis of axonal damage in the optic nerves in neuromyelitis optica (NMO) spectrum disorder (NMOsd). (A–D) Axonal pathology in the optic nerves in NMOsd was quantitatively assessed by immunohistochemistry for SMI-31 (A, B), voltage-dependent anion channel -1 (VDAC-1; C), and transient receptor potential melastatin 4 (TRPM4; D). Axonal damage was classified as normal axons (score 0; green), swollen axons with or without spheroids (score 1; yellow), and fragmented axons (score 2; red), as modified from a previous report<sup>35</sup> (B). The density of SMI-31<sup>+</sup> axons was significantly reduced (A), and percentages of swollen axons (score 1; yellow) and fragmented axons (score 2; red; B) were significantly increased in plaques and periplaque white matter (PPWM) in NMOsd and multiple sclerosis (MS) compared to controls. Swollen or fragmented axons with no significant reduction in axonal density were present in normal-appearing white matter (NAWM) in NMOsd, whereas both types of morphological changes and reduced axonal density were evident in NAWM in MS (A, B). Quantitative analysis of the mitochondrial volume in axons demonstrated that the VDAC-1 $^+$  mitochondrial content in SMI-31 $^+$  axons was significantly increased in swollen axons of PPWM and NAWM, and in areas of Wallerian degeneration (Wld) in NMOsd compared to controls (C). In contrast, quantitative analysis of the mitochondrial volume in axons demonstrated that the VDAC-1<sup>+</sup> mitochondrial content in SMI-31<sup>+</sup> axons was not increased in plaques, PPWM, or NAWM in MS (C). Quantification of axonal TRPM4 expression revealed significantly more TRPM4 immunoreactivity in areas of plaques and in swollen axons of PPWM and NAWM in NMOsd and MS compared to controls, in which we detected only a small proportion of TRPM4<sup>+</sup> axons (D). TRPM4<sup>+</sup> axons were more evident in swollen axons than in nonswollen axons in NMOsd and MS (D). (E) When the severity of axonal damage was compared between NMOsd and MS, more severe axonal damage, including swollen axons, increased axonal mitochondrial content, and aberrant TRPM4 cation channel expression, was prominent in plaques, PPWM, and NAWM in NMOsd compared to MS, except that reduced axonal density and swollen axons were more evident in NAWM in MS compared to NMOsd. These qualitative (see Figs 5 and 6) and quantitative (current figure) data suggest that the accumulation of mitochondria with abnormal morphology in the axons was more extensive in NMOsd compared to controls and depended on morphological changes in axons (swollen axons) and the disease stage (active stage) from the lesion center to NAWM in NMOsd. \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001, \*\*\*\*p < 0.0001; vs control in A–D, MS vs NMO in E.

oxygen species and consequently play important roles in apoptosis, mitophagy, and oxygen sensing.<sup>22</sup> Intriguingly, in the EAE model, focal axonal degeneration/swelling

(FAD) with focal intra-axonal accumulation of dysmorphic and swollen mitochondria, but with intact myelin sheaths, is the earliest ultrastructural sign of damage,



FIGURE 8.
which progresses to axonal fragmentation and demyelination.<sup>35</sup> These axonal changes that are consistent with FAD are present in acute MS lesions.<sup>35</sup> In addition, lack of mitochondrial respiratory chain complex IV activity in a proportion of  $Na^+/K^+$  ATPase  $\alpha$ -1<sup>+</sup> demyelinated axons supports axonal dysfunction as a contributor to neurological impairment in MS.36 Second, TRPM4 contributes crucially to inflammation-induced neurodegeneration including glutamate excitotoxicity, which is downstream of glutamatergic Ca<sup>2+</sup> influx and depends on calcium-activated TRPM4-mediated inward currents. This excitotoxicity mediates axonal swelling and neuronal cell death in the EAE model and MS.24 Third, Wld, which was traditionally thought to result from passive degeneration of axons due to a lack of cell body-derived nutrients,<sup>37</sup> is a prominent early feature of most neurodegenerative disorders, including amyotrophic lateral sclerosis. However, analysis of the slow Wallerian degeneration (Wld<sup>3</sup>) mutant mouse has recently elucidated that Wld is mediated by an active autodestruction program via axon death signaling pathways, including the Toll-like receptor adaptor SARM1.<sup>38</sup> Early during Wld, mitochondria swell, fragments accumulate at paranodes, and mitochondria lose their membrane potential.<sup>38</sup> In the EAE model, Wld<sup>S</sup> mutant mice show a modest attenuation of axonal loss, suggesting that EAE-associated axonal damage may occur by a mechanism that is partially similar to Wld.<sup>39</sup> In early MS, Wld is a major component of axonal pathology in plaques and PPWM.<sup>34</sup> Collectively, degenerative mitochondria, accumulation of TRPM4, and Wld may be mutually interrelated and mediate processes of severe neurodegeneration in MS and EAE. In this study, we showed that more severe and widespread neurodegeneration, including Wld with accumulation of degenerative mitochondria and TRPM4 channels, was evident in NMOsd compared to MS. These factors may contribute to clinical features such as the profound and persistent visual loss and chronic progressive deterioration seen in ON in NMOsd.

We here propose that the pathogenesis of the AVP in NMOsd may be caused by several neurodegenerative processes, including not only secondary anterograde (Wld) or retrograde changes after ON, but also in vivo primary neuroaxonal pathology via AQP4 antibodymediated pathology of astrocytes/Müller cells, regardless of ON history. The presence of neurodegeneration in AQP4-deficient regions including (1) myelin-preserved PPWM adjacent the optic nerve plaques and (2) the retina, which is physiologically devoid of myelin, in NMOsd may indicate that demyelination is not necessarily essential for processes of neurodegeneration in NMOsd. A marked reduction in the astrocytic Na<sup>+</sup>-

FIGURE 8: Retinal nerve fiber layer (RNFL) thinning and mild loss of calbindin<sup>+</sup> horizontal cells with unique dynamics of Müller cells in the retina of neuromyelitis optica (NMO) spectrum disorder (NMOsd). (F) The retina consists of 2 major parts: the retinal pigment epithelium and the neural retina. The neural retina can be divided into 9 layers: (1) photoreceptors (rods [r] and cones [c]); (2) external limiting membrane (attachment sites of adjacent photoreceptors and Müller cells [m]); (3) outer nuclear layer (ONL; nuclei of photoreceptors); (4) outer plexiform layer (OPL; axonal extensions of photoreceptors), which contains the middle limiting membrane (desmosomelike attachments of photoreceptor synapse expansions); (5) inner nuclear layer (INL; nuclei of bipolar [b], horizontal [h], amacrine [a], and Müller cells); (6) inner plexiform layer (IPL; mostly synapses of bipolar cells, ganglion cells [g], and amacrine cells); (7) ganglion cell layer (GCL); (8) RNFL (axons of ganglion cells); and (9) internal limiting membrane (basement membrane of Müller cells). In addition to Müller cells, astroglial (as) and microglial cells (mi) are found in the inner retina. In normal conditions, aquaporin-4 (AQP4) water channels are expressed by astrocytes within the RNFL and by Müller cell membranes especially within the RNFL and OPL, and the cell soma of Müller cells lies within the INL. The calcium-binding protein calbindin is a known marker of cone photoreceptors, horizontal cells, and subpopulations of bipolar cells and amacrine cells in the human retina. The human retina is devoid of myelin. Ganglion cell complex (GCC) scanning using a Fourier/spectrum domain optical coherence tomography system encompasses the 3 layers, including the RNFL, GCL, and IPL in the retina. We uncovered 3 important findings in NMOsd retinas. (A) First, all NMOsd and multiple sclerosis (MS) autopsy cases showed severe thinning of the RNFL with an abundance of glial fibrillary acidic protein (GFAP)<sup>+</sup> and AQP4<sup>+</sup> astrocytes, and neuronal loss in the GCL in the retina, suggesting the presence of secondary retinal degeneration after optic neuritis (ON). (B, D) Second, we observed scattered loss of AOP4 immunoreactivity (IR) in the INL, OPL, and ONL of Müller cells and mild to severe reduction in Müller cell nuclei with glutamine synthetase (GS)-IR-rich cytoplasm in the INL of NMOsd cases. Conversely, the GS<sup>+</sup> cell soma of Müller cells lay within the INL in control and MS retinas (arrowheads). (C) Third, all retinas in the NMOsd and MS patients showed mild loss of calbindin<sup>+</sup> horizontal cells (arrows) in the INL. (E) Schematic lesion maps of the retina in each case with NMOsd are shown. Lesions with loss of AQP4 IR in Müller cells in the INL, OPL, and ONL are indicated in red, lesions with loss of soma of GS<sup>+</sup> Müller cells in the INL are indicated in blue, and lesions with loss of calbindin<sup>+</sup> horizontal cells in the INL are indicated in green. All NMOsd patients showed mild neuronal loss in the INL with unique dynamics in Müller cells. Iba-1<sup>+</sup> microglia were present in the inner retina of NMOsd cases, but CD45RO<sup>+</sup> T cells, CD20<sup>+</sup> B cells, and perivascular deposition of complement, C9neo, were not observed (data not shown). These data suggest that the characteristics of the retinal pathology observed in NMOsd cases were strikingly distinct not only from those of the retina in MS cases but also from those of myelitis or ON in NMOsd cases (see Fig 4). Therefore, the retina in NMOsd cases was characterized by primary as well as secondary neuronal and axonal loss with unique dynamics of Müller cells. The data shown are representative of NMOsd, MS, and control retinas (A–D). HE = hematoxylin and eosin; I = INL; O = ONL; R = RNFL.

dependent excitatory amino acid transporter 2,<sup>40,41</sup> and downregulation of the NMDA-receptor subunit NR1,<sup>41</sup> are present in AQP4-deficient regions in NMOsd patients and a rat passive transfer model using AQP4 antibodies, even when myelin IR is preserved.<sup>41</sup> These data suggest that disruption of glutamate homeostasis<sup>40,41</sup> may play a role in primary neurodegeneration via glia– neuron interactions in NMOsd pathogenesis. Further research is required to investigate all aspects of NMOsd pathogenesis, including modulation of synaptic function/ network activity and/or myelin maintenance caused by astrocytopathy in NMOsd.

The basic effector mechanisms of autoantibody binding to its cellular target may be: (1) functional block of the target, (2) target internalization, 3) complementdependent cytotoxicity (CDC), and (4) antibodydependent cell-mediated cytotoxicity (ADCC) in autoimmune disorders. Previous studies in rodent models and in vitro assays have shown indirect evidence that CDC,<sup>42</sup> ADCC,<sup>43</sup> and internalization of AQP4 channels<sup>44</sup> may play important roles in NMOsd pathogenesis. CDC is the most important immune mechanism of AQP4 antibodies in NMOsd, because a hallmark of NMOsd pathology is pattern-specific loss of AQP4 IR on astrocytes with complement activation. This hallmark was prominent at 2 major sites, the optic nerves (see Fig 4) and spinal cord.<sup>9,21</sup> Conversely, the pathology in the retina (see Fig 8) and cortical gray matter<sup>12,45</sup> in NMOsd is distinct from that in the optic nerves and spinal cord. The retinal pathology in NMOsd was characterized by secondary retrograde degeneration of RNFL axons with gliosis (an abundance of AQP4<sup>+</sup> astrocytes) following ON, loss of horizontal cells in the INL, and scattered loss of AQP4 IR on Müller cells, but no deposition of complement in situ. The cortical gray matter pathology in NMOsd is characterized by substantial cortical neuronal loss in layers II, III, and IV with nonlytic reaction of AQP4-negative astrocytes in layer I, but no cortical demyelination nor deposition of complement.<sup>12</sup> Thus, the pathological processes in the retina and cortical gray matter in NMOsd may consist of an entirely different mechanism than CDC or ADCC in the spinal cord and optic nerves. These region-specific pathologies in NMOsd may depend on the following factors: (1) anatomical structures, including the glial framework and BBB integrity; (2) the complement regulators CD46, CD55, and CD59, which protect the brain from complement-mediated damage<sup>46</sup>; and (3) the cluster size of OAPs containing AOP4 tetramers, which is influenced by maturational differences in the ratio of the AQP4 M1 isoform to M23. OAPs contribute to effective complement activation on astrocytic membranes,44 because OAP assemblies are required for

AQP4 antibodies to recognize AQP4.<sup>47</sup> The binding of AQP4 antibodies may cause incomplete internalization of AQP4 into the endolysosomal compartment in astrocytes, followed by disruption of water and/or glutamate homeostasis with excitotoxic consequences for neurons in the retina and cortical gray matter.

This study has some limitations. The subjects who were evaluated for neuro-ophthalmological assessments were different from those who were pathologically examined, and the cohort of individuals who were pathologically assessed was small. Our study has intrinsic limitations owing to the limited availability of autopsied samples and the partial postmortem detachment that occurs in the retina in NMOsd, similar to the situation in previous reports describing MS eyes.<sup>48</sup> In the future, prospective, long-term follow-up and large cohort studies are needed to further characterize NMOsd pathogenesis.

In summary, our findings are consistent with a new concept in which severe neurodegeneration via abnormal AQP4 dynamics in astrocytes/Müller cells is prominent in NMOsd pathogenesis. Based on this study, future therapeutic strategies designed to limit the deleterious effects of not only AQP4 antibodies and damage to astrocytes but also neurodegeneration via glia-neuron interactions (eg, mitochondrial protection and replenishment strategies for degenerative axonal mitochondria<sup>33</sup>; TRPM4 inhibition strategies for accumulation of TRPM4<sup>24</sup>; and *Wld<sup>S</sup>*/nicotinamide mononucleotide adenylyltransferase-mediated axonal protection strategies for Wld<sup>38</sup>) are worthy of further investigation.

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# Author Contributions

M.H. and A.Y. contributed equally to this work. Conception and design of the study: M.N., I.K. Acquisition and analysis of data: all authors. Drafting the text or preparing the figures: M.H., A.Y., M.A., E.S., K.Y., M.N., I.K.

## **Potential Conflicts of Interest**

Nothing to report.

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**RESEARCH ARTICLE** 

# Effects of Alda-1, an Aldehyde Dehydrogenase-2 Agonist, on Hypoglycemic Neuronal Death

Tetsuhiko Ikeda<sup>1</sup>, Tetsuya Takahashi<sup>1</sup>, Mika Tsujita<sup>2</sup>, Masato Kanazawa<sup>1</sup>, Masafumi Toriyabe<sup>1</sup>, Misaki Koyama<sup>1</sup>, Kosuke Itoh<sup>2</sup>, Tsutomu Nakada<sup>2</sup>, Masatoyo Nishizawa<sup>1</sup>, Takayoshi Shimohata<sup>1</sup>\*

1 Department of Neurology, Brain Research Institute, Niigata University, Niigata, Japan, 2 Department of Center for Integrated Human Brain Science, Brain Research Institute, Niigata University, Niigata, Japan

\* t-shimo@bri.niigata-u.ac.jp

# Abstract

Hypoglycemic encephalopathy (HE) is caused by a lack of glucose availability to neuronal cells, and no neuroprotective drugs have been developed as yet. Studies on the pathogenesis of HE and the development of new neuroprotective drugs have been conducted using animal models such as the hypoglycemic coma model and non-coma hypoglycemia model. However, both models have inherent problems, and establishment of animal models that mimic clinical situations is desirable. In this study, we first developed a short-term hypoglycemic coma model in which rats could be maintained in an isoelectric electroencephalogram (EEG) state for 2 min and subsequent hyperglycemia without requiring anti-seizure drugs and an artificial ventilation. This condition caused the production of 4-hydroxy-2nonenal (4-HNE), a cytotoxic aldehyde, in neurons of the hippocampus and cerebral cortex, and a marked increase in neuronal death as evaluated by Fluoro-Jade B (FJB) staining. We also investigated whether N-(1,3-benzodioxole-5-ylmethyl)-2,6-dichlorobenzamide (Alda-1), a small-molecule agonist of aldehyde dehydrogenase-2, could attenuate 4-HNE levels and reduce hypoglycemic neuronal death. After confirming that EEG recordings remained isoelectric for 2 min, Alda-1 (8.5 mg/kg) or vehicle (dimethyl sulfoxide; DMSO) was administered intravenously with glucose to maintain a blood glucose level of 250 to 270 mg/dL. Fewer 4-HNE and FJB-positive cells were observed in the cerebral cortex of Alda-1-treated rats than in DMSO-treated rats 24 h after glucose administration (P = 0.002 and P = 0.020). Thus, activation of the ALDH2 pathway could be a molecular target for HE treatment, and Alda-1 is a potentially neuroprotective agent that exerts a beneficial effect on neurons when intravenously administered simultaneously with glucose.

# Introduction

Transient hypoglycemic episodes occur during treatment of diabetes mellitus with insulin or oral hypoglycemic drugs and in cases involving insulinoma, alcoholism, anorexia nervosa, or

others. Although many patients recover with appropriate treatment, transient hypoglycemic episodes can result in coma, seizures, and myriad other global and focal neurological deficits [1-3]. In an analogy to hypoxic encephalopathy, this syndrome has been termed hypoglycemic encephalopathy (HE). The only treatment for HE is blood glucose (BG) correction by glucose administration, and no neuroprotective drugs have been developed as yet.

Studies on the pathogenesis of HE and the development of neuroprotective drugs have been conducted using animal models. There are two conventional animal models of HE: one in which an isoelectric electroencephalogram (EEG) is maintained for a long period (hypoglycemic coma model) [4, 5] and the other involving euthanization or glucose administration before the isoelectric EEG (hypoglycemic non-coma model) [6-8]. In the hypoglycemic coma model, the isoelectric EEG manifesting after the BG decrease is maintained for at least 30 min to ensure that the brains are in a state of severe hypoglycemia. However, once changes in the brain due to hypoglycemia become apparent, seizures and respiratory arrest that cause additional neuronal damage are unavoidable. Model animals must therefore be treated with an anti-seizure drug and an artificial ventilator beforehand. Regarding a prolonged, markedly hypoglycemic state with an anesthetic agent and an artificial ventilator use, the pathogenesis of HE in animal models may differ from that of human HE experienced in clinical practice. However, the hypoglycemic non-coma model can also be problematic in that the various indices used by researchers as alternatives to isoelectric EEG, to indicate degrees of severity and courses, vary considerably, reflecting differences in individual responses to hypoglycemic loads. Thus, in order to elucidate the pathogenesis of HE and develop neuroprotective drugs, there is a need to establish animal models using isoelectric EEG as a quantitative index of hypoglycemic loads, without the use of anti-seizure drugs and an artificial ventilation.

Regarding the pathological conditions of HE, neuronal death in animal models is reportedly induced by oxidative stress generated after glucose administration [5, 9, 10]. This phenomenon is termed "glucose reperfusion injury", and its severity increases with higher BG levels after BG correction [10]. In this context, we recently confirmed higher BG levels after correction in 47 consecutive patients who experienced hypoglycemic episodes ( $271.1 \pm 128.5 \text{ mg/dL}$ ) [3]. Therefore, an animal model that experiences hypoglycemia followed by similar higher BG levels is preferred.

Studies on the development of neuroprotective drugs for HE have aimed to improve hypoenergetic conditions associated with hypoglycemia or to inhibit oxidative stress caused by glucose reperfusion injury. Among factors causing oxidative stress, 4-hydroxy-2-nonenal (4-HNE), a cytotoxic aldehyde that is a lipid oxidation product, has been intensively investigated in myocardial ischemia [11, 12]. It has been shown that 4-HNE is produced from  $\omega$ -6 polyunsaturated fatty acids, such as arachidonic acid, and can cause mitochondrial disorders to potentially induce neuronal death [13]. Interestingly, *N*-(1,3-benzodioxole-5-ylmethyl)-2,6-dichlorobenzamide (Alda-1), an aldehyde dehydrogenase 2 (ALDH2) agonist that increases the activity of mitochondrial ALDH2, inhibits myocardial damage caused by 4-HNE after ischemia [12, 14]. Therefore, this raises the possibility that Alda-1 may inhibit oxidative stress caused by glucose reperfusion injury via suppression of 4-HNE levels.

Based on the aforementioned observations, this study had the following three objectives: (1) to establish a novel animal model of HE that can be maintained in an isoelectric EEG state for a short period without the use of anti-seizure drugs and an artificial ventilation, enabling the isoelectric EEG to be used as an index of HE (we termed this model "a short-term hypoglycemic coma model"), (2) to confirm whether oxidative stress and neuronal death occur in this model, and (3) to reveal whether Alda-1 exerts protective effects against neuronal death associated with glucose reperfusion injury thereby demonstrating its potential as a neuroprotective drug.

## **Materials and Methods**

The study protocol was approved by the Niigata University Administrative Panel on Laboratory Animal Care. All operations concerning animals were performed according to ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines [15]. All efforts were made to minimize the number of animals used and their suffering. Alda-1 and vehicle (dimethyl sulfoxide, DMSO) were coded and administered by a blinded observer.

# EEG recordings

Male Sprague-Dawley rats (260-310 g, 8-10 weeks old, Charles River Japan, Inc.) were used. After sebum had been removed from both the shaved head and anterior neck regions by scrubbing with cotton balls soaked in alcohol, both areas were abraded with EEG abrasive gel (Skin Pure, Nihon Kohden Corporation). EEG disc electrodes (LEAD110A, BIOPAC Systems, Inc.) to which EEG disc electrode paste had been applied (Elefix, Nihon Kohden Corporation) were then attached to the forehead (near the nose) and the occiput (between the porus acusticus) of the rat, for use as EEG recording electrodes; the former served as the reference. Another disc electrode was also attached to the anterior neck to be used as the earth electrode. After the electrodes were attached, we confirmed with an alternating current (AC) resistance measuring instrument (Model 1089ES Checktrode, UFI Inc.) that the contact resistance between each electrode was 10 k $\Omega$  or less and that the variation in contact resistance at each electrode was 3 k $\Omega$  or less. An EEG amplifier (EEG100C, BIOPAC Systems, Inc.) was used for EEG amplification, and an analog-digital (A/D) converter (MP100System, BIOPAC Systems, Inc.) and waveform analysis software (AcqKnowledge, BIOPAC Systems, Inc., version 3.7.2) were used to collect and analyze the data. A bandpass of 0.1 to 100 Hz was used to filter the amplified EEG, and the sampling frequency of A/D conversion was 200 Hz.

## Short-term hypoglycemic coma model

The aforementioned rats were used. Each rat was fed a restricted diet of 20 g/day [16] for at least three consecutive days and then fasted, being allowed only water, for one day before the operation. To prevent changes in food consumption due to stress caused by an increase or decrease in the number of rats in each cage [17], the number per cage was fixed at three. The remaining amount of food was measured once a day before feeding to confirm that there were no changes in food consumption.

Hypoglycemic loads were applied under anesthesia maintained with a mixed gas of 70% nitrous oxide and 30% oxygen with 1.0% to 1.5% halothane added while rectal temperature was monitored. The rectal temperature was maintained at  $37.0 \pm 0.5$  °C with a temperature control mat, a fan, and a heating lamp. Under direct visualization using a surgical microscope, the left inguinal region was incised to expose the left femoral vein. A polyethylene catheter (Clay Adams Intramedic polyethylene tube PE50, Becton Dickinson & Co.) filled with 1% heparinized saline was then placed in the vein and used to collect blood samples for BG measurement. A BG monitoring instrument (Ascensia BREEZE 2, Bayer Health Care) was used for BG measurement. Next, the left cervical region was incised to expose the left anterior facial vein. Another tube, as described above, was placed in the vein and used to administer glucose and experimental drugs. After a resting EEG had been recorded, rapid-acting insulin (Novolin R 100 IU/mL Injection, Novo Nordisk Pharma Co., Ltd.) was intraperitoneally administered at a dose of 15 IU/kg [5, 18], and blood samples were then collected every 15 min. If the EEG did not become isoelectric by more than 180 min after insulin administration, the experiment was discontinued. After 2 or 10 min of isoelectric EEG state, 50% glucose was intravenously injected at a dose of 0.2 mL (Fig 1A). Then, 50% glucose was intravenously infused at a rate of





**Fig 1. Short-term hypoglycemic coma model.** (A) Protocol for hypoglycemic loading and induction of the hyperglycemic state. Rapid-acting insulin was intraperitoneally administered at a dose of 15 IU/kg. After 2 or 10 min of the presence of an isoelectric electroencephalogram (EEG), 50% glucose was intravenously injected at a dose of 0.2 mL and intravenously infused at a rate of 2.2 mL/h. Rats were sacrificed after 24 h of glucose reperfusion. (B) Alda-1 treatment protocol. After 2 min of an isoelectric EEG state, Alda-1 was administered intravenously at a dose of 8.5 mg/kg with 50% glucose (Alda-1 group) or the same dose of dimethyl sulfoxide (DMSO) was administered (control group).

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2.2 mL/h and again intravenously injected at a dose of 0.2 mL three times every 5 min. Furthermore, while blood samples were collected every 15 min, an additional 50% glucose solution was administered at doses of 0.2 mL if necessary, to maintain BG levels at the target of 250 to 270 mg/dL for 3 h to create a hyperglycemic state that would cause glucose reperfusion injury in HE. BG levels after correction by glucose administration were determined by reference to our clinical trials [3]. Disc electrodes and catheters were removed 3 h after the start of glucose administration.

#### Intravenous administration of an aldehyde dehydrogenase agonist

An ALDH2 agonist (Alda-1, Merck KGaA), an activator of aldehyde dehydrogenase, was dissolved in DMSO at a concentration of 15 mg/mL. At the end of the isoelectric EEG period, Alda-1 was administered to the Alda-1 group animals at a dose of 8.5 mg/kg with glucose into the left anterior facial vein, and the same dose of DMSO was administered to the control group (Fig 1B). The dose of Alda-1 was determined according to the optimal dosage described in previous reports of myocardial ischemia in rats [12, 14]. Alda-1 and DMSO were administered to randomly selected rats.

# Immunostaining for 4-HNE, a lipid oxidation product

Rats were euthanized with halothane 24 h after glucose administration. The left ventricle was perfused with 200 mL of cold saline and 100 mL of 4% paraformaldehyde (PFA) [19]. Removed whole brains were fixed in 4% PFA at 4°C for 12 h [20]. After rinsing with 0.1 M phosphate buffered saline (PBS) at 4°C overnight, brains were immersed in 15% sucrose/0.1 M PBS at 4°C overnight and then 30% sucrose/0.1 M PBS at 4°C for 24 h. Four coronal sections were collected from each animal starting 4.0 mm posterior to Bregma. They were frozen in isopentane, embedded in optimal cutting temperature (OCT) compound (Tissue-Tek, Sakura Finetek Inc.), and stored at -80°C. Brain samples were cut with a cryostat (Cryotome E Electronic Cryostat, Thermo Electron Co.) into sections of 20 µm thickness, placed on slides, and dried for 2 h. Samples were immersed in 0.3% hydrogen peroxide dissolved in absolute methanol for 30 min to inhibit peroxidase activity. After rinsing with PBS, sections were incubated with mouse monoclonal anti-4-HNE antibody (HNEJ-2, JalCA, 1:50) at 4°C overnight. The secondary antibody (#BA-2000, Vector Laboratories, Inc.) was applied at room temperature for 2.5 h. After incubation with an ABC Elite kit (Vectastain ABC, Vector Laboratories, Inc.), sections were developed in 3,3'-diaminobenzidine. Cerebral cortices (hippocampus level) from each rat were viewed using a light microscope (Eclipse E600W, Nikon Co.). The number of 4-HNE-positive cells was counted in five non-overlapping areas of 0.25 mm<sup>2</sup> in both parietal and temporal cortical sections, and findings were confirmed in triplicate. Cells were counted in both hemispheres by a blinded observer.

# Assessment of neuronal death by Fluoro-Jade B

The 25-µm sections were placed on slides, dried, then immersed in 0.1 M PBS, dried at 50°C or higher for 30 min, and immersed in 80% ethanol containing 1% sodium hydroxide. Next, samples were immersed in 70% ethanol solution for 2 min and in purified water for 2 min. After immersion in 0.06% potassium permanganate solution for another 10 min, samples were rinsed with purified water for 2 min. After immersion in degenerating neuron binding fluorescent derivative staining solution (Fluoro-Jade B [FJB], Histo-Chem Inc., 0.0004%) for 20 min, a 1-min rinse with purified water was repeated three times; samples were then dried at 50°C for 15 min. Next, samples were immersed in ethanol and xylene, dehydrated, and mounted with DPX (Fluka; Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany). Cerebral cortices (hippocampus level) from each rat were observed with a fluorescence microscope (excitation wavelength, 480 nm; fluorescence wavelength, 525 nm). The number of FJB-positive cells was counted in five non-overlapping areas of 0.25 mm<sup>2</sup> in both parietal and temporal cortical sections, and findings were confirmed in triplicate. Cells were counted in both hemispheres by a blinded observer.

# Statistical analysis

Results are expressed as means  $\pm$  standard deviation (SD). A *t*-test was used for statistical analyses. A *P* value < 0.05 was considered statistically significant.

# Results

# Changes in BG levels before and after insulin administration

We first measured BG levels of rats before and after insulin administration. The mean BG level before insulin administration was  $112.4 \pm 26.1 \text{ mg/dL}$  (Fig 2). After insulin administration, BG levels gradually decreased and stabilized around 20 mg/dL 60 min after insulin administration. Since their EEGs did not become isoelectric more than 180 min after insulin administration,



**Fig 2. Temporal changes in blood glucose levels after administration of insulin and glucose.** Blood glucose levels were measured every 15 min before and after injection of rapid-acting insulin (from -15 min to 120 min; *n* = 48) as well as after injection of 50% glucose (from 15 min to 180 min and 24 h; *n* = 9). doi:10.1371/journal.pone.0128844.g002

the experiment was discontinued in 3 of 30 rats to which glucose was to be administered 2 min after an isoelectric EEG state was observed (2-min isoelectric EEG group) and 4 of 48 rats to which glucose was to be administered after 10 min of an isoelectric EEG state (10-min isoelectric EEG group). After glucose administration, BG levels immediately increased and remained

in the 250 to 270 mg/dL range 30 min after the start of glucose administration and beyond.

# Changes in EEG and neurological findings

We next investigated EEG findings and neurological signs associated with hypoglycemia and glucose reperfusion (Fig 3). Resting EEGs before insulin administration exhibited amplitudes of 10 to 50  $\mu$ V and a frequency range of 4 to 8 Hz. There were no pupil abnormalities, and the light reflex was rapid. High-amplitude slow waves (amplitude, 100–200 µV; frequency, 0.1–4 Hz) occurred 69  $\pm$  29 min after insulin administration. At this time, BG levels were 21.4  $\pm$ 4.9 mg/dL, pupil diameter was increased, and the light reflex was slow. Mean EEG amplitude and frequency then both gradually decreased, and the EEG became isoelectric  $92 \pm 33$  min after insulin administration. At this time, BG levels were  $18.3 \pm 7.8$  mg/dL, pupils were dilated, and the light reflex disappeared. When glucose was administered after isoelectric EEG, the appearance of the EEG was similar to that at rest (recovering EEG). Following glucose administration, BG levels were  $245.8 \pm 92.7$  mg/dL, dilatation of the pupils disappeared, and the light reflex was rapid. Since pupil diameter and light reflexes were not maintained at a constant level during each phase, these findings were not suitable for predicting precise BG levels. Frequencies of seizure and subsequent death in the isoelectric EEG 2-min group were 6.7% (2/30) and 16.7% (5/30), respectively. By contrast, these were 100% (48/48) and 87.5% (42/48), respectively, in the 10-min isoelectric EEG group.



Time after insulin administration [min]	Blood glucose level [mg/dL]	EEG pattern		Neurological symptoms			
				Pupil size [mm]	Light reflex	Seizure %	
						Iso-EEG 2-min group	Iso-EEG 10-min group
N/A	112.4±26.1	Resting EEG (Before insulin administration)	mmmmmm	1.5-2.5 (Normal)	Brisk	0	0
69±29	21.4±4.9	High amplitude and low frequency EEG (Blood glucose falling period)	mmmm	2.0-5.0	Sluggish	0	o
92±33	18.3±7.8	Isoelectric-EEG (Severe hypoglycemia)		4.5-6.0 (Mydriasis)	Absent	6.7	100
117±53	245.8±92.7	Normalized EEG (After glucose infusion)	1 [sec] 200 [µV]	1.5-2.5 (Normal)	Brisk	0	22.9

Fig 3. Temporal changes in electroencephalogram findings and neurological signs. EEG; Electroencephalogram, N/A; not applicable. Data are means ± SD. Since pupil diameter and light reflexes were not maintained at a constant level during each phase, these findings were not suitable for predicting precise BG levels.

# Assessment of 4-HNE-positive cells in the short-term hypoglycemic coma model

The number of 4-HNE-positive cells was compared among the sham group (to which insulin was not administered), the 2-min isoelectric EEG group, and the 10-min isoelectric EEG group (Fig 4). There were no significant differences in body weight, food consumption, or BG levels before or after the experiment among the three groups. In the sham group, no 4-HNE-positive cells were detected in any of the brain regions examined. In the 2-min isoelectric EEG group, 4-HNE-positive cells were detected in the frontal to parietal-temporal cortex and in the hippocampus. The number of 4-HNE-positive cells in the hippocampus was less than that in the cerebral cortex. The frequency of 4-HNE-positive cells in the parietal-temporal cortex at the level of the hippocampus was significantly higher in the 10-min isoelectric EEG group than in the 2-min isoelectric EEG group (217.9 ± 27.1 cells/0.25 mm<sup>2</sup> vs. 104.8 ± 8.9 cells/0.25 mm<sup>2</sup>; P < 0.001).

# Assessment of FJB-positive cells in the short-term hypoglycemic coma model

The frequency of hypoglycemia-induced neuronal death, evaluated by FJB staining of the parietal-temporal cortex at the level of the hippocampus, was significantly higher in the 10-min isoelectric EEG without glucose reperfusion group than in the 2-min isoelectric EEG without glucose reperfusion group (109.9 ± 45.4 cells/0.25 mm<sup>2</sup> vs. 53.3 ± 15.9 cells/0.25 mm<sup>2</sup>; P = 0.009, S1 Fig).

Next, the number of FJB-positive cells was compared among the sham, 2-min isoelectric EEG, and 10-min isoelectric EEG groups (Fig 5). In the sham group, there were no FJB-positive cells in any of the brain regions examined. In the 2-min isoelectric EEG group, FJB-positive

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Fig 4. Distribution of 4-HNE-positive cells in the short-term hypoglycemic coma model. (A) Representative images of 4-HNE-positive cells in the sham group, the 2-min isoelectric EEG (iso-EEG) group, and the 10-min isoelectric EEG group. Higher magnification views of 4-HNE-positive cells are shown in the small frames. Scale bar: 100  $\mu$ m. (B) The number of 4-HNE-positive cells in the sham group, the 2-min isoelectric EEG group, and the 10-min isoelectric EEG group. Data are means ± SD (n = 15 fields). \*P < 0.001.

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cells were observed in the frontal to parietal-temporal cortex and hippocampus. The number of FJB-positive cells in the hippocampus was less than that in the cerebral cortex. The frequency of FJB-positive cells in the parietal-temporal cortex at the level of the hippocampus was significantly higher in the 10-min isoelectric EEG group than in the 2-min isoelectric EEG group (150.7  $\pm$  19.8 cells/0.25 mm2 vs. 89.9  $\pm$  6.8 cells/0.25 mm2; P < 0.001).

## Effects of Alda-1 on 4-HNE-positive cells

To examine the effects of Alda-1 on glucose reperfusion-associated 4-HNE expression, the number of 4-HNE-positive cells was examined in rats to which Alda-1 had been administered after 2 min of isoelectric EEG (Alda-1 group) and in rats to which DMSO, a solvent, had been administered (control group). There were no significant differences in body weight, food consumption, or BG levels between the two groups. The number of 4-HNE-positive cells was significantly lower in the Alda-1 group than in the control group (101.5 ± 5.6 vs. 92.9 ± 8.1 cells/ 0.25 mm<sup>2</sup>; P = 0.002) (Fig 6). We did not examine the effect of Alda-1 in the 10-min isoelectric EEG group because a large number of rats developed seizures before administration of glucose and Alda-1.





**Fig 5.** Fluoro-Jade B-positive cells in the short-term hypoglycemic coma model. (A) Representative images of Fluoro-Jade B-positive cells in the sham group, the 2-min isoelectric EEG (iso-EEG) group, and the 10-min isoelectric EEG group. Scale bar:  $100 \mu$ m. (B) The number of Fluoro-Jade B-positive cells in the sham group, the 2-min isoelectric EEG group, and the 10-min isoelectric EEG group. Data are means  $\pm$  SD (n = 15 fields). \*P < 0.001.

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#### Effects of Alda-1 on FJB-positive cells

Finally, to ascertain whether Alda-1 exerts protective effects against glucose reperfusion-associated neuronal death, the number of FJB-positive cells was compared in the Alda-1 and control groups. Notably, the number of FJB-positive cells was significantly lower in the Alda-1 group than in the control group ( $50.9 \pm 11.1 \text{ vs. } 41.1 \pm 6.3 \text{ cells}/0.25 \text{ mm}^2$ ; P = 0.020) (Fig.7).

## Discussion

In this study, we first attempted to develop a short-term hypoglycemic coma model that could be maintained in an isoelectric EEG state for a short period. The isoelectric EEG period was reduced from 30 min, which is the isoelectric period used in a conventional hypoglycemic coma model, to 2 min. We confirmed that seizures did not occur in this condition. The advantages of this model are as follows. (i) The degree of severity induced by the hypoglycemic load is relatively uniform, while measurements of the light reflex and pupil diameter lack objectivity and quantitativity. (ii) BG levels during the hyperglycemic state are determined on the basis of actual human clinical data [3]. (iii) This model does not require the use of anti-seizure drugs and an artificial ventilator. (iv) The severity of oxidative stress and neuronal death are quantifiable by measuring 4-HNE- and FJB-positive cells, respectively.





Next, we investigated the characteristics of glucose reperfusion injury using this model, and noted the following observations. (i) We found that the appearance of 4-HNE-positive cells in the brain even in the 2-min isoelectric EEG group. This suggests that the conditions present in the 2-min isoelectric EEG group are enough to cause glucose reperfusion injury. Since oxidative stress is not caused by hypoglycemic loads alone [7, 10], this raises the possibility that hyperglycemia associated with glucose administration causes glucose reperfusion injury. (ii) We observed fewer 4-HNE-positive cells in the 2-min isoelectric EEG group than in the 10-min isoelectric EEG group, although BG levels during the hyperglycemic state were similar. This finding suggests that the severity of glucose reperfusion injury may be influenced by the degree of severity of the hypoglycemic load-induced neuronal damage. However, to our knowledge, no reports have determined the effect of the severity of the hypoglycemic load on glucose reperfusion injury. Future studies need to address this issue. (iii) We observed fewer 4-HNE-positive cells in the cerebral cortex. Previous studies demonstrated similar findings in the non-coma model [7, 8], although the mechanism underlying the difference in 4-HNE production in different brain regions remains unknown.

Finally, we showed that administration of Alda-1 inhibits both the production of 4-HNE and neuronal death associated with glucose reperfusion injury, similar to previous reports in





which Alda-1 inhibits myocardial damage caused by 4-HNE after myocardial ischemia [12, 14]. Previous studies show that treatments devised to attenuate oxidative stress associated with glucose reperfusion injury may be therapeutic candidates, including therapeutic hypothermia [19], administration of nitric oxide synthase inhibitors [21], and nicotine adenine dinucleotide phosphate oxidase inhibitors [10]. In addition, Alda-1 may be a therapeutic candidate. Activation of ALDH2 by Alda-1 increases detoxification of reactive aldehydes, such as 4-HNE, a cytotoxic aldehyde that accumulates during glucose reperfusion injury as a by-product of ROS-induced lipid peroxidation [14]. Enhanced ALDH2 activity reduces 4-HNE protein adduct formation and increases cell survival. We suggest that activation of the ALDH2 pathway could be a molecular target for HE treatment, and that Alda-1 is a potentially neuroprotective agent that exerts a beneficial effect on neurons when intravenously administered simultaneously with glucose.

This study has a number of limitations. First, with regard to the FJB-positive cells in the 10-min isoelectric EEG group, the seizures associated with a prolonged hypoglycemic state may affect the results. Second, we did not investigate the effect of other neuroprotective drugs that inhibit oxidative stress on neuronal death. Future studies are required to determine the most suitable drug for treatment of glucose reperfusion injury.

In conclusion, we established a short-term hypoglycemic coma model. In this model, we confirmed both the appearance of 4-HNE-positive cells and neuronal death associated with glucose reperfusion injury. Furthermore, with this model we demonstrated that Alda-1 inhibits 4-HNE production and exerts a protective effect against neuronal death. Thus, Alda-1 may be a candidate neuroprotective agent for cases involving HE.

# **Supporting Information**

S1 Fig. Hypoglycemia-induced neuronal death evaluated by Fluoro-Jade B staining. (A) Representative images of Fluoro-Jade B-positive cells in the sham group, the 2-min isoelectric EEG (iso-EEG) without glucose reperfusion group, and the 10-min isoelectric EEG without glucose reperfusion group. Scale bar: 100  $\mu$ m. (B) The number of Fluoro-Jade B-positive cells in the sham group, the 2-min isoelectric EEG without glucose reperfusion group, and the 10-min isoelectric EEG without glucose reperfusion group, and the 10-min isoelectric EEG without glucose reperfusion group, and the 10-min isoelectric EEG without glucose reperfusion group. Data are means  $\pm$  SD (n = 15 fields). \*P = 0.009.

(TIF)

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# **Author Contributions**

Conceived and designed the experiments: TI TT TS. Performed the experiments: TI TT M. Kanazawa M. Toriyabe M. Koyama. Analyzed the data: TI TT M. Kanazawa TS. Contributed reagents/materials/analysis tools: M. Tsujita KI TN. Wrote the paper: TI MN TS.

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RESEARCH ARTICLE

# Reduced CSF Water Influx in Alzheimer's Disease Supporting the β-Amyloid Clearance Hypothesis

Yuji Suzuki<sup>1</sup>, Yukihiro Nakamura<sup>1</sup>, Kenichi Yamada<sup>1</sup>, Hironaka Igarashi<sup>1</sup>, Kensaku Kasuga<sup>2</sup>, Yuichi Yokoyama<sup>3</sup>, Takeshi Ikeuchi<sup>2</sup>, Masatoyo Nishizawa<sup>4</sup>, Ingrid L. Kwee<sup>5</sup>, Tsutomu Nakada<sup>1,5</sup>\*

Center for Integrated Human Brain Science, Brain Research Institute, University of Niigata, Niigata, Japan,
Department of Molecular Genetics, Brain Research Institute, University of Niigata, Niigata, Japan,

3 Department of Psychiatry, Faculty of Medicine, University of Niigata, Niigata, Japan, 4 Department of Neurology, Brain Research Institute, University of Niigata, Niigata, Japan, 5 Department of Neurology, University of California Davis, Davis, California, United States of America

\* tnakada@bri.niigata-u.ac.jp

# Abstract

# Objective

To investigate whether water influx into cerebrospinal fluid (CSF) space is reduced in Alzheimer's patients as previously shown in the transgenic mouse model for Alzheimer's disease.

# Methods

Ten normal young volunteers (young control, 21-30 years old), ten normal senior volunteers (senior control, 60-78 years old, MMSE  $\geq$  29), and ten Alzheimer's disease (AD) patients (study group, 59-84 years old, MMSE: 13-19) participated in this study. All AD patients were diagnosed by neurologists specializing in dementia based on DSM-IV criteria. CSF dynamics were analyzed using positron emission tomography (PET) following an intravenous injection of 1,000 MBq [ $^{15}$ O]H<sub>2</sub>O synthesized on-line.

# Results

Water influx into CSF space in AD patients, expressed as influx ratio,  $(0.755 \pm 0.089)$  was significantly reduced compared to young controls  $(1.357 \pm 0.185; p < 0.001)$  and also compared to normal senior controls  $(0.981 \pm 0.253, p < 0.05)$ . Influx ratio in normal senior controls was significantly reduced compared to young controls (p < 0.01).

## Conclusion

Water influx into the CSF is significantly reduced in AD patients.  $\beta$ -amyloid clearance has been shown to be dependent on interstitial flow and CSF production. The current study indicates that reduction in water influx into the CSF may disturb the clearance rate of  $\beta$ -amyloid, and therefore be linked to the pathogenesis of AD.



# G OPEN ACCESS

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### **Trial Registration**

UMIN Clinical Trials Registry UMIN000011939

### Introduction

The brain lacks conventional lymphatics for clearing interstitial fluid of solutes not absorbed across capillaries. The cerebrospinal flow (CSF) system has long been suggested to play a role equivalent to the systemic lymphatic system. Nevertheless, the role of CSF as the brain's lymphatic system has not received much attention. Recently, however, the classic CSF circulation theory where CSF is almost exclusively produced by choroid plexus is found to be incomplete. It is now understood that influx from the peri-capillary (Virchow-Robin) space into the CSF system, classically referred to as interstitial flow, plays a major role in CSF production [1–3]. Furthermore, a number of studies have now shown that this interstitial flow plays a critical role in the clearance of  $\beta$ -amyloid [4–8]. Prealbumin (transthyretin) in the CSF has been identified to play the role of chaperon to  $\beta$ -amyloid, and prevents  $\beta$ -amyloid's natural tendency to aggregate and form plaques [9]. It is highly conceivable, therefore, that disturbance of interstitial flow may play a significant role in the pathogenesis of senile plaque (SP) formation.

Water homeostasis of the Virchow-Robin space and, therefore, interstitial flow is regulated by aquaporin-4 (AQP-4) [2,3,10], an isoform of water channels abundant in the brain. Using JJ vicinal coupling proton exchange (JJVCPE) imaging [11,12], a novel non-invasive magnetic resonance imaging (MRI) method capable of tracing exogenously applied substrates in a manner similar to positron emission tomography (PET), we had previously demonstrated that water influx into the CSF system is significantly reduced in senile plaque bearing transgenic AD model mice to the extent similar to AQP-4 knockout mice [12,13]. Using PET, we confirmed in this study that water influx into CSF is also reduced in AD patients.

## **Materials and Methods**

#### Subjects

Ten normal young volunteers (10 males, ages 21-30 years), ten normal senior volunteers (7 males and 3 females, ages 60-78 years), and ten Alzheimer's disease (AD) patients (6 males and 4 females, ages 59-84 years) participated in this study. Participant recruitment was completed between October 1, 2013, and April 4, 2014 (Fig 1). All volunteers were free of any significant medical conditions such as hypertension, diabetes, chronic pulmonary diseases, and were not taking any prescribed, over the counter, or herbal medications. AD patients were recruited from the Memory Clinic, Niigata University Hospital. The diagnosis of AD was determined based on DSM-IV criteria by neurologists specializing in dementia. The neurologists further determined whether the potential subject had decision making capacity to consent for the study. Age-matched senior volunteers were assessed to have no functional and no cognitive impairment (Mini-Mental State Examination (MMSE) score  $\geq$ 29), and had no neurological disease. In compliance with the Institutional Review Board of University of Niigata, the study protocol was explained in detail to all potential subjects, or their proxy where appropriate. Written informed consent was obtained from all subjects or, where the Alzheimer's subject was deemed to have no decision making capacity to consent for the study, by his/her proxy. All imaging studies were performed between October 2013 and April 2014. The study was approved by the Ethics Review Board of the Internal Review Board of University of Niigata. The project



# **CONSORT 2010 Flow Diagram**



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was registered at the UMIN Clinical Trials Registry as UMIN000011939 (http://www.umin.ac. jp/ctr/index.htm). Participants were provided with contact names and telephone numbers in the event of any adverse event related to the study. All participants were followed up within one month of the study to further confirm the absence or occurrence of adverse events related to the study, not otherwise self-reported by the study participant or proxy to the study coordinator. Alzheimer's patients are additionally followed up annually at the Memory Clinic.

# **PET** imaging

PET imaging was performed using a combined PET/CT scanner (Discovery ST Elite, GE Healthcare, Schenectady NY, USA) with a 15 cm field of view (FOV) positioned in the region of the cerebrum. To correct for photon attenuation, low-dose CT imaging was acquired in helical mode. The subject's head rested on a foam cushioned headrest. A head strap was applied to minimize head movement.

1000 MBq [<sup>15</sup>O]H<sub>2</sub>O, synthesized on-line ([<sup>15</sup>O]CO<sub>2</sub> + 4H<sub>2</sub>  $\rightarrow$  2[<sup>15</sup>O]H<sub>2</sub>O + CH<sub>4</sub>) was injected intravenously into an antecubital vein via an automatic water injection system (AM WR01, JFE Technos, Yokohama, Japan). The system delivered a 10 ml bolus over 10 seconds at 1 ml/sec with both pre- and post- flush of an inert saline solution. Immediately after starting the administration, emission data were acquired over 20 minutes in three-dimensional list mode with a 25.6 cm axial FOV and sorted into 40 time frames (40 × 30 seconds).

All emission scans were normalized for detector inhomogeneity and corrected for random coincidences, dead time, scattered radiation, and photon attenuation. For optimization of image quality, the 40 frames of the dynamic emission scans were reconstructed using 3D-OSEM (Ordered-Subset Expectation Maximization) with 2 iterations and 28 subsets. The resultant image quality allowed manual identification of regions of interest (ROIs). For the reconstruction algorithms, the data were collected in a  $128 \times 128 \times 47$  matrix with a voxel size of  $2.0 \times 2.0 \times 3.27$ mm.

# Data Analysis

The CT and PET image data were transferred to a Xeleris 1.1 workstation (GE Healthcare) for PET data analysis. Manually defined ROIs on the attenuation corrected axial images and CT images (lateral and third ventricle, cortex of occipital lobe) were used to obtain the time-activity data of the scans of each subject. The tissue activity concentration in each ROI was expressed as the standardized uptake value (SUV, g/ml), corrected for the subject's body weight and administrated dose of radioactivity. Each tissue time activity concentration was determined by fitting the data using the following equation:

$$\mathbf{y}(t) = \mathbf{y}_0 + a\mathbf{e}^{-bt}$$

where  $y_0$  is the baseline SUV. Subsequently, the ratio between SUVs of ventricle and cortex was defined as the influx ratio. The numerical data were subjected to the Mann-Whitney-Wilcoxon rank sum test for group analysis using IBM SPSS version 19.5 (IBM Corporation, Armonk, NY, USA).

# Results

Results are shown in Table 1 and summarized in Fig 2. Water influx into CSF space in AD patients, expressed as influx ratio (0.755  $\pm$  0.089), was significantly reduced compared to young controls (1.357  $\pm$  0.185, p < 0.001) and senior controls (0.981  $\pm$  0.253, p < 0.05). Furthermore, the influx ratio of senior controls compared to that of young controls was significantly reduced

	Age	Influx Ratio
Young	21	1.10620
	21	1.35608
	21	1.45009
	21	1.14260
	22	1.16180
	22	1.62079
	22	1.24990
	24	1.41213
	24	1.48959
	30	1.57873
Senior	60	0.68299
	63	0.92728
	63	1.03987
	63	1.05720
	65	1.44195
	68	0.94050
	68	1.27526
	69	0.81396
	74	1.03050
	78	0.59892
AD	59	0.69261
	61	0.79321
	63	0.86105
	63	0.62796
	71	0.70088
	71	0.73065
	73	0.74510
	79	0.66232
	80	0.84389
	84	0.88935

#### Table 1.

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(p < 0.01) as well. The observed large range of influx ratio (0.599–1.442) in the senior controls suggested that the reduction in water influx into CSF represented an aging process.

## Discussion

Fluid-filled canals surrounding perforating arteries and veins in the brain parenchyma were recognized in the early era of modern medicine. They became known as the Virchow Robin space, so named after the first two scientists who described the structure in detail, namely, Rudolph Virchow and Charles Philippe Robin [14,15]. It was soon recognized that fluid in the Virchow Robin space may play a role similar to systemic lymphatics [4–8,16]. Studies based on modern technologies disclosed some of the physiological roles of the Virchow Robin space and interstitial fluid flow, ranging from the regulation of regional blood flow to  $\beta$ -amyloid clearance during sleep [17–19].

The unique anatomical features of the brain's vascular system ensure a properly functioning blood brain barrier (BBB) and are a result of modification of the systemic circulation system. A critical property of the BBB is prevention of free water permeability across capillary walls (tight

Reduction of Water Influx into CSF in Alzheimer's Disease



**Fig 2. Schematic presentation of the results with mean (circle) and standard deviation (bar).** Water influx into CSF space is expressed as influx ratio (IR): the ratio between the standardized uptake value (SUV, g/ml) of the ventricle to that of cortex. IR in Alzheimer's disease patients (AD) is significantly reduced compared to both young controls (p < 0.001) and senior controls (p < 0.05), Mann-Whitney-Wilcoxon rank sum test. Note that there is no overlap in data points between AD and young controls. Reduction of influx ratio in senior controls compared to that in young control is found to be significant (p < 0.01) as well. A large range of influx ratio in senior controls suggests that the observed reduction likely represents one of the aging processes.

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endothelium). This is in strong contrast to systemic capillaries which possess a leaky endothelium. The molecular basis of the tight endothelium is the formation of tight junctions. Various tight junction proteins such as claudin and occludin play the role of "gating control" of paracellular transport. Passive water movement across brain capillaries is strongly restricted by these tight junctions. In systemic capillaries, active water transport across endothelial cells is accomplished by the water channel aquaporin 1 (AQP-1). In brain capillaries, such water movement is totally abolished through the active suppression of AQP-1 [20]. Accordingly, water motion between capillary lumen into the peri-capillary Virchow Robin space is highly limited to restricted passive movement.

In contrast to the above situations, the peri-capillary Virchow Robin space receives constant water influx through AQP-4, another isoform of the aquaporin family, abundantly expressed in the perivascular endfeet of astrocytes [21]. Virchow Robin space water homeostasis and, hence, interstitial flow is primarily dependent on water influx through AQP-4 [2,3,10]. Accordingly, brain interstitial flow, which plays a role equivalent to the systemic lymphatic system, is now considered to be an AQP-4 dependent system [13]. The basic function of lymphatics is drainage of cellular debris that has been subjected to molecular scrutiny before being returned to venous circulation.  $\beta$ -amyloid has been shown to be essential for synaptic formation [22]. Disturbance in the proper clearance of  $\beta$ -amyloid, and negative balance between its clearance and production has been implicated to play a significant role in senile plaque formation and ultimately the pathogenesis of Alzheimer's disease. The various components of amyloid homeostasis have become a target of therapeutic strategies [23-25]. Since under physiological conditions interstitial flow plays a critical role in the clearance of  $\beta$ -amyloid [4–8], disturbance in AQP-4 functionality and resultant reduction in interstitial flow could cause significant  $\beta$ amyloid accumulation. Transgenic mice lacking endothelial cell expression of Agrin are shown to have reduced AQP4 while BBB remained intact. These mice have significant accumulation of  $\beta$ -amyloid[26].

Although it is difficult to determine whether the observed reduction in water influx into the CSF system in AD patients is the primary abnormality or merely related to  $\beta$ -amyloid deposits in the brain, the clear cut differences between young normal volunteers and AD patients unequivocally indicated that AD patients have significant reduction in interstitial flow. Furthermore, the theoretical possibility of contribution by choroid plexus or the leptomeningeal vasculature to the water detected in the CSF compartment cannot be totally excluded. Nevertheless, the observed large range of influx ratios in senior controls without cognitive dysfunction, strongly suggests that reduction of water influx into the CSF itself is not a sufficient factor for the pathogenesis of AD, a situation similar to senile plaque formation. There must be additional factors that eventually lead to neural death and cortical dysfunction.

Drainage of  $\beta$ -amyloid by interstitial flow through the Virchow Robin space into CSF is essential for maintaining proper homeostasis between  $\beta$ -amyloid production and clearance. The balance between  $\beta$ -amyloid production and its clearance appears to be vital for maintaining proper neural function. Disruption in  $\beta$ -amyloid homeostasis may play a critical role in the pathogenesis of senile plaque formation, and, hence, the development of Alzheimer's disease. Assessment of the dynamic indices of production and clearance of  $\beta$ -amyloid in various cohort groups, including mild cognitive impairment (MCI), is warranted.

## **Supporting Information**

**S1 TREND Checklist.** (PDF)

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### **Author Contributions**

Conceived and designed the experiments: TN. Performed the experiments: YS YN KY KK YY TI. Analyzed the data: YS HI MN ILK TN. Wrote the paper: YS ILK TN.

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# The Molecular Mechanisms of Neural Flow Coupling: A New Concept

#### Tsutomu Nakada

From the Center for Integrated Human Brain Science, Brain Research Institute, University of Niigata, Niigata, Japan.

## ABSTRACT

The phenomenon known as neural flow coupling (NFC) occurs at the capillary level where there are no known pressure controlling structures. Recent developments in advanced magnetic resonance imaging technologies have made possible in vivo direct investigations of water physiology that have shed new insight on the water dynamics of the cortical pericapillary space and their complex functionality in relation to NFC. Neural activities initiate a chain of events that ultimately affect NFC. First, neural activities generate extracellular acidification. Extracellular acidosis in turn produces inhibition of aquaporin-4 (AQP-4) located at the end feet of pericapillary astrocytes, the water channel which regulates water influx into the pericapillary space and, hence, interstitial flow. Reduction of pericapillary water pressure results in a negative balance between pericapillary and intraluminal capillary pressure, allowing for capillary caliber expansion. Proton permeability through the tight junctions of the blood brain barrier is significantly high owing to the Grotthuss proton "tunneling" mechanism and, therefore, carbonic anhydrase (CA) type IV (CA-IV) anchored to the luminal surface of brain capillaries functions as scavenger of extracellular protons. CA-IV inhibition by acetazolamide or carbon dioxide results in the accumulation of extracellular protons, causing AQP-4 inhibition and a secondary increase in rCBF.

Keywords: neuro flow coupling, aquaporin 4, interstitial flow, protons, neural activation.

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**Correspondence**: Address correspondence to Tsutomu Nakada, MD, PhD, FAAN, FANA, Center for Integrated Human Brain Science, Brain Research Institute, University of Niigata, 1-757 Asahimachi, Niigata, 951-8585, Japan. E-mail: tnakada@bri.niigata-u.ac.jp.

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#### Introduction

Recent studies have shown that the classic circulation model of cerebrospinal fluid (CSF) is incomplete.<sup>1</sup> Production of CSF is not only dependent on choroid plexus but also on water flux in the pericapillary (Virchow Robin) space.<sup>2,3</sup> Historically, CSF flow through the pericapillary space is known as interstitial flow, and is considered to play a role equivalent to the systemic lymphatic system.4-10 Advancements in modern magnetic resonance imaging (MRI) technologies have allowed for the noninvasive investigation of water flow in vivo. These studies revealed that water dynamics of the pericapillary space, ie, interstitial flow, is controlled by aquaporin-4 (AQP-4), the main subset of the aquaporin water channel family in the brain.<sup>11-13</sup> It has also been demonstrated that inhibition of AQP-4 is strongly coupled with an increase in regional cerebral blood flow (rCBF).14 These observations have led to a better understanding of the architectural significance and functionality of the cerebrovascular system.<sup>15,16</sup> This article is a concise review of the modern concept of neural flow coupling (NFC) and its relationship to water dynamics in the pericapillary space.5-10,16

#### **Cerebral Autoregulation: Upstream Control**

Cerebral autoregulation signifies an intrinsic ability of the cerebral vasculature to maintain cerebral blood flow at a relatively constant rate of approximately 50 ml per 100 g brain tissue per minute in the face of blood pressure changes.<sup>17–22</sup>

Autoregulation generally functions between mean blood pressures of 60 to 150 mmHg. It is maintained in parasympathetically and/or sympathetically denervated animals,<sup>21</sup> and the system is independent from extrinsic neural control. Instead, intrinsic neural nitric oxide (NO) control<sup>22</sup> and release of vasoactive substrates by the brain are believed to play essential roles in maintaining constant cerebral perfusion.<sup>19,20</sup> Perfusion is held constant by means of the cerebral vasculature smooth muscle that constricts and dilates in response to elevated and decreased systemic pressure, respectively.<sup>17–22</sup> Although this "upstream" control of inflow pressure appears to be rather straightforward, the physiologic mechanisms underlying NFC, neural activity-associated rCBF increase, were until now poorly understood.

#### Virchow Robin Space and Interstitial Flow: Cerebral Lymphatic Equivalent

Fluid-filled canals surrounding perforating arteries and veins in the parenchyma of the brain were recognized in early modern medicine and described in detail by Rudolph Virchow and Charles Philippe Robin.<sup>23,24</sup> The space is commonly referred to as the Virchow Robin space. It is now clearly understood that the fluid in the Virchow Robin space constitutes interstitial flow that drains into the CSF system (Fig 1). Virchow Robin interstitial flow is believed to play a role similar to systemic lymphatics.<sup>2-10</sup>

The basic function of systemic lymphatics is drainage of cellular debris subjected to molecular scrutiny before returning

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**Fig 1.** Virchow Robin space and interstitial flow. The ventricles and subarachnoid space represent the cerebrospinal fluid (CSF) space in the brain. The Virchow Robin space is a continuous canal surrounding penetrating vessels. Interstitial flow runs within the Virchow Robin space and drains into the subarachnoid space. Contrary to the classical concept of CSF flow, water CSF within the subarachnoid space is now believed to be dependent on interstitial flow in the Virchow Robin space. Although not as yet mainstream, the Virchow Robin space likely surrounds the medullary veins and subependymal veins as well. As shown in Figure 3, water influx from the systemic circulation into CSF is strongly dependent on interstitial flow in the Virchow Robin space through aquaporin-4 (AQP-4).

to the venous circulation. Although there is no conventional lymphatic system in the brain, interstitial flow of the Virchow Robin space constitutes its equivalent, and plays an essential role in clearing toxic proteins from brain parenchyma. Interstitial flow, and, hence, CSF circulation, eventually drains into the venous system through the cerebral dural sinuses (Pacchionian bodies), the latter playing a role similar to the thoracic duct of the systemic lymphatic system.

An important and intriguing example of interstitial flow protein clearance is  $\beta$ -amyloid clearance.<sup>3,5,7,8,14-16</sup>  $\beta$ -amyloid is essential for synaptic formation.<sup>25</sup> Nonetheless, excess  $\beta$ -amyloid can result in aggregation of the protein and senile plaque formation. Drainage of  $\beta$ -amyloid by interstitial flow through the Virchow Robin space into CSF is likely critical for maintaining proper homeostasis of  $\beta$ -amyloid production and clearance. The role of prealbumin, abundant in CSF, as  $\beta$ -amyloid chaperon and in preventing  $\beta$ -amyloid's natural tendency to plaque formation, further supports the  $\beta$ -amyloid clearance hypothesis.<sup>26</sup>

#### Pericapillary Water Dynamics and AQP-4

The aquaporin family is a large collection of integral membrane proteins that enable the movement of water across biological membranes. Three isoforms, namely AQP-1, AQP-4, and AQP-9, have been identified in brain in vivo. Expression of AQP-1 within CNS capillaries is actively suppressed, and AQP-1 in the brain is uniquely found in the choroid plexus epithelium. AQP-9 is only scarcely expressed in the CNS and is considered to have no significant role.<sup>12,27</sup> By contrast, AQP-4 is expressed abundantly in the brain and has a specific distribution: the subpial and perivascular endfect of astrocytes.<sup>2,10-14</sup>

Active suppression of AQP-1 expression in brain capillaries is essential for proper maintenance of the blood brain barrier, preventing excessive movement of water across capillary walls.<sup>27,28</sup> Active water influx into the CSF system from the blood stream has been shown to be regulated by AQP-4, not AQP-1, indicating that interstitial flow plays an important role in CSF dynamics.<sup>2</sup>

As cerebral equivalent of the systemic lymphatic system, interstitial flow dysfunction can be expected to result in reduction of  $\beta$ -amyloid clearance. Indeed, senile plaque bearing transgenic mice showed significant decline of water influx into the CSF system, to the extent similar to that found in AQP-4 knockout mice.<sup>29</sup> Positron emission tomography studies in AD patients have shown virtually identical results.<sup>14</sup>

#### NFC and rCBF

Increased rCBF associated with brain activation is a well-recognized phenomenon that is known as NFC. Since this is a micro-, rather than macroenvironmental event occurring within an area limited to 250  $\mu$ m around the site of neural activity, the regulatory mechanism for NFC should be within the capillaries.<sup>30</sup>

Considering blood flow to be steady, laminar flow within a long cylindrical pipe (Fig 2), the Hagen-Poiseuille equation gives volumetric blood flow,  $\Phi$ , as

$$\Phi = \frac{\pi}{8\eta} \frac{\Delta P}{L} R^4$$

where  $\Delta P$  is pressure loss (differences in inflow and outflow pressure), *L* is the length of the vessel tube,  $\eta$  is blood viscosity, and *R* is the radius of the vessel.<sup>31</sup>

Given that steady inflow pressure is rigorously controlled by upstream arterial autoregulation<sup>17,18</sup> and constant venous pressure, under physiological conditions, cerebral blood flow is virtually determined by the radius of the vessel and increases parallel to its fourth power

$$\Phi \sim R^4$$

The relationship implies that even small changes in capillary caliber have significant effects on rCBF.

Capillaries are devoid of muscle and, hence, are not under neural control. The perforating vessels of the cerebral cortex are surrounded by a fluid-filled perivascular (Virchow Robin) space. At the capillary level, fluid pressure within the vessel lumen is directly opposed by pericapillary fluid pressure. Therefore, the parameter that determines capillary caliber is the pressure balance between luminal (intracapillary) and outer (pericapillary) fluid pressures. Since intracapillary pressure reflects the inner pressure of arterioles, and is therefore a function of upstream arterial autoregulation,<sup>17,18</sup> it is the fluid pressure of the pericapillary space that inversely determines cerebral capillary caliber changes and, hence, rCBF, as follows:

$$\Phi \sim R^4 \sim {}^1/{}_{P_{peri-capillary}}$$

AQP-4 controls the water dynamics of the pericapillary space in the brain.<sup>2,10,14</sup> Therefore, AQP-4 activities play a role in controlling rCBF. Simply put, under physiological conditions, rCBF correlates inversely to AQP-4 activities.

rCBF 
$$\sim 1/AQP4$$
 activities

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Fig 2. Vessel diameter is determined by tension of smooth muscle in artery, arteriole, venule, and vein (Brain Vessels). Capillaries are devoid of muscle and in capillaries with tight endothelium such as brain capillaries, capillary caliber is determined by the pressure balance between luminal and outer fluid pressures (Brain Capillary). For capillaries with leaky endothelium (Common Capillary), pressure balance is quickly equalized without capillary caliber changes.



**Fig 3.** Pericapillary water dynamics. Water permeability of brain capillaries is restricted due to the tight endothelium, presence of tight junctions and active suppression of AQP-1. By contrast, significant water flow is present in the Virchow Robin space (interstitial flow) and is supported by active water inflow through AQP-4. Although it has not been clearly confirmed (?), interstitial flow may similarly be present along the medullary and subependymal veins.

Indeed, reduced AQP-4 activities by its inhibitor TGN-020 effectively increased rCBF in mice.<sup>13</sup> Under physiological conditions, AQP-4 is believed to be inhibited by extracellular protons similar to other AQP isoforms.<sup>32</sup> Therefore, rCBF is predicted to correlate with extracellular proton density (Figs 3 and 4).

$$CBF \sim [H^+]_{extra}$$

The steps outlined above provide a fresh understanding of the underpinnings of NFC.

#### **Neural Activities and Extracellular Acidosis**

Since the original description by Urbanics et al<sup>33</sup> extracellular (interstitial) acidification associated with neural activities has been extensively studied by various investigators.<sup>34-36</sup> Modern MRI technologies demonstrated unequivocally that regional neural activities in humans are accompanied by extracellular acidosis found in a virtually identical distribution as the neural activity-induced increase in rCBF detected by blood oxygenation level-dependent contrast.<sup>37</sup> Therefore, at least from a phenomenological stand point, neural activity-induced extracellular acidification plays a role in NFC. Although the precise underlying mechanisms remain to be elucidated, it appears clear that neural activity-induced interstitial acidification, and the resultant inhibition of AQP-4, is indeed a main mediator of neural activity-associated rCBF increase. Further support for this concept comes from the "Diamox effect." Acetazolamide (Diamox) is a carbonic anhydrase (CA) inhibitor and a powerful agent for increasing rCBF. This "Diamox effect" is well known to be accompanied by interstitial acidosis in the brain.38

Within the large CA family, CA type IV (CA-IV) represents the dominant CA in the cerebral cortex and is anchored to the luminal surface of cerebral capillaries.<sup>39</sup> It has been shown that interstitial CA activity in the brain is attributable to CA-IV.<sup>40</sup> The human NBC1 sodium bicarbonate cotransporter directly interacts with CA-IV. The tethering of intracellular CA type II (CA-II) and extracellular CA-IV in proximity to the NBC1 HCO<sub>3</sub><sup>-</sup> transport site maximizes the transmembrane HCO<sub>3</sub><sup>-</sup> gradient local to NBC1 and thereby activates the transport rate.<sup>41</sup>

Since proton permeability through the tight junctions is significantly higher than for other small molecules, owing to the Grotthuss proton tunneling mechanism,<sup>42</sup> capillary CA-IV with NBC1 and CA-II effectively function as scavenger of extracellular protons generated by neural activation (Fig 5). CA inhibition by acetazolamide or excess of carbon dioxide (CO<sub>2</sub>) in capillary blood results in accumulation of extracellular protons which in turn inhibit water flux through AQP-4. The resultant negative pressure relation with respect to intraluminal capillary pressure affects capillary dilatation and an increase in rCBF.



**Fig 4.** Neural activation. Neural activation produces extracellular acidification accompanied by increase in rCBF and astrocyte swelling. Proton inhibition of AQP-4 results in a reduction of water flow from astrocytes into the pericapillary Virchow Robin space, astrocyte swelling and capillary expansion due to reduction of pericapillary fluid pressure.



**Fig 5.** CA-IV system. Complex of CA-IV anchored to luminal surface of cerebral capillary, human NBC1 sodium bicarbonate cotransporter and intracellular CA-II. Their proximity maximizes the transmembrane  $HCO_3^-$  gradient local to NBC1 and thereby activates the transport rate. Because of the high proton permeability through tight junctions, capillary CA-IV with NBC1 and CA-II effectively function as scavenger of extracellular protons generated by neural activation. CA inhibition by acetazolamide or excess of  $CO_2$  in capillary blood results in accumulation of extracellular protons.

#### Summary: A New Concept

Technological advancements, especially those in the field of MRI, have led to a new level of understanding of the physiologic underpinnings of neural activation-induced rCBF increase, a phenomenon known as NFC. The main player mediating NFC is the proton. Neural activities produce interstitial acidification. Excess protons inhibit AQP-4 activities in the pericapillary Virchow Robin space, resulting in a reduction in the pericapillary pressure. The negative balance between pericapillary and intraluminal capillary pressure induces dilatation of capillaries and an increase in rCBF. Acetazolamide or excess  $CO_2$  blocks active clearance of interstitial protons which are ordinarily highly permeable through the tight junctions and, similar to neural activities, causes interstitial acidification and an increase in rCBF.

The precise molecular mechanism of extracellular acidification associated with neural activities remains to be elucidated. Such acidification has been shown to be associated with

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intracellular alkalinization of astrocytes.<sup>36</sup> Active proton extrusion by astrocytes appears to be the most attractive explanation.<sup>14,36</sup> The functional significance of NFC has been linked to elimination of heat production brought about by neural activities. A heat-sensitive voltage-gated proton channel similar to neutrophil Hv1 may play a role, although much remains to be investigated.<sup>14,43</sup>

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### REVIEW

# N-acetylaspartate Decrease in Acute Stage of Ischemic Stroke: A Perspective from Experimental and Clinical Studies

Hironaka Igarashi<sup>1\*</sup>, Yuji Suzuki<sup>1</sup>, Vincent J. HUBER<sup>1</sup>, Masahiro Ida<sup>2</sup>, and Tsutomu Nakada<sup>1</sup>

<sup>1</sup>Center for Integrated Human Brain Science, Brain Research Institute, University of Niigata 1–757 Asahimachi-dori, Chuo-ku, Niigata City 951–8585, Japan <sup>2</sup>Department of Radiology, Tokyo Metropolitan Ebara Hospital (Received March 25, 2014; Accepted July 16, 2014; published online December 15, 2014)

N-acetylaspartate (NAA) appears in a prominent peak in proton magnetic resonance spectroscopy (<sup>1</sup>H-MRS) of the brain. Exhibition by NAA of time-dependent attenuation that reflects energy metabolism during the acute stage of cerebral ischemia makes this metabolite a unique biomarker for assessing ischemic stroke. Although magnetic resonance (MR) imaging is a powerful technique for inspecting the pathological changes that occur during ischemic stroke, biomarkers that directly reflect the drastic metabolic changes associated with acute-stage ischemia are strongly warranted for appropriate therapeutic decision-making in daily clinical settings. In this review, we provide a brief overview of NAA metabolism and focus on the use of attenuation in NAA as a means for assessing the pathophysiological changes that occur during the acute stage of ischemic stroke.

Keywords: brain, ischemia, magnetic resonance spectroscopy, N-acetylaspartate

#### Introduction

N-acetylaspartate (NAA) is the acetylated derivative of L-aspartate<sup>1</sup> and the second most abundant amino acid-related metabolite in the mammalian brain after glutamate.<sup>2,3</sup> Although the reason for its abundance in the brain remains to be elucidated, NAA exists mainly in neurons at sufficient concentrations to be visible in proton magnetic resonance spectra (<sup>1</sup>H-MRS) of normal brain, where the methyl group of NAA shows a prominent peak at 2.01 ppm (Fig. 1). Furthermore, NAA levels attenuate in many of the pathological states relating to impaired cellular metabolism and neuronal loss. Pathological conditions characterized by acute failure of energy metabolism, i.e., ischemic stroke, hypoxia, and traumatic brain injury, exhibit drastic NAA attenuation from within minutes to hours following the specific initiating event.<sup>4,5</sup> This attenuation during the acute stage may initially involve a deterioration of NAA synthesis, acceleration of NAA catabolism, or their combination. These pathologic conditions often lead to a combination of brain tissue necrosis and neuronal loss within several days to a week following the ischemic event unless energy metabolism is resumed within the first several hours.

Ischemic stroke has been the most widely studied of pathological conditions that exhibit acute NAA attenuation in both animal models and humans, primarily because of its high worldwide incidence. <sup>1</sup>H-MRS is useful for visualizing the progression of those pathological conditions following severely compromised cerebral blood flow resulting from vascular occlusion. Lactate appears within minutes of the occlusion due to an acceleration of anaerobic glycolysis, which compensates for hampered mitochondrial oxidation. Because NAA synthesis is adenosine triphosphate (ATP)-dependent (energydependent),<sup>6</sup> impaired energy availability initiates a continuous decline in levels of NAA, the rate of which depends in part on severity, within hours of the ischemic event. Although magnetic resonance (MR) imaging, especially diffusion-weighted images (DWI) and perfusion images (PI), is a powerful technique for visualizing pathological changes in vivo that can be applied to ischemic stroke, we believe observation of biomarkers that directly reflect

<sup>\*</sup>Corresponding author, Phone: +81-25-227-0676, Fax: +81-25-227-0822, E-mail: higara@bri.niigata-u.ac.jp



Fig. 1. Chemical structure of N-acetylaspartate (NAA) and representative proton magnetic resonance spectroscopy (<sup>1</sup>H-MRS). The methyl group of NAA (**a**) shows the most prominent singlet peak at 2.01 ppm in <sup>1</sup>H-MRS of normal brain (**b**). NAA reduces and lactate appears (**c**) in the acute ischemic lesion delineated by diffusion-weighted imaging (DWI) (**d**). tCHO, total choline compounds; tCr, sum of creatine and phosphorcreatine.

metabolic changes during the acute stage will be highly valuable for making the most appropriate therapeutic decisions. In this review, we present a brief overview of NAA metabolism with subsequent focus on the use of NAA visualization to assess pathophysiological changes during the acute stage of ischemic stroke.

#### Distribution, Biochemistry, and Functions

#### Distribution of NAA

Human studies using MRS show a relatively homogenous distribution of NAA throughout the brain, with slightly higher concentrations found in the occipital cortex, thalamus, and cerebellar hemisphere.<sup>7,8</sup> These results agree with those of previous immunohistochemical studies using rodent brain.9 Additional immunohistochemical studies have demonstrated the presence of NAA predominately in neuronal cell bodies and their processes.4,9,10 Further confirmation of this distribution was obtained with high performance liquid chromatography (HPLC) and high resolution nuclear magnetic resonance (NMR) studies.<sup>11-13</sup> Oligodendrocyte type-2 astrocyte progenitor cells (O-2A progenitors, NG2 cells)<sup>14</sup> were also found to express NAA at easily detectable levels.<sup>12,13</sup> NG2 cells in postnatal brain have the potential to differentiate into oligodendroglia in gray and white matter<sup>15</sup> and have been recently confirmed to make up about 5% of all cells in the adult central nervous system (CNS).<sup>16</sup> Although the expression of NAA by NG2 cells in the adult brain has not been confirmed, some caution is warranted in interpreting changes in NAA concentration as a pure neuronal marker.

#### Tricellular compartment metabolism of NAA

NAA and its glutamate dipeptide derivative, Nacetylaspartylglutamate (NAAG), which supposedly works as a cotransmitter with glutamate and  $\gamma$ aminobutyric acid (GABA),<sup>17</sup> form the unique tricellular compartment metabolism involving neurons, oligodendrocytes, and astrocytes (Fig. 2).<sup>18</sup> Both NAA and NAAG are synthesized in the neuron, but neither are catabolized in the neuron itself. NAA is either released from neurons or is transported to oligodendrocytes, where it is metabolized into acetate and aspartate by aspartoacylase (ASPA).<sup>19</sup> NAA-derived acetate in the oligodendrocytes is converted to acetyl coenzyme A (acetyl CoA) with acetyl CoA synthetase, which serves primarily as a precursor in myelin lipid synthesis.<sup>20,21</sup> NAAG is released from the axon terminal in response to neuronal activity, and most is subsequently hydrolyzed



**Fig. 2.** Tricompartment metabolism of N-acetylaspartate (NAA) and N-acetylaspartylglutamate synthetase (NAAG). Both NAA and NAAG are synthesized in neurons. NAA is mainly released from neurons or transported to oligodendrocytes, where it is metabolized into acetate and aspartate by aspartoacylase. NAA-derived acetate in oligodendrocytes is mainly used as a precursor of myelin lipid synthesis. NAAG is released from axonal terminals in concordance with neuronal activity, and most of them are hydrolyzed into NAA and glutamate with glutamate carboxypeptidase II (GCPII) at the surface of astrocytes. AAT, aspartate aminotransferase; acetyl CoA, acetyl coenzyme A;  $\alpha$ -KG,  $\alpha$ -keto glutarate; APSA, aspartoacylase; Asp-NAT, L-aspartate N-acetyltransferase; GS, glutamine synthetase; OAA, oxaloacetate.

into NAA and glutamate at the surface of the astrocyte membrane by glutamate carboxypeptidase II (GCPII).<sup>22</sup>

#### Synthesis, catabolism, and functions

The majority of NAA is thought to be synthesized in neuronal mitochondria<sup>23</sup> from aspartate and acetyl-CoA.<sup>24,25</sup> Aspartate is converted in the mitochondria from malate, which enters the mitochondria through the mitochondrial transporter, aralar1.<sup>26</sup> The conversion of malate to aspartate occurs via aspartate aminotransferase (AAT) and part of the tricarboxylic acid (TCA) cycle (Fig. 2).<sup>27</sup> Finally, NAA is synthesized by membrane-bound L-aspartate Nacetyltransferase (Asp-NAT)<sup>28</sup> in an energy-dependent manner.<sup>6,12,29</sup> Partly because of that energy dependence and the involvement of the TCA cycle in the conversion of malate to aspartate in mitochondria, NAA biosynthesis is very susceptible to energy failure.<sup>6</sup> Once synthesized, NAA is transported to the cytosol by the dicarboxylic acid transporter.<sup>30</sup> A recent report identified the synthesis of a small but distinct amount of NAA in cytosol.<sup>31,32</sup> In this secondary pathway, Asp-NAT in the cytosol is thought to utilize aspartate sequestered from the mitochondria through aralar1.<sup>33</sup> However, the significance of the 2 distinct NAA synthetic pathways remains unknown.

NAA synthesized in the neuron is transported to either the extracellular space (ECS) or oligodendroglia. Because it exists as a dianion at physiological pH, the nonspecific diffusion of NAA through the cell membrane is unlikely. No transporter of NAA has been identified, but Fujita and Ganapathy suggest that Na<sup>+</sup>/carboxylate cotransporter 3 (NaC3) is a likely candidate.<sup>34</sup> It has been estimated that approximately 5% of the total NAA in the CNS is excreted from the ECS into the blood presumably via putative NAA transporters in the astrocytes.<sup>35</sup>

NAA is hydrolyzed by aspartoacylase, the distribution of which is restricted to oligodendrocytes.<sup>36</sup> Aspartoacylase activity is reported to increase in parallel with white matter myelination during development,<sup>37</sup> and NAA is hydrolyzed into aspartate

and acetate, the latter being an important component of myelin lipid. Therefore, it is believed that part of the NAA synthesized in the neuron serves as a source of building blocks for myelin synthesis in oligodendrocytes.<sup>20,21,38</sup>

Other roles for NAA in the brain are still debated. However, there is evidence that NAA is important in maintaining the microenvironment of the brain parenchyma.<sup>4</sup> Two tissues outside the CNS, peritoneal mast cells<sup>39</sup> and ocular lenses,<sup>40</sup> have been found to contain NAA, and in both examples, NAA appeared to play an important role in osmoregulation and the control of water distribution. Likewise, NAA is also thought to participate in controlling osmolality and water distribution in the CNS.<sup>41</sup> In this context, Baslow claimed that NAA may be released from neurons in some controlled fashion, which can differ from that of neurotransmitters, and serve in intercellular signaling.<sup>19</sup> A high concentration of NAA has also been considered to compensate for the anion deficit in the CNS.<sup>2,4</sup> Nakada suggested that the negatively charged NAA in neuronal cytosol can facilitate passive diffusion of high energy phosphates from the mitochondria to the cell membrane to fulfill the high energy demands of neuronal cell membranes.42,43

# NAA Attenuation in Acute-stage Ischemic Stroke

### Concept of experimental ischemic stroke

Experimental models of cerebral ischemia have been developed to assess pathophysiological changes resulting from ischemia. The universal feature of the various models is the interruption of cerebral blood flow that feeds a part of the brain, which, in turn, elicits a depletion of oxygen and glucose and disrupts tissue metabolism.<sup>44</sup> At present, rodent models are widely used because their preparation is convenient, and multiple means of analysis are available.<sup>45</sup>

Rodent models of ischemia can be divided into 2 classes, global and focal. The global ischemia model is characterized by temporary pan-forebrain ischemia of relatively short duration, typically 5 to 30 min, that is induced by occlusion of the bilateral internal carotid arteries<sup>46,47</sup> alone or with the simultaneous occlusion of the bilateral vertebral arteries.<sup>48</sup> This model elicits a profound ischemia of short duration over the forebrain, so it is considered to be a model of short-duration cardiac arrest or severe hypotension. Infarction is not the primary result of this insult, which instead leads within a week of the ischemic injury to patchy neuronal loss with several degrees of reactive gliosis in vulnera-

ble regions, such as the hippocampus, striatum and cortex.

Focal ischemia models are used to mimic clinical ischemic stroke. In these models, permanent (no reperfusion) or transient (reperfusion typically within several hours) ischemia are induced in a certain vascular territory, commonly that of the middle cerebral artery (MCA), by arterial occlusion using a silicon-coated suture, blood clot, or clipping. In this model, energy failure from severe blood cessation in the core of the circumscribed lesion (ischemic core) produces irreversible biological changes within a short time. Reduction of blood flow is relatively mild in the rim of the lesion, and that tissue can be salvaged if blood flow can be restored (reperfusion) within a few hours.<sup>49</sup> In this salvageable area, called the "penumbra" after the half-shaded area of an eclipse, blood flow is too low to maintain electric activity but remains sufficient to preserve the function of energy-dependent ion channels.<sup>50</sup> The term "penumbra" is now generally used to define ischemic but salvageable cerebral tissue that requires immediate reperfusion.<sup>51</sup>

# *MRS findings and gradual attenuation of NAA in the acute stage of ischemic stroke*

Typical chronological changes visible by MRS during acute ischemic stroke have been identified in several animal studies<sup>52-55</sup> and subsequently confirmed in human clinical studies.<sup>56-59</sup> Typically, chronological changes of four MRS visible metabolites can be seen following cerebral ischemia, namely rise of lactate, and attenuations of NAA, creatine and phosphocreatine (Cr), and choline compounds (Cho). Lactate appears immediately after cerebral blood flow is compromised and usually reaches a concentration plateau within 24 hours depending on several factors, such as the severity of hypoperfusion and plasma glucose concentrations. Attenuation of NAA levels becomes evident within an hour and continues almost linearly during the first 24 hours (Fig. 3).<sup>54,56–59</sup> After 24 hours, lactate tends to cover the entire topographical area of hypoperfusion, including the penumbra. Part of the "tissue at risk" can be identified as areas in which lactate is present but there is no attenuation of NAA (Fig. 3b).<sup>60</sup> Respective peaks caused by Cr and Cho begin to gradually fall after cell death begins, and tissue necrosis becomes obvious, typically several hours following ischemia.

The changes in relaxation affect the attenuations of metabolite peaks mainly in subacute to chronic stage of ischemia. In experimental study, van der Toorn and associates measured  $T_1$  and  $T_2$  of the metabolites and concluded that relaxations of NAA



**Fig. 3.** Chronological changes in N-acetylaspartate (NAA) of rat brain with permanent focal ischemia. In a diffusion-weighted imaging (DWI)-delineated area of ischemia (**a**), NAA visually decreased on proton magnetic resonance spectroscopy (<sup>1</sup>H-MRSI), and lactate appeared in the area at risk (**b**). NAA declined more steeply in the ischemic core (lateral caudo-putamen [LCP] and somatosensory cortex [SSC]) than the ischemic rim (MCS). MCP, medial caudo-putamen; R, reference area.

did not change within 24 hours after the induction of MCA occlusion in the ischemic core of the rat brain.<sup>61</sup> These results agree with the clinical study which concluded that the  $T_2$  of NAA decreased during the period between 5 and 20 days after the insult but did not change earlier.<sup>62</sup>

Our laboratory confirmed a linear decrease in NAA levels within the ischemic core and rim in studies utilizing both rodent models and clinical cases.55,63 In both studies, no reperfusion was attempted at the point where MRS imaging (MRSI) was carried out. The observation of a steeper slope in the decline in NAA in the core than the penumbra (Figs. 3, 4) indicates that the rate of NAA attenuation in acute-stage ischemia depends in part on the severity of hypoperfusion. Some of the data in the human study deviated lower (open circle, Fig. 4), and most of those cases demonstrated hyperglycemia (>120 mg/dL), which adversely affects metabolic deterioration<sup>64</sup> and stroke outcome.<sup>65</sup> Therefore, we undertook a partial correlation of the normalized NAA concentration, time from stroke onset, and initial plasma glucose concentration. That correlation revealed that both the amount of time elapsed from the ischemic event and the plasma

concentration of glucose in the ischemic core contributed to the rate of NAA decline (Fig. 4b). The reason NAA declines faster in response to hyperglycemia remains unknown. However, hyperglycemia is thought to facilitate the release of cytoplasmic amino acids and their derivatives into the ECS during ischemia,<sup>66</sup> thereby raising the intracellular concentration of calcium ions, which can, in turn, activate aspartoscylase.<sup>67</sup> These mechanisms may affect the acceleration of NAA decline.

In our human study, the rate of NAA attenuation was calculated as  $0.67 \,\mu g/kg/h$ ; this included compensation from the concentration of plasma glucose in the brain tissue, which was calculated with an NAA concentration of 9.2 mmol/g (Fig. 2b) revealed by <sup>13</sup>C-MRS study in human.<sup>68</sup> The result was somewhat larger than the rate of NAA synthesis in normal adult human brain ( $0.55 \,\mu g/kg/h$ ) calculated from a human <sup>13</sup>C NMR study that measured the turnover of NAA in brain tissue.<sup>42,68</sup> These results imply the occurrence of either NAA catabolism or its excretion and cleavage products, namely aspartate and acetate, during ischemic stroke, even if NAA production has failed completely. The <sup>13</sup>C NMR study in rats demonstrated that NAA syn-


**Fig. 4.** Chronological changes in N-acetylaspartate (NAA) in clinical ischemic stroke. As observed in rat brain with permanent focal ischemia, NAA declines more steeply in the ischemic core than the ischemic rim (**a**). A closed circle shows the result from euglycemic cases ( $\leq 120 \text{ mg/dL}$ ), and an open circle denotes the results from hyperglycemic (> 120 mg/dL) cases in the ischemic core. NAA decline was partly determined with time and plasma glucose concentration in the ischemic core (**b**).

thesis continued within the ischemic core at 120 min after induction of the MCA occlusion, albeit at a significantly impaired level.<sup>69</sup> A massive NAA release into the ECS was observed in rat models of permanent focal ischemia, with the concentration of NAA in the ECS increasing to 10 times that of a normal rat. However, NAA was undetectable in the blood with ischemic stroke,<sup>70</sup> so the direct excretion of NAA into the residual blood flow within the ischemic core can be expected to play only a minor role on NAA attenuation in acute sgate of ischemia. Furthermore, no rise in NAA levels were found in the CSF of rat models of focal ischemia<sup>71,72</sup> despite its massive release into the ECS. These results suggest a previously unknown NAA catabolic pathway in brain tissue that is activated during ischemia. Indeed, complete global ischemia achieved by decapitation, in which there is a lack of residual blood flow, leads to a reciprocal accumulation of aspartate and acetate over NAA loss.72 Aspartacylase activated by Ca<sup>2+</sup> accumulated in cytoplasm as a result of ischemia may play the dominant role in NAA catabolism in complete global ischemia.68 Paradoxically, the levels of both aspartate and acetate decreased in the ischemic lesion during focal ischemia.<sup>72,73</sup> One important difference between these 2

models of ischemia is the complete absence of blood flow in the global model and the maintenance of residual blood flow to the ischemic core of the focal model.<sup>74</sup> A massive amount of aspartate is also released into the ECS during ischemia.75 Excretion of the aspartate from the ECS into the blood can occur through alanine-serine-cysteine transporter 2 (ASCT2) channels located on the basal membranes of the vascular epithelium, which are activated by ischemic acidosis.76 Indeed, serum concentrations of aspartate were reported to rise during focal ischemia.<sup>77</sup> From these reports, we can ascertain that part of aspartate is released into residual blood flow from the CNS. Acetate may also be released into blood or further metabolized by surviving cells, possibly astrocytes that exclusively metabolize external acetate under normal physiological conditions.<sup>78</sup> Though the fate of acetate is unclear compared to that of aspartate, we confirmed that during focal ischemia, the NMR signal from acetate was detectable in the center of the ischemic core (Fig. 5a-c). In summary, attenuation of NAA during ischemic stroke can result from suppression of NAA synthesis, acceleration of NAA catabolism, excretion of NAA-related derivatives into blood, and further metabolism (Fig. 5d, e).



**Fig. 5.** Magnetic resonance spectroscopy (MRS) at 10 hours after induction of focal ischemia (**a-c**) and suspected metabolic state of N-acetylaspartate (NAA) and its derivatives (**d**,**e**). Proton MRS (<sup>1</sup>H-MRS) (**a**, dashed line), fitted curve (**a**, solid line, and **c**) and fitted residual (**d**). An acetate peak was seen at 1.9 ppm (arrow), but a suspected aspartate peak (2.8 ppm) was absent (arrowhead). 1, total choline compounds (tCho); 2, creatine and phosphocreatine (Cr); 3, glutamate; 4, NAA; 5, acetate; 6, lactate. Reduction of NAA in ischemic stroke can result in the suppression of NAA synthesis, acceleration of NAA catabolism, release into blood of the derivatives, and further metabolism (**d**,**e**).

# Association and Dissociation between NAA Attenuation and DWI Findings

#### Cross-sectional studies: topographical NAA concentration as a complement of DWI

Diffusion-weighted imaging is a powerful modality for visualizing focal ischemic lesions in acute ischemic stroke. It can be used to delineate the regional reduction of the apparent diffusion coefficient (ADC) of water, which can be calculated from several DWI images. Though energy failure from ischemia reduces both ADC and NAA, ADC is reduced primarily in the areas in which cellular structure is altered by  $Na^+/K^+$  ATP pump failure, which may only reflect an indirect disruption of energy metabolism. On the other hand, a reduction of NAA directly reflects metabolic changes, specifically energy failure, in brain tissue during acute-stage ischemia. Based on that difference, we can expect NAA levels to reflect the condition of the ischemic area more sensibly than ADC. However, because NAA metabolism is comparatively slow, the delineation of the ischemic lesion based on NAA attenuation relating to metabolic energy failure lags behind the reduction of ADC. In clinical cases of proximal MCA occlusion, Nicoli reported that within 7 hours from onset, MRSI could indicate a large heterogeneity in NAA concentration in ischemic areas showing even the lowest ADC values.<sup>79</sup> Moreover, some reports suggest a wide range of NAA concentrations, from 40 to more than 100% of the normal range, in acute-stage ischemic lesions having an unperturbed DWI.<sup>59,80</sup> Although it is difficult at present to ascribe a threshold NAA concentration for making an accurate tissue prognosis, i.e., whether it will infarct or be salvageable, during the acute stages of ischemia, it has been suggested that salvageable areas might be characterized by the presence of lactate with no or only subtle NAA attenuation.<sup>80</sup> In cases with hemispheric transient ischemic attack (TIA), NAA was observed to attenuate about 10 to 15% in the acute stage, which recovered in 12 to 18 months after the incidents.<sup>81</sup> Only the report of a case with occlusion of the brachiocephalic artery described the reversibility of NAA in the acute stage of ischemia, with the ratio of NAA to Cr attenuated 10% at the initial measurement normalized after recanalization.<sup>82</sup> Though a larger study is warranted for confirmation, these reports suggest that NAA attenuation by less than 10

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**Fig. 6.** Chronological changes of proton magnetic resonance spectroscopy (<sup>1</sup>H-MRS), apparent diffusion coefficient (ADC) map, and regional cerebral blood flow (rCBF) map before, during, and after short-term focal ischemia.<sup>92</sup> ADC restored at one hour after recirculation, but N-acetylaspartate (NAA) demonstrated continuous attenuation after the induction of ischemia. Open square delineates the region of interest for magnetic resonance spectroscopy (MRS).

to 15% may be reversible in the acute stage of cerebral ischemia.

# Longitudinal studies: perpetual NAA attenuation against temporary restoration of ADC

NAA levels decline at an almost linear rate over time from the onset of ischemia. This time-dependent decline distinguishes NAA as a useful biomarker for the imaging of acute-stage ischemic stroke. Cvoro described a moderate correlation between the degree of NAA reduction and the time from onset.<sup>59</sup> Because the rate at which NAA levels decline is largely governed by the time from onset, residual blood flow and initial plasma glucose concentration, which are thought to be the most important factors determining the fate of ischemic tissue,<sup>83</sup> NAA levels can be used to predict the fate of tissue with an acute ischemic lesion independently. Combined DWI and PI studies may not be sufficient to predict tissue outcome successfully<sup>83,84</sup> except in some limited situations.<sup>85</sup> Thus, a study combining DWI, PI, and MRSI can be expected to improve the prediction of tissue outcome, particularly given that NAA findings complement those of DWI and PI.<sup>86</sup>

A representative situation in which dissociation between NAA and ADC is often observed is the case of transient ADC restoration following early reperfusion in both experimental models<sup>87,88</sup> and clinical<sup>89,90</sup> settings. Following early reperfusion within a few hours of onset, DWI suggested resolution of the ischemia within several hours of reperfusion; however, the area re-emerged several hours later on DWI and became infarcted within 24 hours. We tested the chronological changes in NAA levels in a rat model of focal ischemia using 90-min MCA occlusion.<sup>91</sup> Though ADC was restored to the normal range within one hour of reperfusion in the part of ischemic temporal cortex and lateral basal ganglia in which ischemic insult should be mild, NAA levels continued to show a decline in the same area (Fig. 6). The mechanism of NAA attenuation despite ADC resolution has not been solved in detail. However, one clue may be found in reports suggesting that energy restoration after recirculation could re-establish membrane potential (which is maintained by  $Na^+/K^+$  ATP pump activity) to a marginally survivable level following ischemic insult and thereby normalize DWI. Nevertheless, energy production following reperfusion may not be sufficient to maintain other energy-dependent mechanisms, such as NAA synthesis, protein synthesis,<sup>92</sup> and DNA repair via poly(ADP-ribose) polymerase (PARP),<sup>93</sup> at a level sufficient to ensure long-term cell survival.

#### Conclusion

Direct reflection of energy metabolism and nearlinear time-dependency during acute-stage cerebral ischemia make NAA a unique biomarker for the clinical assessment of ischemic stroke. Despite the challenges remaining for the application of MRS(I) in the daily clinical setting, the levels of NAA with those of lactate measured in the CNS can provide one of the most promising biomarkers available for the assessment of pathophysiological changes that occur during acute-stage ischemic stroke.

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# Reduced plasma desmosterol-to-cholesterol ratio and longitudinal cognitive decline in Alzheimer's disease

Yoshiaki Sato<sup>a</sup>, Francois Bernier<sup>a</sup>, Yasukazu Yamanaka<sup>b</sup>, Ken Aoshima<sup>a</sup>, Yoshiya Oda<sup>c</sup>, Martin Ingelsson<sup>d</sup>, Lars Lannfelt<sup>d</sup>, Akinori Miyashita<sup>e</sup>, Ryozo Kuwano<sup>e</sup>, Takeshi Ikeuchi<sup>e</sup>,\*

> <sup>a</sup>Eisai Co., Ltd., Tsukuba, Ibaraki, Japan <sup>b</sup>EIDIA Co., Ltd., Inashiki-gun, Ibaraki, Japan

<sup>c</sup>Eisai Inc., Andover, MA, USA

<sup>d</sup>Geriatrics, Department of Public Health and Caring Sciences, Uppsala University, Uppsala, Sweden <sup>e</sup>Department of Molecular Genetics, Brain Research Institute, Niigata University, Niigata, Japan

Abstract	<b>Background:</b> We here examined whether plasma desmosterol-to-cholesterol ratio (DES/CHO) is decreased in patients with Alzheimer's disease (AD) and investigated the association between plasma DES/CHO and longitudinal cognitive decline.
	<b>Methods:</b> Plasma DES/CHO of AD patients and age-matched controls in a Japanese cross-sectional cohort was determined. Plasma DES/CHO at baseline and follow-up visits was assessed in relation to cognitive decline in Japanese and Swedish longitudinal cohorts.
	<b>Results:</b> Plasma DES/CHO was significantly reduced in Japanese AD patients and significantly correlated with Mini-Mental State Examination (MMSE) score. The longitudinal analysis revealed that plasma DES/CHO in AD patients shows a significant decrease at follow-up intervals. The decline in plasma DES/CHO is larger in the AD group with rapid progression than in that with slow progression. The changes in plasma DES/CHO significantly correlated with changes in the MMSE score. <b>Conclusion:</b> Plasma DES/CHO is decreased in AD patients and may serve as a longitudinal surro-
	gate marker associated with cognitive decline. © 2015 The Alzheimer's Association. Published by Elsevier Inc. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).
Keywords.	Alzheimer's disease: Mild cognitive impairment: Blood-based biomarker: Desmosterol: Longitudinal biomarker

#### 1. Introduction

Alzheimer's disease (AD) is one of the most common and debilitating neurodegenerative disorders of the aging population. AD manifests itself as a progressive decline in memory accompanied by other cognitive and functional disabilities [1]. From the viewpoint of clinical practice and therapeutic clinical trials in AD, biomarkers are becoming increasingly important particularly when disease-modifying drugs will become available. Numerous studies have shown that tau, phosphorylated tau, and amyloid- $\beta$  (A $\beta$ ) 42 in cerebrospinal

E-mail address: ikeuchi@bri.niigata-u.ac.jp

fluid (CSF) are reliable biomarkers for AD diagnosis [2–4]. However, the CSF examination of AD patients has not been broadly applied in general clinical practice because lumbar puncture to obtain CSF is relatively invasive and time consuming. Moreover, these CSF markers do not seem to be associated with longitudinal cognitive decline in patients with AD [5]. Thus, there is a compelling need to establish a noninvasive biomarker for AD that follows the disease progression. Efforts to find reliable blood-based biomarkers for AD have met with little success [6]. Several reports have been published describing altered levels of proteins, peptides, or metabolites in patients with AD, but those blood-based biomarkers have proven difficult to replicate in independent studies [6], highlighting the importance of multiple validations.

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<sup>\*</sup>Corresponding author. Tel.: +81-25-227-2343; Fax: +81-25-227-0793.

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Table 1 Demographic characteristics of AD patients and age-matched cognitively normal controls in the Japanese cross-sectional cohort

^		
Variable	Control $(n = 201)$	AD $(n = 200)$
Female (%)	72	75
Age, mean (SD)		
Age at examination (y)	77.6 (4.7)	77.6 (5.4)
Age at onset (y)	n/a	73.5 (5.0)
MMSE, mean (SD)	28.7 (1.5)	17.0 (5.2)**
APOE genotype		
2*3	15	6
2*4	2	1
3*3	151	71
3*4	32	99
4*4	1	23
DES/CHO $(10^{-6})$ , mean (SD)	456 (119)	357 (134)**

Abbreviations: AD, Alzheimer's disease; n/a, not available; MMSE, Mini-Mental State Examination; DES/CHO, desmosterol-to-cholesterol ratio.

\*\*P < .01.

In our previous report, we found that the plasma desmosterol-to-cholesterol ratio (DES/CHO) is significantly decreased in Caucasian patients with AD and subjects with mild cognitive impairment (MCI) [7]. Desmosterol is the most abundant precursor but rarely exceeds 1% of total brain sterols because the conversion from desmosterol to cholesterol is tightly regulated by the enzyme 3-hydroxysterol 24-reductase (DHCR24) [8]. A substantially higher desmosterol concentration in the hippocampus could be attributed to neurogenesis and synaptic plasticity that take place in the adult dentate gyrus [9]. Conversely, a decrease in desmosterol level in the hippocampus could at least in part correlate with the reduced number of progenitor cells differentiating into neurons [10]. These reports suggest an important role of desmosterol in the brain.

With this background, we here measured plasma DES/ CHO of samples from a large Japanese cohort to extend our previous result that plasma DES/CHO is decreased in patients with AD in a different ethnic group. Furthermore, we

Table 2

Demographic characteristics of subjects in the longitudinal study

performed longitudinal studies to determine the association between plasma DES/CHO and cognitive decline in patients with AD over time.

#### 2. Materials and methods

#### 2.1. Subjects

For cross-sectional analysis, plasma samples of 200 patients with AD and 201 age-matched cognitively normal elderly individuals (older than 65 years) were collected from seven clinical institutions in Japan (Table 1). The diagnosis of AD was made on the basis of the criteria of the National Institute of Neurological and Communicative Diseases and the Stroke–Alzheimer's Disease and Related Disorders Association [11]. Each participant was asked to complete the Mini-Mental State Examination (MMSE) [12]. *APOE* genotyping was performed as previously reported [13].

For longitudinal analysis, we used 17 subjects with AD (Japanese longitudinal cohort collected at Niigata University Hospital) and 28 subjects (Swedish longitudinal cohort consisting of 6 control, 12 MCI, and 10 AD subjects collected at Uppsala University Hospital), whose blood was drawn at two different time points (Table 2). Additional longitudinal plasma samples of 30 subjects at least at 3 different time points were obtained from Uppsala University Hospital (AD, n = 6; MCI, n = 6; control, n = 2) or purchased from PrecisionMed, Inc. (AD, n = 12; control, n = 4) (San Diego, CA, USA). The criteria of Petersen et al. [14,15] were used for the diagnosis of MCI. To be considered as having MCI, the patients had to be free of significant underlying medical, neurologic, or psychiatric illness and meet the following criteria: (1) subjective memory complaint, (2) objective signs of decline in any cognitive domain, (3) intact activities of daily living, and (4) clinical features not fulfilling the DSM-IV/ICD-10 criteria for dementia [16]. The two AD/MCI groups with slow and rapid progression were classified on the basis of

	Japanese cohort	Swedish cohort			Combined cohort
Variable	AD $(n = 17)$	Control $(n = 6)$	MCI (n = 12)	AD $(n = 10)$	MCI/AD ( $n = 39$ )
Female (%)	71	75	33	30	56
Age, mean (SD)	68 (8)	67 (9)	62 (8)	66 (10)	66 (9)
Follow-up time, y, mean (SD)	2.0 (1.0)	2.7 (0.8)	1.8 (0.9)	2.2 (0.9)	2.1 (1.0)
MMSE, mean (SD)					
Baseline	20 (5)	30 (1)	28 (2)	25 (3)	24 (6)
Follow-up	16 (7)	30 (0)	28 (2)	22 (3)	21 (7)
ΔMMSE	-4 (4)**	0(1)	0 (2)	-3 (3)*	-2 (4) **
DES/CHO $(10^{-6})$ , mean (SD)					
Baseline	329 (103)	654 (146)	660 (200)	556 (232)	489 (225)
Follow-up	290 (79)	661 (130)	607 (237)	487 (276)	438 (237)
% Change	-10 (14)*	1.7 (5.1)	-8.3 (21)	-18 (27)	-11 (20)**

Abbreviations: AD, Alzheimer's disease; MCI, mild cognitive impairment; MMSE, Mini-Mental State Examination; DES/CHO, desmosterol-to-cholesterol ratio.

\*P < .05, \*\*P < .01.



Fig. 1. Decreased plasma DES/CHO in patients with AD. (A) Comparison of plasma desmosterol-to-cholesterol ratio (DES/CHO) between 201 control and 200 AD subjects from a large Japanese cohort. (B) Comparison of plasma DES/CHO among male control (M\_Cont), female control (F\_Cont), male AD (M\_AD), and female AD (F\_AD) subjects. (C) Comparison of plasma DES/CHO between control subjects without *APOE*  $\varepsilon 4$  (nonE4\_Cont) and with *APOE*  $\varepsilon 4$  (E4\_Cont), and AD patients without *APOE*  $\varepsilon 4$  (nonE4\_AD) and with *APOE*  $\varepsilon 4$  (E4\_AD). (D) Comparison of plasma DES/CHO among groups classified as MMSE groups. \*P < .05; \*\*P < .01.

the mean change in MMSE score (change from the baseline) as threshold. Written informed consent was obtained from each of the participants (or the respective legal guardian); the study was approved by the appropriate university, hospital, and company institutional ethics committees.

#### 2.2. Blood sampling and laboratory measurement

Peripheral blood samples were obtained from each participant using the commercially available blood collection tubes (Terumo Venoject for Japanese cohort and BD Vacutainer for Swedish cohort) containing EDTA as the anticoagulant. Plasma was separated by centrifugation at 1500 g for 15 minutes at room temperature before being aliquoted and stored at  $-80^{\circ}$ C until analysis.

The concentrations of desmosterol and cholesterol were measured with a liquid chromatography mass spectrometer (LC/MS), as described previously [7]. Briefly, 25  $\mu$ L of plasma was spiked with cholesterol-25,26,26,26,27,27,27-D7 and desmosterol-26,26,26,27,27,27-D6 as internal standards. Fifty percent potassium hydroxide was then added to the solution, which was then mixed thoroughly and incu-

bated at 70°C for 60 minutes. After the incubation, 2 mL of hexane and 0.5 mL of phosphate-buffered saline (pH 6.8) were added and mixed well. The solution was centrifuged for 10 minutes at 2000 g, and the upper organic phase was transferred to a new tube. The lower layer was extracted with an additional 1 mL of hexane, which was also added to the organic-phase extract. The solvents were evaporated to dryness under a nitrogen gas stream at 40°C, and the obtained pellet was reconstituted in ethanol and the resulting solution was subjected to liquid chromatography/atomospheric pressure chemical ionization-mass spectrometer analysis as described previously [7].

#### 2.3. Statistical analysis

Values are shown as mean  $\pm$  standard deviation. Correlations between different variables were assessed using the Pearson correlation coefficient on log-transformed data. The *t* test or analysis of variance was carried out to determine differences between two or more groups. Nonparametric tests (Mann-Whitney *U* test) were carried out when the variables were not normally distributed. The paired *t* test was



Fig. 2. Change in plasma DES/CHO between baseline and follow-up visits. AD/MCI subjects were classified into two groups, namely, those with slow progression (A, D, G) and those with rapid progression (B, E, H) on the basis of the mean  $\Delta$ MMSE between baseline and follow-up visits in a Japanese cohort (A–C), a Swedish cohort (D–F), and a combined cohort (G–I). Plasma DES/CHO remains stable in the group with slow progression (A, D, G) whereas in the group with rapid progression, a significant decline over time was found (B, E, H). The change in plasma DES/CHO was significantly larger in the group with rapid progression than in the group with slow progression (C, F, I). \**P* < .05; \*\**P* < .01.

carried out to see the difference in the individual values between baseline and follow-up visits. The statistical significance was set at P < .05.

#### 3. Results

#### 3.1. Cross-sectional study of plasma desmosterol

The characteristics of the AD and age-matched control subjects included in this cross-sectional study are listed in Table 1. A significant decline in plasma DES/CHO was observed in patients with AD compared with control subjects (P < .01; Fig. 1A). The decline in plasma DES/CHO in AD patients was significant regardless of gender or *APOE*  $\varepsilon$ 4 status (Fig. 1B and C). There was a significant decline in plasma DES/CHO in AD patients with both the *APOE*  $\varepsilon$ 3/3 and  $\varepsilon$ 3/4 genotypes (data not shown; see Supplementary Fig. 1A and B for review). Plasma DES/

CHO of female subjects in both the AD and control groups was significantly lower than that of male subjects (Fig. 1B, P < .01). No significant correlation of plasma DES/CHO with age was observed (data not shown). A significant correlation between plasma DES/CHO and MMSE score in both males and females was observed (data not shown; see Supplementary Fig. 1C and D). We divided subjects of this cohort into four MMSE score groups: high MMSE score  $(30 \ge MMSE \ge 26)$ , middle MMSE score  $(25 \ge MMSE)$  $\geq$  20), low MMSE score (19  $\geq$  MMSE  $\geq$  10), and very low MMSE score (9  $\geq$  MMSE  $\geq$  0). We then compared DES/CHO among these groups. A significant difference between the high MMSE score group and the other MMSE score groups was observed (Fig. 1D). The linear trend analysis revealed that there was also a significant change showing that groups with lower MMSE scores had lower DES/CHO (trend *t* test: P < .01).



Fig. 3. Comparison of longitudinal change in plasma DES/CHO between control and AD subjects. (A) Longitudinal changes in plasma DES/CHO at two different points were compared between control subjects and AD patients from the combined cohort. \*P < .05. (B) Longitudinal changes in plasma DES/CHO at multiple points were compared between control subjects and AD patients from the Swedish cohort and a commercially available resource. \*\*P < .01.

#### 3.2. Longitudinal analysis

Longitudinal plasma samples were collected from two clinical institutes, namely, Niigata University Hospital (Japanese cohort) and Uppsala University Hospital Memory Clinic (Swedish cohort). Forty-seven participants composed of 17 subjects in the Japanese cohort and 30 subjects in the Swedish cohort were included, and the demographic characteristics of these subjects are listed in Table 2. At baseline, DES/CHO in Japanese AD patients was significantly lower than that of Swedish AD patients (P < .01).

In the Japanese longitudinal cohort, the average change in the MMSE score ( $\Delta$ MMSE) between the baseline and follow-up visits was  $-4 \pm 4$  with a change in DES/CHO



Fig. 4. Correlation between longitudinal changes in MMSE and plasma DES/CHO (%) in AD patients, MCI patients, and normal subjects. There were 122 points for blood collection, consisting of 30 baselines and 92 follow-ups (AD, 52 follow-ups; MCI, 13 follow-ups; and control, 27 follow-ups). There was a significant correlation between changes in MMSE and plasma DES/CHO (r = 0.37, P < .01).

of  $-10 \pm 14\%$  (Table 2). Both MMSE score and plasma DES/CHO significantly decreased between the two visits in the Japanese cohort (paired *t* test, *P* < .05). In the Swedish cohort, the MMSE score ( $-3 \pm 3$ ) decreased significantly in AD patients between the baseline and follow-up visits (*P* < .05). However, in this cohort, there was no significant change in DES/CHO in both the AD patients and MCI subjects (Table 2). In the combined AD/MCI cohort, MMSE score ( $-2 \pm 4$ ) and plasma DES/CHO ( $-11 \pm 20\%$ ) decreased significantly between the two visits (Table 2).

Next, we divided the AD/MCI subjects into two groups, namely, those with slow or rapid progression, on the basis of their mean  $\Delta$ MMSE in the Japanese (cutoff score, -4) and Swedish cohorts (cutoff score, -2) and compared the longitudinal change in plasma DES/CHO between groups (Fig. 2). Although the AD/MCI group with slow progression did not show any significant change in plasma DES/CHO (Fig. 2A, D, and G), groups with rapid progression showed a significant decrease in plasma DES/CHO between the baseline and follow-up visits (Fig. 2B, E, and H). In addition, the change in plasma DES/CHO was significantly larger in the AD/MCI group with rapid progression than in the group with slow progression in the Japanese, Swedish, and combined cohorts (Fig. 2C, F, and I).

We further compared the longitudinal change in plasma DES/CHO in control subjects and AD patients (Fig. 3). The change in DES/CHO between the two visits was  $-12.8 \pm 19.7$  in the AD patients, which was significantly larger than that in the control subjects ( $1.7 \pm 5.3$ ; Fig. 3A). These results suggest that although plasma DES/CHO in normal subjects remained stable, plasma DES/CHO in AD patients tended to decline over time.

Finally, we performed another longitudinal study to determine the association between plasma DES/CHO and  $\Delta$ MMSE in 30 participants, including 18 AD, 6 MCI, and 6 control subjects from either the Swedish cohort or from PrecisionMed. Blood samples were collected every year or

every 6 months from the participants. There were 122 points for blood collection, consisting of 30 baselines and 92 follow-ups. The correlation between the  $\Delta$ MMSE and the change in plasma DES/CHO (compared with baseline) determined using all 92 follow-up points is shown in Fig. 4. There was a significant correlation between the longitudinal  $\Delta$ MMSE and the change in DES/CHO from the baseline (P = .01, r = 0.37). All the changes in MMSE score and plasma DES/CHO in each of the participants are shown (see Supplementary Fig. 2). In addition, AD patients showed a significant decrease in plasma DES/CHO at follow-up intervals ( $-7.7 \pm 36.8\%$ ) compared with the control subjects ( $6.4 \pm 12.3\%$ ) in this cohort (Fig. 3B).

#### 4. Discussion

The present cross-sectional study using a Japanese cohort was undertaken to replicate our previous finding that plasma DES/CHO is decreased in Caucasian patients with AD. The following points were confirmed in the present Japanese and previously reported Caucasian cross-sectional cohorts: (1) plasma DES/CHO was decreased in patients with AD in comparison with control subjects, (2) the decrease in plasma DES/CHO in AD patients was independent of gender and *APOE* genotype, (3) female subjects tended to have a lower plasma DES/CHO than male subjects, and (4) plasma DES/ CHO correlated significantly with the MMSE score. Taken together, the results suggest that plasma DES/CHO may be a potential diagnostic biomarker reflecting cognitive dysfunction in AD patients.

Recently, Popp et al. [17] have reported that the plasma desmosterol level does not change in AD patients. The discrepancy in finding between that and the present study may be explained by differences in the analytical methods used to determine the concentration of desmosterol. We previously showed that the LC/MS method that we used in the present study enables the purification of desmosterol in plasma more efficiently than the gas chromatography method used in the study by Popp et al. [7,17]. Thus, the LC/MS method is likely to be more suitable for the measurement of plasma desmosterol concentration.

Here, we determined for the first time the longitudinal change in plasma DES/CHO and examined a possible association with concurrent cognitive decline in AD/MCI patients. Our results revealed (1) that plasma DES/CHO was relatively stable over time in cognitively normal controls, whereas it significantly decreased in AD patients; (2) a more pronounced decline in plasma DES/CHO in the AD/ MCI group with rapid progression than in the group with slow progression; and (3) that the longitudinal change in plasma DES/CHO positively correlated with the change in the MMSE score. These results suggest that the plasma DES/CHO change is associated with the cognitive decline in AD and might be used to monitor the progression of cognitive decline in patients with AD. It will be interesting to clarify the usefulness of monitoring plasma DES/CHO as a surrogate marker for evaluating the effects of clinical drug trials in patients with AD.

Our longitudinal study suggests that the plasma DES/CHO changes before the appearance of clinical symptoms, as determined by MMSE in some cases (see Supplementary Fig. 2 for review; subjects A, C, and H). The result obtained from the subject with MCI that converted to AD (see Supplementary Fig. 2 for review; subject S) may suggest that plasma DES/CHO is useful as a progression marker to monitor the conversion from MCI to AD. In this connection, recent lipidomic analysis showed that the quantification of several lipid metabolites in plasma, such as phosphatidylcholine and acylcarnitine, is useful for predicting phenoconversion to amnestic MCI or AD in cognitively normal subjects [18].

There is now accumulating evidence that cholesterol metabolism may be relevant to the production and clearance of A $\beta$  and thus to the A $\beta$ -related toxicity in the pathogenesis of AD [19]. The strongest genetic risk factor for sporadic AD is the  $\varepsilon 4$  allele of *APOE*, which encodes apolipoprotein E (apoE), with a crucial role in cholesterol metabolism [20]. The presence of APOE  $\varepsilon 4$  may contribute to the pathologic accumulation and deposition of cerebral AB at early preclinical disease stages [21]. A recent study has shown that CSF apoE levels are decreased in patients with AD and that MCI in subjects with a low CSF apoE level will more likely convert to AD [22]. An interaction between APOE genotype and plasma desmosterol level may be postulated because desmosterol is the immediate precursor of cholesterol. However, the plasma desmosterol level was not clearly associated with APOE genotype in this study.

It is of particular interest that the level of desmosterol in the AD brain was found to be lower than that of control brain [7,23]. It has been demonstrated that the levels of steroid hormones (e.g., progesterone, pregnenolone, and 17aOHprogesterone) that exhibit inhibitory activity against DHCR24 are decreased in the AD brain, particularly in the vicinity of plaques and neurofibrillary tangles [24]. Notably, the concentration of desmosterol is 100-fold higher in the rat brain than in the rat liver [25], which implies that most of the desmosterol in the blood might originate from the brain. Taken together, it could be speculated that brain desmosterol level may decrease with an increase in DHCR24 activity in the AD brain; this may subsequently result in a change in plasma DES/CHO. The question of why plasma DES/CHO decreases in patients with AD and is associated with longitudinal cognitive decline in the course of the disease warrants further investigation.

Although our findings, using samples from cross-sectional and longitudinal cohorts, are interesting, our study has some limitations. We did not analyze the samples from other types of dementia, including dementia with Lewy bodies, frontotemporal dementia, and vascular dementia. An additional cross-sectional study that includes samples from other types of dementia will be necessary. The number of samples from longitudinal cohorts in this study is relatively small. Longitudinal studies with a prospective design using a larger number of samples should be performed to confirm the utility of plasma DES/CHO as a longitudinal biomarker. Moreover, it is important to understand how early plasma DES/CHO starts to decline using longitudinal samples from asymptomatic AD subjects with amyloid deposition confirmed by amyloidpositron emission tomography imaging. Although our findings need to be validated in independent cohorts, our data suggest that the use of plasma desmosterol as a blood biomarker can be useful in the diagnosis of AD and also in monitoring disease progression.

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#### Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.dadm.2014.11.009.

#### **RESEARCH IN CONTEXT**

- 1. Systemic review: There is a compelling need to establish blood-based biomarker to diagnose Alzheimer's disease (AD) and monitor the disease progression. A previous study reported that plasma desmosterol-to-cholesterol ratio (DES/CHO) is significantly decreased in Caucasian patients with AD.
- Interpretation: We found that plasma DES/CHO was significantly reduced in Japanese AD patients. The longitudinal study revealed (1) that plasma DES/CHO was relatively stable in normal controls, whereas it significantly decreased in AD patients; (2) a more pronounced decline in plasma DES/CHO in the AD/MCI group with rapid progression than in that with slow progression; and (3) that the longitudinal change in plasma DES/CHO positively correlated with the change in MMSE score.
- 3. Future directions: A future cross-sectional study that includes samples from other types of dementia and longitudinal studies with a prospective design using a larger number of samples need to be performed to confirm the utility of plasma DES/CHO.

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# Figure legend Supplementary Fig. 1:

(A) Comparison of plasma DES/CHO between control subjects and AD patients with APOE  $\varepsilon 3/3$ . \*\*, p<0.01. (B) Comparison of plasma DES/CHO between control subjects and AD patients with APOE  $\varepsilon 3/4$ . \*\*, p<0.01. (C) Correlation between plasma DES/CHO and MMSE score in males (n=105).
(D) Correlation between plasma DES/CHO and MMSE score in females (n=281).



### Supplementary Fig. 2. Sato et al. (for review)

#### Figure legend for Supplementary Fig. 2:

Changes in plasma DES/CHO and MMSE scores in AD patients (A-R), MCI subjects (S-X), and normal subjects (Y-IV). The longitudinal change in plasma DES/CHO (red line) was compared with the baseline value. Longitudinal MMSE scores are shown in blue.



## Supplementary Figure 2. Sato et al. (continued)

#### SHORT COMMUNICATION

# Systematic review and meta-analysis of Japanese familial Alzheimer's disease and FTDP-17

Kensaku Kasuga<sup>1,2,6</sup>, Masataka Kikuchi<sup>1,3,6</sup>, Takayoshi Tokutake<sup>4</sup>, Akihiro Nakaya<sup>1,5</sup>, Toshiyuki Tezuka<sup>4</sup>, Tamao Tsukie<sup>1,3</sup>, Norikazu Hara<sup>1</sup>, Akinori Miyashita<sup>1</sup>, Ryozo Kuwano<sup>1</sup> and Takeshi Ikeuchi<sup>1</sup>

Mutations in APP. PSEN1 and PSEN2 as the genetic causes of familial Alzheimer's disease (FAD) have been found in various ethnic populations. A substantial number of FAD pedigrees with mutations have been reported in the Japanese population; however, it remains unclear whether the genetic and clinical features of FAD in the Japanese population differ from those in other populations. To address this issue, we conducted a systematic review and meta-analysis of Japanese FAD and frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17) by literature search. Using this analysis, we identified 39 different PSEN1 mutations in 140 patients, 5 APP mutations in 35 patients and 16 MAPT mutations in 84 patients. There was no PSEN2 mutation among Japanese patients. The age at onset in Japanese FAD patients with PSEN1 mutations was significantly younger than that in patients with APP mutations. Kaplan-Meier analysis revealed that patients with MAPT mutations showed a shorter survival than patients with PSEN1 or APP mutations. Patients with mutations in different genes exhibit characteristic clinical presentations, suggesting that mutations in causative genes may modify the clinical presentations. By collecting and cataloging genetic and clinical information on Japanese FAD and FTDP-17, we developed an original database designated as Japanese Familial Alzheimer's Disease Database, which is accessible at http://alzdb.bri. niigata-u.ac.jp/.

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Mutations in APP, PSEN1 and PSEN2 as the genetic causes of familial Alzheimer's disease (FAD) have been found in various ethnic populations.<sup>1,2</sup> In addition, patients with mutations in MAPT associated with frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17) have been shown to exhibit Alzheimer's disease (AD)-like phenotypes.<sup>3,4</sup> Although a substantial number of FAD pedigrees have been reported in Japan, it is not yet clear whether the genetic and clinical features of FAD in the Japanese population differ from those in other ethnic populations. To characterize the genetic and clinical features of Japanese FAD and FTDP-17, we here performed a systematic review and meta-analysis, and developed an original database of Japanese FAD and FTDP-17.

To comprehensively review the previously reported Japanese FAD and FTDP-17 cases, we performed a systematic search for publications in PubMed and Ichushi, a bibliographic database of medical literature in Japanese. The terms 'familial Alzheimer', 'familial AD', 'FTDP-17', 'presenilin', 'PSEN1', 'PSEN2', 'APP' and 'MAPT' were used to search in PubMed, and the equivalent terms in Japanese were used to search in Ichushi. From the literature searches we found 60 English and 29 Japanese articles and/or abstracts that reported on Japanese FAD and FTDP-17 pedigrees bearing the causative mutations (Supplementary Table 1). Using the information obtained by the systematic literature review, we developed an original database for Japanese FAD and FTDP-17 designated as Japanese Familial Alzheimer's Disease database (JFADdb). In the database, each of the mutations in APP, PSEN1/2, MAPT and GRN was described in accordance with the reference sequences.<sup>5</sup> Information on age at onset, clinical manifestations, age at death and APOE genotype were included in the database (Supplementary Figure 1).

We identified 39 different PSEN1 mutations in 140 patients, 5 APP mutations in 35 patients and 16 MAPT mutations in 84 patients (Table 1). Among them, 10 PSEN1 mutations, 5 APP mutations and 11 MAPT mutations were not included in the well-known Alzheimer Disease and Frontotemporal Dementia Mutation database (http:// www.molgen.ua.ac.be/ADMutations/).6 No PSEN2 mutation has been found in Japanese FAD. The frequency of mutated genes in FAD patients in the Japanese population was not significantly different from those in other populations ( $\chi^2$ , P = 0.99).<sup>6</sup> Most FAD pedigrees show autosomal dominant inheritance; however, an APP  $\Delta$ E693 mutation was responsible for a recessively inherited FAD.<sup>7</sup> Sporadic occurrences of mutations were observed: five patients with PSEN1 mutations, one patient with APP mutation and two patients with MAPT mutations.

<sup>&</sup>lt;sup>1</sup>Department of Molecular Genetics, Brain Research Institute, Niigata University, Niigata, Japan; <sup>2</sup>Center for Transdisciplinary Research, Niigata University, Niigata, Japan; <sup>3</sup>Research Association for Biotechnology, Tokyo, Japan; <sup>4</sup>Department of Neurology, Brain Research Institute, Niigata University, Niigata, Japan and <sup>5</sup>Department of Genome Informatics, Graduate School of Medicine, Osaka University, Osaka, Japan <sup>6</sup>These authors contributed equally to this work.

Correspondence: Dr T Ikeuchi, Department of Molecular Genetics, Brain Research Institute, Niigata University, 1-757 Asahimachi, Niigata 951-8585, Japan. E-mail: ikeuchi@bri.niigata-u.ac.jp

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Table 1 Summary of genetic features of Japanese FAD and FTDP-17

Disease	Genes	Number of mutations	Number of pedigrees	Number of patients
FAD	PSEN1	39	40	140
	PSEN2	0	0	0
	APP	5 <sup>a</sup>	13	35
FTDP-17	MAPT	16	29	84
	GRN	2	2	2

Abbreviations: FAD, familial Alzheimer's disease; FTDP-17, frontotemporal dementia with parkinsonism linked to chromosome 17.

<sup>a</sup>APP duplication was included.

In our analysis, the majority of mutations (73%) was observed in a single small pedigree. Considering that novel mutations in FAD tend to be reported rapidly, note that there may be publication bias in the frequency of mutations in the database. Although rare, there were two *GRN* mutations in patients with primary progressive aphasia and frontotemporal lobar degeneration (FTLD).<sup>8</sup> Because the number of *GRN* mutations was too small, the patients with *GRN* mutations were excluded from further meta-analysis.

The ages at onset were  $44 \pm 8$  years (mean  $\pm$  s.d.) in patients with PSEN1 mutations (n=87),  $54 \pm 9$  years in APP mutations (n=23)and  $45 \pm 10$  years in *MAPT* mutations (n = 51). These ages at onset of FAD in our analysis are consistent with those reported in other ethnic populations.<sup>6,9</sup> The age at onset in patients with APP mutations was significantly older than those in patients with PSEN1 or MAPT mutations (Figure 1a). The clinical phenotypes of patients with MAPT mutations were classified into three subgroups: FTLD,10 AD-like3,4 and progressive supranuclear palsy (PSP) phenotypes.8 The age at onset in patients with the FTLD or PSP phenotype was significantly younger than that with the AD-like phenotype (Supplementary Figure 2). APOE genotypes did not significantly modify the age at onset in patients with causative mutations (Supplementary Figure 3). There was no significant difference in age at death among the patients with mutations in the three genes (Figure 1b). The disease duration from age at onset to death in patients with MAPT mutations was significantly shorter than that with PSEN1 mutations (Figure 1c). The survival of patients after the onset was analyzed by Kaplan-Meier estimation, which revealed that patients with MAPT mutations showed a shorter survival than patients with PSEN1 or APP mutations (Supplementary Figure 4).

The clinical diagnosis of AD before the genetic testing was performed in 96% of patients with *PSEN1* mutations and 97% of patients with *APP* mutations (Supplementary Table 2). Notably, only 57% of patients with *MAPT* mutations were clinically diagnosed as having FTLD; 19% and 12% of patients with *MAPT* mutations were clinically diagnosed as having AD and PSP, respectively (Supplementary Table 2). This finding suggests that mutational screening of clinically diagnosed FAD patients should not only include *APP* and *PSEN1/2* mutations but also include *MAPT* mutations.

We next analyzed the frequency of each of the clinical manifestations including psychiatric symptoms, mood disorders, spastic paraparesis, parkinsonism and epilepsy/seizure (Figure 2). As expected, the frequencies of psychiatric symptoms and parkinsonism were significantly higher in patients with *MAPT* mutations. Spastic paraparesis, which is a characteristic symptom of 'variant AD with cotton-wool plaque pathology'<sup>11,12</sup> was observed in 15% of patients with *PSEN1* mutations, whereas none of the patients with *APP* mutations exhibited spastic paraplegia. Epilepsy/seizure was described in 8% of patients with *PSEN1* and 6% of patients with *APP* mutations,



**Figure 1** Age at onset and death, and disease duration in Japanese FAD and FTDP-17 patients. (a) Age at onset for patients grouped on the basis of *PSEN1, APP* and *MAPT* mutations. The horizontal line in the box indicates the median, the lower and upper boundaries of the box represent the lower and upper quartile boundaries, respectively, and whiskers are 1.5 times the interquartile range. Patients with *PSEN1* and *MAPT* mutation showed significantly younger age at onset than patients with *APP* mutations (\*\*P<0.01, ANOVA with *post hoc* Tukey's test). (b) Age at death of three groups with gene mutations. There was no significant difference in age at death among the groups. (c) Disease duration was defined as the period from age at onset to death. The disease courses of patients with *MAPT* mutations (7 ± 4 years, mean ± s.d.) were significantly shorter than those with *PSEN1* mutations (11±5) (\*P<0.05, ANOVA with *post hoc* Tukey's test). ANOVA, analysis of variance; FAD, familial Alzheimer's disease; FTDP-17, frontotemporal dementia with parkinsonism linked to chromosome 17.



**Figure 2** Frequency of each clinical manifestation in patients with Japanese FAD and FTDP-17. We investigated the presence or absence of psychiatric symptoms, mood disorders, spastic paraplegia, parkinsonism and epilepsy/seizure by careful reading of the original papers. We determined the frequency of each of the clinical manifestations by counting the number of patients for whom the presence of the manifestation was described in literature. In case there was no description of the manifestation, the patient was not counted as manifesting the manifestation. The observed frequencies of causative gene mutation were significantly different from the expected frequencies determined by residual analyses for  $\chi^2$  statistical analysis (\**P*<0.05, \*\**P*<0.01). FAD, familial Alzheimer's disease; FTDP-17, frontotemporal dementia with parkinsonism linked to chromosome 17.

whereas none of the patients with *MAPT* mutations exhibited epilepsy/seizure. Previous studies showed that the frequency of seizure was relatively high in patients with early onset of AD,<sup>13,14</sup> and low in patients with *MAPT* mutations.<sup>15</sup> These findings suggest that epilepsy/seizure is closely associated with amyloid pathology, and that tauopathy alone may not be sufficient to cause epilepsy. Taken together, mutations in causative genes may modify the clinical presentations in patients with familial dementia.

In summary, we have comprehensively collected, cataloged and systematically meta-analyzed the data from currently available data on Japanese FAD and FTDP-17. We made all the results publicly available on the online database 'JFADdb'. The database may provide information useful for estimating the age at onset and the natural course of disease in future preventive or therapeutic trials of Japanese FAD.

#### CONFLICT OF INTEREST

The authors declare no conflict of interest.

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# ApoE-isoform-dependent cellular uptake of amyloid- $\beta$ is mediated by lipoprotein receptor LR11/SorLA





Ryuji Yajima<sup>a</sup>, Takayoshi Tokutake<sup>a</sup>, Akihide Koyama<sup>b</sup>, Kensaku Kasuga<sup>a,b,c</sup>, Toshiyuki Tezuka<sup>a,c</sup>, Masatoyo Nishizawa<sup>a</sup>, Takeshi Ikeuchi<sup>c,\*</sup>

<sup>a</sup> Department of Neurology, Brain Research Institute, Niigata University, Niigata, Japan

<sup>b</sup> Center for Transdisciplinary Research, Niigata University, Niigata, Japan

<sup>c</sup> Department of Molecular Genetics, Brain Research Institute, Niigata University, Niigata, Japan

#### A R T I C L E I N F O

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#### ABSTRACT

The formation of senile plaques composed of  $\beta$ -amyloid (A $\beta$ ) in the brain is likely the initial event in Alzheimer's disease (AD). Possession of the APOE  $\varepsilon$ 4 allele, the strong genetic factor for AD, facilitates the Aβ deposition from the presymptomatic stage of AD in a gene-dosage-dependent manner. However, the precise mechanism by which apoE isoforms differentially induce the AD pathology is largely unknown. LR11/SorLA is a type I membrane protein that functions as the neuronal lipoprotein endocytic receptor of apoE and the sorting receptor of the amyloid precursor protein (APP) to regulate amyloidogenesis. Recently, LR11/SorLA has been reported to be involved in the lysosomal targeting of extracellular amyloid- $\beta$  (A $\beta$ ) through the binding of A $\beta$  to the vacuolar protein sorting 10 (VPS10) protein domain of LR11/SorLA. Here, we attempted to examine the human-apoE-isoform-dependent effect on the cellular uptake of  $A\beta$  through the formation of a complex between an apoE isoform and LR11/SorLA. Cell culture experiments using Neuro2a cells revealed that the cellular uptake of secreted apoE3 and apoE4 was enhanced by the overexpression of LR11/SorLA. In contrast, the cellular uptake of apoE2 was not affected by the expression of LR11/SorLA. Co-immunoprecipitation assay revealed that apoE-isoform-dependent differences were observed in the formation of an apoE-LR11 complex (apoE4 > apoE3 > apoE2). ApoEisoform-dependent differences in cellular uptake of FAM-labeled Aβ were further investigated by coculture assay in which donor cells secrete one of the apoE isoforms and recipient cells express FL-LR11. The cellular uptake of extracellular  $A\beta$  into the recipient cells was most prominently accentuated when cocultured with the donor cells secreting apoE4 in the medium, followed by apoE3 and apoE2. Taken together, our results provide evidence for the mechanism whereby human-apoE-isoform-dependent differences modulate the cellular uptake of A<sup>β</sup> mediated by LR11/SorLA.

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#### 1. Introduction

Alzheimer's disease (AD) has emerged as the most prevalent form of dementia in adults. The cardinal pathological features of AD brain are the loss of synapses and neurons as well as abnormal accumulation of misfolded proteins such as  $\beta$ -amyloid (A $\beta$ ) and

http://dx.doi.org/10.1016/j.bbrc.2014.11.111 0006-291X/© 2014 Elsevier Inc. All rights reserved. phosphorylated tau. The deposition of A $\beta$  in the brain is considered to be the earliest event in AD [1] and is substantially affected by the *APOE* genotype, which has been shown to be a strong genetic risk factor for AD in various ethnic populations [2,3]. The presence of the *APOE*  $\varepsilon$ 4 allele markedly increases the risk of developing AD and decreases the age at onset by 10 to 15 years; in contrast, the  $\varepsilon$ 2 allele confers protection against AD development [4,5]. Although there have been numerous studies attempting to elucidate the mechanism underlying the increase or decrease in the risk of AD posed by different apoE isoforms [6], the exact mechanisms are still not completely understood. Thus, to address the differential effects of apoE isoforms on the amyloid pathology and A $\beta$  metabolism is important in the elucidation of the AD pathogenic pathway.

LR11, also known as SorLA, is a type I membrane protein that has homology to members of the LDL receptor family and vacuolar

Abbreviations: AD, Alzheimer's disease; A $\beta$ , amyloid- $\beta$ ; APP, amyloid precursor protein; VPS10, vacuolar protein sorting 10; TACE, tumor necrosis factor- $\alpha$ -converting enzyme; FL, full-length; CSF, cerebrospinal fluid; PCR, polymerase chain reaction; GFP, green fluorescent protein; HEK, human embryonic kidney; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; co-IP, coimmunoprecipitation.

<sup>\*</sup> Corresponding author at: Department of Molecular Genetics, Brain Research Institute, Niigata University, 1-757 Asahimachi, Niigata 951-8585, Japan. Fax: +81 25 227 0793.

E-mail address: ikeuchi@bri.niigata-u.ac.jp (T. Ikeuchi).

protein sorting 10 (VPS10) protein family. LR11/SorLA is predominantly expressed on neurons in the cerebral cortex and hippocampus [7]. Reduced expression of LR11/SorLA in the brains of AD patients and subjects with mild cognitive impairment has been reported [8]. Furthermore,  $A\beta$  deposition is more prominent in LR11 knockout mice crossed with a mouse AD model [9]. These findings suggest the protective role of LR11/SorLA against AD pathology. In addition, some of the single-nucleotide polymorphisms (SNPs) in SORL1 encoding LR11/SorLA were found to be associated with a risk of sporadic AD in various ethnic backgrounds [3]. Full-length (FL) LR11/SorLA is cleaved by tumor necrosis factor- $\alpha$ -converting enzyme (TACE) to generate soluble LR11 [10]. We previously reported that the level of soluble LR11/SorLA was significantly high in the cerebrospinal fluid (CSF) of patients with AD [11]. The APOE E4 carriers among AD patients showed higher levels of soluble LR11/SorLA than the  $\varepsilon$ 4 noncarriers [11]. This suggests that the level of soluble LR11/SorLA in CSF is associated with AD in an apoE-isoform-dependent manner.

LR11/SorLA functions as the neuronal lipoprotein endocytic receptor of ApoE [12] and the sorting receptor of the amyloid precursor protein (APP) to regulate amyloidogenesis in endosomal and Golgi compartments [13]. LR11/SorLA interacts with newly synthesized APP in the Golgi apparatus and prevents the trafficking of APP into the cellular compartment where secretases reside. Consequently, the overexpression of LR11/SorLA in cultured cells reduces amyloidogenic processing [14]. Very recently, a novel function of LR11/SorLA has been reported. Caglayan et al. reported that LR11/SorLA plays a role in the lysosomal targeting of newly generated  $A\beta$  through the binding of  $A\beta$  to the amino-terminal VPS10 protein domain of LR11 [15]. This binding enhances Aβ clearance in lysosomes by internalization of extracellular  $A\boldsymbol{\beta}$  through the receptor LR11/SorLA. With this as a background, we here attempted to determine the apoE-isoform-dependent effects on the cellular uptake of  $A\beta$  through the formation of a complex between an spoE isoform and LR11/SorLA.

#### 2. Materials and methods

#### 2.1. cDNA cloning and construction of expression plasmids

Complementary DNA (cDNA) of human full-length LR11 was amplified by PCR using pCMV6 plasmid cDNA containing human LR11 (Origene, Rockville, USA) and cloned into the pcDNA3.1 vector (Life Technologies, Carlsbad, USA). Human APOE  $\varepsilon$ 3 cDNA was cloned and inserted into the pcDNA3.1 vector. We generated two allelic variants of human APOE, namely, APOE  $\varepsilon$ 2 and  $\varepsilon$ 4, by mutagenesis. Each isoform of APOE was cloned into the pEGFP vector (Clontech, Mountain View, USA). All constructs generated from PCR products were verified by DNA sequencing.

#### 2.2. Cell culture and transfection

Mouse neuroblastoma Neuro2a (N2a) and human embryonic kidney (HEK)293T cells were cultured as previously described [16]. Transient transfection of plasmid DNA into cells was carried out by using Lipofectamine 2000 (Life Technologies). To generate stable cell lines, N2a cells transfected with cDNA encoding human apoE2, apoE3, apoE4 or FL-LR11 were selected using G418. Peptides of Aβ40 were purchased from Wako (Tokyo, Japan) and FAM-labeled Aβ40 from AnaSpec (Fremont, USA). They were dissolved in 1% ammonium hydroxide.

#### 2.3. Coculture system

Coculture experiments were performed essentially as previously described [16]. Donor HEK293T cells transiently transfected

with cDNA encoding human apoE2, apoE3, apoE4, apoE2-GFP, apoE3-GFP, or apoE4-GFP were cultured on a dish with a 1.0  $\mu$ m filter insert (BD Bioscience, San Diego, USA) for 24 h. The donor HEK293T cells on the dish were placed in a six-well culture dish containing the recipient N2a cells mock-transfected or transiently transfected with the FL-LR11 cDNA construct. After 24 h of coculture, the lysate of the recipient cells and the medium were subjected to Western blot analysis.

#### 2.4. Western blot analysis

The cells were solubilized using a lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 0.5% NP-40, 0.5% sodium deoxycholate, and 5 mM EDTA). The protein concentration of the detergentextracted lysates was determined using a bicinchoninic acid protein assay kit (Pierce, Rockford, USA). Equal amounts of protein were boiled in Laemmli sample buffer and then subjected to Tris-glycine or Tris-tricine SDS-PAGE system. The separated proteins were transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, USA) and then incubated with appropriate primary antibodies. FL-LR11 and sLR11 were detected using the monoclonal antibody 48/LR11 (BD Biosciences), which recognizes the extracellular domain of LR11. ApoE was visualized by the monoclonal antibody E6D7 (Sigma, St. Louis, USA). Aβ polypeptides were detected by the monoclonal antibody 6E10 (Covance, Berkeley, USA). GFP-fusion proteins were detected by the monoclonal antibody1E4 (MBL, Japan). Actin was detected using the goat anti-actin antibody I-19 (Santa Cruz Biotechnology, Dallas, USA). Immunoreactive bands were detected using an Immobilon Western Chemiluminescent HRP substrate (Millipore). The band intensities were quantified using a LAS system (GE Healthcare, Pittsburgh, USA).

#### 2.5. Co-immunoprecipitation assays

HEK293T cells were cotransfected with the cDNA encoding apoE2, apoE3, or apoE4 and with FL-LR11 cDNA. The cells were solubilized using co-IP buffer (1% CHAPS, 150 mM NaCl, 50 mM HEPES, pH 7.5). The supernatant was incubated with either an anti-LR11 antibody (48/LR11) or an anti-apoE antibody (AB947, Millipore) at 4 °C for 16 h. To immunoprecipitate the protein complex, 25  $\mu$ L of protein G Mag Sepharose beads (GE Healthcare) was added to the mixture, and the mixture was incubated on a rotator at 4 °C. The beads were collected on a magnetic stand and washed three times with the solubilization buffer. The immunoprecipitated proteins were released from the beads by incubation in Laemmli sample buffer. The obtained samples were analyzed by SDS–PAGE followed by Western blot analysis.

# 2.6. Fluorescence confocal laser scanning microscopy and image analysis

N2a cells treated with GFP-labeled apoE3 or FAM-labeled A $\beta$ 40 were cultured. All images were obtained using an inverted microscope (TE-300NT, Nikon, Japan) and a confocal microscope (CSU-10, Yokogawa Electric Corp, Japan). The obtained images were further analyzed with a quantitative analysis system (AquaCosmos, Hamamatsu Photonics, Japan).

#### 2.7. Statistical analyses

Data are shown as the mean  $\pm$  standard error of the mean (SEM). Statistical comparison between two groups was carried out by the Student *t*-test. For statistical comparison among several groups, we used one-way analysis of variance (ANOVA) followed

by the Tukey post hoc test. The statistical analyses were performed using SPSS ver. 12.0 J (SPSS Japan).

#### 3. Results

#### 3.1. Isoform-dependent change in level of secreted apoE by expression of LR11/SorLA

We first determined whether the expression of FL-LR11/SorLA affects the levels of apoE in the lysate and medium. N2a cells stably expressing human apoE3 were mock-transfected or transiently transfected with human FL-LR11 cDNA. The levels of apoE3 in the medium and lysate were determined using the anti-apoE antibody at different time points from 2 to 24 h after the medium was replaced. The level of apoE3 migrating at ~34 kDa and secreted into the medium increased after the medium replacement in a

time-dependent manner (Fig. 1A). The level of apoE3 in the medium was significantly lower in N2a cells transfected with FL-LR11 24 h after the medium change compared with mock-transfected cells, whereas the level of apoE in the lysate was comparable between the mock- and FL-LR11-transfected cells (Fig. 1B).

Next, we investigated whether apoE-isoform-dependent differences are observed in this study. N2a cells stably expressing human apoE2, apoE3, or apoE4, were mock-transfected or transiently transfected with FL-LR11, and the levels of apoE in the medium and lysate were determined by immunoblot analysis. The apoE ratio (medium/lysate) significantly decreased in apoE3and E4-expressing cells that were transiently transfected with FL-LR11 (Fig. 1C and D), whereas the apoE ratio (medium/lysate) was unchanged in apoE2-expressing cells transfected with FL-LR11. These findings suggest that the effect of LR11/SorLA on apoE ratio (medium/lysate) is dependent on the apoE isoform.



**Fig. 1.** Human-apoE-isoform-dependent differences in apoE ratio mediated by LR11/SorLA. (A) N2a cells stably expressing human apoE3 were mock-transfected or transiently transfected with human FL-LR11. The levels of apoE3 in the medium and cell lysate were determined by immunoblot analysis at different time points ranging from 2 to 24 h after the medium change. Note that the level of apoE in the medium of cells expressing FL-LR11 was decreased compared with mock-transfected cells. The equivalence of protein loading is shown in the  $\beta$ -actin blot. (B) Results of semiquantitative analysis by densitometry of apoE level. The apoE ratio (medium/lysate) was defined as the ratio of the level of apoE in the medium to that in the cell lysate. The apoE ratio (medium/lysate) significantly decreased 24 h after the medium change in cells transfected with FL-LR11 compared with mock-transfected cells. Results of three independent experiments are shown as mean ± SEM. \*p < 0.05 by Student *t*-test. (C) N2a cells stably expressing human apoE3, or apoE4 were mock-transfected or transiently transfected with human FL-LR11. The levels of apoE in the medium and the lysate of cells expressing each of the apoE isoforms were determined 24 h after the medium change. (D) Results of semiquantitative analysis of the apoE ratio (medium/lysate). The apoE ratio (medium/lysate) was significantly decreased in apoE3- or apoE4-expressing cells transfected with FL-LR11, whereas the apoE ratio (medium/lysate). \*p < 0.05.



**Fig. 2.** ApoE isoform-dependent cellular uptake of apoE by LR11/SorLA in coculture system. (A) The recipient N2a cells mock-transfected or transiently transfected with FL-LR11 were cocultured with the donor HEK293T cells that were transiently transfected with apoE2, apoE3, or apoE4 for 24 h. Cellular uptake of secreted apoE from the medium into the recipient N2a cells was examined by Western blot analysis. (B) Results of semiquantitative analysis of cellular uptake of apoE are shown. The apoE ratio (medium/lysate) significantly decreased in the recipient cells transfected with FL-LR11 compared with the mock-transfected recipient cells when the recipient cells were cocultured with the donor HEK293T cells expressing apoE3 or apoE4 (*upper panels*). The apoE level normalized by β-actin level in the lysate of the recipient cells transfected with FL-LR11 showed a significant increase compared with that of mock-transfected recipient cells were cocultured with the donor HEK293T cells expressing apoE3 or apoE4 (*upper panels*). The apoE level normalized by β-actin level in the lysate of the recipient cells transfected with FL-LR11 showed a significant increase compared with that of mock-transfected recipient cells were cocultured with the donor HEK293T cells expressing apoE3 or apoE4 (*lower panels*). \*p < 0.05; \*\*p < 0.01. (C) The recipient N2a cells mock-transfected or transiently transfected with FL-LR11 were cocultured with the donor HEK293T cells apoE3 or apoE4 (*lower panels*). \*p < 0.05; \*\*p < 0.01. (D) Results of semiquantitative analysis of cellular uptake of apoE-GFP in the medium for 24 h. Cellular uptake of apoE form the medium into the recipient N2a cells was examined. (D) Results of semiquantitative analysis of cellular uptake of apoE-GFP normalized by β-actin level for the apoE isoforms are shown. (*lower panels*). \*p < 0.05; \*\*p < 0.01.



**Fig. 3.** Human-apoE-isoform-dependent differences in formation of complex between apoE and LR11/SorLA examined by co-IP assay. (A) HEK293T cells were cotransfected with each of the human apoE isoforms and human FL-LR11. The formed complex between apoE and LR11/SorLA was immunoprecipitated using the anti-apoE antibody (AB947) or anti-LR11 antibody (48/LR11), followed by immunoblot analysis. The lysates (*lanes* 1–3) and immunoprecipitated samples (*lanes* 4–7) were run on the same gel. (B) Results of semiquantitative analysis of immunoprecipitated apoE and LR11/SorLA. \*p < 0.05; \*\*p < 0.01 by Tukey test following ANOVA.

# 3.2. Cellular uptake of apoE examined by fluorescence confocal laser microscopy

The finding that the level of apoE in the medium of cells transfected with FL-LR11 decreased raises the possibility that apoE in the medium was taken up into cells by the lipoprotein receptor LR11/SorLA. To explore this possibility, N2a cells were incubated with the medium prepared from cells that transiently expressed the apoE3-GFP fusion protein. Fluorescence confocal microscopy revealed apoE-GFP signals inside the cells transfected with FL-LR11 and incubated with the medium of cells expressing apoE3-GFP (Supplementary Fig. 1B). This finding suggests that the cellular uptake of apoE from the medium was enhanced by the expression of LR11/SorLA. 3.3. ApoE-isoform-dependent cellular uptake of apoE in coculture system

We next examined whether the enhanced uptake of apoE by FL-LR11 is modified by apoE isoforms using the coculture system. First, we attempted to confirm whether apoE3 and GFP-labeled apoE3 secreted from the donor HEK293T cells are taken up into the recipient cells. This experiment revealed that apoE3 and GFP-labeled apoE3 were detected in the lysate of the recipient cells (Supplementary Fig. 2B), whereas GFP alone without apoE was not detected in the recipient cells.

Having established that apoE secreted from the donor cells is taken up into the recipient cells, we next asked if isoforms of apoE differently affect the cellular uptake of apoE in the recipient N2a cells mock-transfected or transiently transfected with FL-LR11. The level of apoE taken up into the recipient N2a cells was determined by immunoblot analysis using the anti-apoE antibody (Fig. 2A). The cellular uptake levels of apoE3 and apoE4 were significantly higher in the recipient cells that express FL-LR11 compared with the mock-transfected cells (Fig. 2B). In a similar experiment using donor cells that express apoE2-GFP, apoE3-GFP, or apoE4-GFP, the cellular uptake levels of apoE3-GFP and apoE4-GFP were increased by the expression of FL-LR11 (Fig. 2C and D). These results suggest that FL-LR11 enhances the cellular uptake of apoE3 and apoE4 but not that of apoE2.

#### 3.4. ApoE-isoform-dependent binding affinity to LR11/SorLA

We speculated that the differential cellular apoE uptake in the recipient cells may be explained by a difference in binding affinity between LR11/SorLA and each of the apoE isoforms. To test this hypothesis, we examined the ability of LR11/SorLA to form a complex with each of the apoE isoforms by co-immunoprecipitation (co-IP) assay. We collected the detergent-extracted lysate of HEK293T cells that were cotransfected with FL-LR11 and each of the apoE isoforms. Co-IP assay using the anti-apoE or anti-LR11 antibody was performed using the medium and cell lysate. This assay revealed that LR11/SorLA bound to each isoform of apoE with different efficiencies (Fig. 3A). ApoE4-expressing cells showed the highest level of the LR11-apoE complex, followed by apoE3- and apoE2-expressing cells (Fig. 3B).

#### 3.5. ApoE-isoform-dependent cellular uptake of $A\beta$

Given the physical interaction between LR11/SorLA and apoE, we next considered the possibility that FL-LR11 may be involved in the cellular uptake of the apoE-A $\beta$  complex, because apoE is known to form a complex with A $\beta$ . To address this issue, N2a cells were incubated with FAM-labeled A $\beta$ 40 at various concentrations of ranging from 0.5 to 3.0  $\mu$ M. By fluorescence confocal laser microscopy, we detected the cellular uptake of FAM-labeled A $\beta$  at concentrations above 1.0  $\mu$ M (Supplementary Fig. 3A). We observed the cellular uptake of FAM-labeled A $\beta$  at a low concentration of 0.25  $\mu$ M when the recipient cells were transfected with FL-LR11 and cocultured with the donor cells expressing apoE4 (Supplementary Figs. 3B, 4A and 4B). The finding suggests that the cellular uptake of A $\beta$  was enhanced in the presence of both FL-LR11 and apoE.

We finally performed coculture experiments in which the recipient N2a cells mock-transfected or transiently transfected with FL-LR11 were cocultured with the donor cells expressing apoE2, E3, or E4 in the presence of the A $\beta$  peptides at 0.25  $\mu$ M. The cellular uptake level of apoE and A $\beta$  were highest when the recipient cells were cocultured with the donor cells expressing apoE4, followed by apoE3 and apoE2 (Fig. 4A and B).



**Fig. 4.** ApoE-isoform-dependent differences in cellular uptake of  $A\beta$  in cells expressing LR11/SorLA. (A) Recipient N2a cells stably expressing human FL-LR11 were cocultured with donor N2a cells stably expressing human apoE2, apoE3, or apoE4 in the presence of  $A\beta40$  peptides at 0.25  $\mu$ M for 24 h. The cellular uptake of  $A\beta$  and apoE into the recipient cells was examined by Western blot analysis. (B) Results of semiquantitative analysis of cellular uptake of  $A\beta$  and apoE are shown as -fold increase compared with the apoE2 isoform. \*p < 0.05; \*\*p < 0.01 by Tukey test following ANOVA.

#### 4. Discussion

Despite significant advances in our understanding of the pathological events occurring in the AD brain, the factors leading to  $A\beta$ accumulation in sporadic AD patients are not well understood. A wealth of evidence has confirmed that APOE E4 is the strongest genetic risk factor for sporadic AD [2,3]. Although it is not fully understood how human apoE isoforms differentially affect the various pathogenic processes implicated in AD, several lines of evidence suggest that the effects of apoE on A<sub>β</sub> accumulation, aggregation, clearance, neurotoxicity, and neuroinflammation may play a role in AD pathogenesis [6]. Hence, understanding how each of the apoE isoforms differentially plays a pathophysiological role in AD is an important question to address. In this regard, this study provided new insights into how apoE isoforms differentially modify the AD pathogenic pathway with particular focus on the cellular uptake of  $A\beta$  via the lipoprotein receptor LR11/SorLA.

First, we demonstrated that the overexpression of LR11/SorLA enhanced the cellular uptake of apoE3 and apoE4. This finding suggests that LR11/SorLA functions as the apoE receptor. This notion was suggested by previous studies showing that FL-LR11 at the cell surface was capable of cellular uptake of apoE [12]. Importantly, we here showed that the cellular uptake of apoE is modified by different apoE isoforms. The cellular uptake of apoE4 and apoE3 was significantly enhanced by the expression of LR11/SorLA. In support of this notion, the co-IP assay revealed that the apoE-LR11/SorLA complex formation showed apoE-isoform-dependent differences in efficiencies, that is, apoE4 > apoE3 > apoE2. Major apoE receptors belong to the LDL receptor family including the LDL receptor and LDLR-related protein (LRP1). Different efficiencies in the formation of complexes between the LDL receptor and the apoE isoforms were observed. Similar to our finding, the cellular uptake levels of apoE3 and apoE4 were previously found to be higher than that of apoE2 in cells expressing the LDL receptor [17].

Secondly, our cell culture experiments revealed that extracellular Aβ was taken up into cells overexpressing LR11/SorLA in an apoE-isoform-dependent manner. A recent study has shown that LR11/SorLA binds to A<sup>β</sup> directly through the VPS10 protein domain of LR11/SorLA [15]. Importantly, we demonstrated that the cellular uptake of extracellular A<sup>β</sup> was apoE-isoform-dependent, that is, apoE4 > apoE3 > apoE2. Although this finding is potentially interesting, how this finding is relevant to AD pathogenesis is not presently understood. Enhancement of cellular uptake of A<sup>β</sup> may have two different consequences. A harmful consequence would be that an increased intracellular AB concentration through the uptake of extracellular  $A\beta$  may provide seeds of  $A\beta$  oligomers, a toxic forms of A $\beta$  [18]. Some studies suggested that intracellular A $\beta$  oligomers may be a major cause of synaptic dysfunction [19]. On the other hand, a beneficial consequence would be that the cellular uptake of extracellular A $\beta$  may enhance the clearance of A $\beta$  in lysosomes [20]. Our finding favors the former possibility, that is, cellular uptake of A<sup>β</sup> may exert a detrimental effect on neurons because this phenomenon is most closely associated with the presence of apoE4. Further studies delineating the precise consequences of increased intracellular Aß level caused by enhanced cellular uptake of A<sup>β</sup> will be required.

ApoE is predominantly generated by astrocytes and microglia in the brain and is subsequently lipidated by ABCA1 to form lipoprotein particles [21]. It has been demonstrated that lipoprotein receptors including LDLR and LRP on the neuronal surface are able to take apoE-Aβ complexes into neurons after apoE is lipidated [22]. The lipidation of apoE has been shown to influence its isoform-specific affinity to  $A\beta$ . The efficiency of complex formation between lipidated apoE and A $\beta$  is in the order of apoE2 > apoE3 > apoE4 [23]. In addition, the variable levels of apoE oxidation may affect the properties of apoE binding to  $A\beta$ . In this study, we did not fully address the lipidation or oxidation status of apoE secreted from HEK293T cells, which should be taken into account when interpreting our results. To minimize confounding factors, it is desirable to use apoE prepared under conditions that preserve apoE lipidation and oxidation status found in the brain. Collectively, our study indicated that LR11/SorLA is capable of cellular uptake of  $A\beta$  in an apoE-isoform-dependent manner; however, its pathophysiological role requires further investigation.

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#### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.11.111.

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#### ORIGINAL ARTICLE

# Dopamine D1 Receptor-Mediated Transmission Maintains Information Flow Through the Cortico-Striato-Entopeduncular Direct Pathway to Release Movements

Satomi Chiken<sup>1,2,†</sup>, Asako Sato<sup>3,4,†</sup>, Chikara Ohta<sup>1,5</sup>, Makoto Kurokawa<sup>5</sup>, Satoshi Arai<sup>3</sup>, Jun Maeshima<sup>3</sup>, Tomoko Sunayama-Morita<sup>4,6</sup>, Toshikuni Sasaoka<sup>2,3,4,7</sup>, and Atsushi Nambu<sup>1,2</sup>

<sup>1</sup>Division of System Neurophysiology, National Institute for Physiological Sciences, Okazaki 444-8585, Japan,
 <sup>2</sup>School of Life Science, SOKENDAI (The Graduate University for Advanced Studies), Okazaki 444-8585, Japan,
 <sup>3</sup>Department of Laboratory Animal Science, Kitasato University School of Medicine, Sagamihara 252-0374, Japan,
 <sup>4</sup>National Institute for Basic Biology, Okazaki 444-8585, Japan, <sup>5</sup>Department of Biological Sciences, Tokyo
 Metropolitan University, Tokyo 192-0397, Japan, <sup>6</sup>Department of Life Sciences, Graduate School of Arts and
 Science, The University of Tokyo, Tokyo 153-8902, Japan and <sup>7</sup>Department of Comparative and Experimental
 Medicine, Brain Research Institute, Niigata University, Niigata 951-8585, Japan

Address correspondence to Atsushi Nambu, Division of System Neurophysiology, National Institute for Physiological Sciences, 38 Nishigonaka, Myodaiji, Okazaki 444-8585, Japan. Email: nambu@nips.ac.jp; Toshikuni Sasaoka, Department of Comparative and Experimental Medicine, Brain Research Institute, Niigata University, Niigata 951-8585, Japan. Email: sasaoka@bri.niigata-u.ac.jp

<sup>†</sup>These authors contributed equally to this work.

#### Abstract

In the basal ganglia (BG), dopamine plays a pivotal role in motor control, and dopamine deficiency results in severe motor dysfunctions as seen in Parkinson's disease. According to the well-accepted model of the BG, dopamine activates striatal direct pathway neurons that directly project to the output nuclei of the BG through D1 receptors (D1Rs), whereas dopamine inhibits striatal indirect pathway neurons that project to the external pallidum (GPe) through D2 receptors. To clarify the exact role of dopaminergic transmission via D1Rs in vivo, we developed novel D1R knockdown mice in which D1Rs can be conditionally and reversibly regulated. Suppression of D1R expression by doxycycline treatment decreased spontaneous motor activity and impaired motor ability in the mice. Neuronal activity in the entopeduncular nucleus (EPN), one of the output nuclei of the rodent BG, was recorded in awake conditions to examine the mechanism of motor deficits. Cortically evoked inhibition in the EPN mediated by the cortico-striato-EPN direct pathway was mostly lost during suppression of D1R expression, whereas spontaneous firing rates and patterns remained unchanged. On the other hand, GPe activity changed little. These results suggest that D1R-mediated dopaminergic transmission maintains the information flow through the direct pathway to appropriately release motor actions.

Key words: basal ganglia, conditional knockdown, dopamine receptor, extracellular recording, mouse

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#### Introduction

In the basal ganglia (BG), dopaminergic transmission plays a pivotal role in the control of voluntary movements and motor learning (Albin et al. 1989; DeLong 1990; Gerfen et al. 1990; Graybiel 2005; Joshua et al. 2009; Enomoto et al. 2011). Dopamine deficiency, as occurs in Parkinson's disease (PD), results in severe motor and nonmotor dysfunctions including bradykinesia, rigidity, tremor, autonomic abnormalities, cognitive dysfunction, and depression (Fahn et al. 2011; Seppi et al. 2011). Dopaminergic inputs from the substantia nigra pars compacta terminate in the striatum and are thought to differentially modulate the excitability of 2 types of striatal projection neurons through different receptors (Albin et al. 1989; DeLong 1990; Mallet et al. 2006) (Fig. 1A, left). One is excitatory effects through dopamine D1 receptors (D1Rs) on direct pathway neurons that directly project to the output nuclei of the BG, the entopeduncular nucleus (EPN), which is homologous to the internal segment of the globus pallidus (GPi) in primates, and substantia nigra pars reticulata (SNr). The other is inhibitory effects through D2 receptors (D2Rs) on indirect pathway neurons that project to the external

segment of the globus pallidus (GPe), which is also called the globus pallidus in rodents. Such differential effects of dopamine through D1Rs and D2Rs were originally proposed based on changes in gene expression, glucose utilization, and receptor binding in these pathways under conditions of dopamine depletion (Gerfen et al. 1990; Hirsch et al. 2000) and have been reinforced by recent in vitro electrophysiological studies (Surmeier et al. 2007; Day et al. 2008; Flores-Barrera et al. 2011; Gerfen and Surmeier 2011; Planert et al. 2013). The striato-EPN/SNr direct and striato-GPe-subthalamo (STN)-EPN/SNr indirect pathways play opposite roles in controlling movements. The signals through the direct pathway reduce activity of the EPN/SNr and increase thalamocortical activity via disinhibition, resulting in release of movements. In contrast, signals through the indirect pathway increase activity of the EPN/SNr, resulting in suppression of movements (Albin et al. 1989; DeLong 1990; Gerfen et al. 1990; Mink 1996; Nambu 2007; Kravitz et al. 2010; Sano et al. 2013). Thus, the loss of dopaminergic inputs to both pathway neurons is considered to increase firing rates of EPN/SNr neurons through the inhibitory striato-EPN/SNr direct and net excitatory



Figure 1. Dopamine D1 receptor (D1R) suppression in D1R knockdown (D1RKD) mice with doxycycline (Dox) treatment. (A) Schematic diagram showing the cortico-basal ganglia pathway and stimulating (Stim.) and recording (Rec.) sites in the electrophysiological experiments (left), along with a typical response pattern (right) in the entopeduncular nucleus (EPN) (homologous to the internal segment of the globus pallidus, GPi) to cortical stimulation (Cx Stim.) with early excitation, inhibition, and late excitation, which are mediated by the i) cortico-subthalamo (STN)-EPN hyperdirect, ii) cortico-striato-EPN direct, and iii) cortico-striato-external pallido (GPe)-STN-EPN indirect pathways, respectively. Red, blue, and green triangles represent glutamatergic excitatory, GABAergic inhibitory, and dopaminergic projections, respectively. D2Rs, dopamine D2 receptors; SNc, substantia nigra pars compacta. (B) Schematic diagram of Dox-regulated D1R expression in D1RKD mice. Before Dox treatment (left), tetracycline transactivator (tTA) binds to the tetracycline responsive element (TRE), and D1Rs and lacZ are transcribed. Dox treatment interferes with tTA binding to TRE (right), and suppresses D1R and lacZ expression (Tet-off system). (C) D1R immunoreactivity in the striatum (Str) of wild-type (WT, top), D1R knockout (D1RKO, middle), and D1RKD (before Dox treatment, bottom) mice shown in frontal sections. The pattern of D1R expression in D1RKD mice was similar to that in WT mice, whereas D1R immunoreactivity was not observed in D1RKO mice. The dorsolateral motor areas of the Str are defined by rectangles (left) and shown mouse (n = 1, number of mice used), D1RKD mice before, during, and after Dox treatment (Days 0, 7, 14, 21, 28, different days after starting Dox treatment; After, 7 days after cessation of Dox treatment for 14 days; n = 3-4 per condition), and WT mice (n = 3).

striato-GPe-STN-EPN/SNr indirect pathways, resulting in the decreased motor activity seen in PD (Albin et al. 1989; DeLong 1990).

However, the exact role of D1R-mediated dopaminergic transmission in vivo is not well understood. Contradicting results have been observed following D1R blockade. D1R knockout (KO) mice show increases in spontaneous locomotor activity, although pharmacological blockade of D1Rs induces locomotor suppression in agreement with the above explanation (Waddington et al. 2005). In the present study, we developed novel D1R knockdown (D1RKD) mice in which the D1Rs can be conditionally and reversibly regulated by doxycycline (Dox) treatment (Fig. 1B). The D1RKD mice enabled us to examine motor behaviors and neuronal activities in the cortico-BG pathways in the presence and absence of D1Rs in the same mice. The mice exhibited decreased spontaneous motor activity and impaired motor ability when D1R expression was suppressed, consistent with the pharmacological blockade study (Waddington et al. 2005). To examine the neural mechanism of the motor deficits, we next recorded neuronal activity in the EPN in awake mice, because the EPN is the main output nucleus of the BG to the thalamus and the target of D1R-expressing striatal neurons. Cortically evoked inhibition in the EPN, which is mediated by the cortico-striato-EPN direct pathway, was mostly lost during suppression of D1R expression, whereas spontaneous firing rates and patterns of EPN neurons remained unchanged. These results indicate that D1R suppression mostly blocks the information flow through the cortico-striato-EPN direct pathway and reduces spontaneous motor activity.

#### **Materials and Methods**

Animal protocols were approved by the Institutional Animal Care and Use Committee of National Institutes of Natural Sciences, Kitasato University and Niigata University, and were conducted according to the guidelines of the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. One to 5 mice were housed in each cage under a 12-h light–dark cycle (lights on at 8:00 AM) and given food and drinking water *ad libitum*.

#### Generation of Conditional and Reversible D1RKD Mice

We developed D1RKD mice in which D1Rs can be conditionally and reversibly regulated by Dox using the Tet-Off system after deletion of the endogenous D1Rs (Fig. 1B). To generate tetracycline transactivator-VP16 (tTA)-expressing mice using the D1R promoter (transactivator line), bacterial artificial chromosome (BAC) clones containing mouse D1R were modified with a 2-step Rec A strategy for BAC modification (Yang et al. 1997). A modification cassette that introduces the tTA into the endogenous D1R coding sequence was constructed (see Supplementary Fig. 1A). A DNA fragment (0.92 kb, A-arm) containing the upstream region of the translation initiation site of D1R was ligated to the DNA fragment (1.5 kb) containing the coding sequence of the tTA and the SV40 poly A signal (poly A) of the pTet-off vector (Clontech), and the resulting fragment was ligated to the DNA fragment (0.96 kb, B-arm) of exon 2 of D1R to generate D1Txx, the modification cassette (3.38 kb). Because the tTA and the SV40 poly A signal sequences were introduced at the initiation codon of D1R, the expression of endogenous D1R was disrupted. To generate the pSV1-RecA-D1T shuttle vector, the DNA fragment of D1Txx was inserted into the pSV1-RecA vector to facilitate homologous recombination in RecA<sup>-</sup> Escherichia coli.

The BAC clone, BAC4-D1R (80 230 bp) (Research Genetics), was used (see Supplementary Fig. 1B). RecA<sup>-</sup> E. coli carrying the original BAC clone were transformed with the pSV1-RecA-D1T shuttle

vector. The resulting clones were subjected to Southern analysis using either a 5' probe or tTA probe to confirm proper recombination (see Supplementary Fig. 1A). The BAC transgenic (Tg) construct, BAC4-tTA, was obtained (see Supplementary Fig. 1B).

The Tg construct, D1R-tetracycline responsive element (D1R-TRE), was generated for tetracycline operator (tetO)-target Tg mouse lines using the following DNA fragments (see Supplementary Fig. 1C): a 9.7-kb fragment including the entire D1R coding sequence, a 0.64-kb fragment encoding the TRE and bidirectional promoter from the pBI Tet vector (Clontech), a 3466-bp fragment encoding *lacZ* from the pBI-GL Tet vector (Clontech), a 0.98-kb fragment containing rabbit beta-globin poly A and SV40 poly A signals from the pBstN plasmid, and a 0.3-kb fragment encoding the chicken beta-globin insulator sequences from the plasmid pUC19 INS240-SNNS.

The BAC4-tTA and D1R-TRE DNAs (10 ng/µL each) were used for Tg mouse generation using standard techniques (Nagy et al. 2002). The BAC4-tTA and D1R-TRE DNAs were independently microinjected into fertilized mouse oocytes isolated from crosses of D1R homozygous knockout (D1RKO) female and male mice (Tran et al. 2008). Three Tg lines for BAC4-tTA and 15 Tg lines for D1R-TRE were independently generated. Each BAC4-tTA Tg line was crossed with each D1R-TRE Tg line. The progeny from crosses of BAC4-tTA and D1R-TRE Tg mice was subjected to analyses of Dox-controllable expression of lacZ using X-gal staining of brain sections using a standard method and expression of D1R in the striatum using western blotting with anti-D1R antibody (Sigma). Two Tg lines harboring BAC4-tTA and D1R-TRE (designated 442-43 and 442-112) were consistently found to exhibit distinct, comparable expression of lacZ in the region where endogenous D1R was expressed and Dox-controllable expression of lacZ and D1R. Therefore, the 442–112 line was used for further analyses as D1RKD mice.

Wild-type (WT) C57BL/6J and D1RKO (Tran et al. 2008) mice were also used for comparison in immunohistochemical, western blot, and behavioral analyses.

#### Dox Treatment

Dox (2.0 mg/mL) was mixed in drinking water containing 5% sucrose and delivered to the D1RKD and WT mice through a water bottle.

#### Immunohistochemistry

We used 4 D1RKD, 3 WT, and 1 D1RKO mice for immunohistochemical analysis. Mice were deeply anesthetized by injection of tribromoethanol (400 mg/kg body weight, i.p.) and perfused transcardially with 4% paraformaldehyde in phosphate buffer (PB, pH 7.4). The brains were postfixed overnight at 4°C, transferred to a 30% sucrose solution, immersed in OCT compound (Sakura Finetek), frozen, and stored at  $-80^{\circ}$ C until use. Frontal sections were cut at 25-µm thickness and stored in PBS at 4°C. Free-floating sections were incubated with 1% bovine serum albumin containing antibody for D1R (1:1000; Frontier Institute) overnight at 4°C. D1R antibody binding was visualized using the Vectastain Elite ABC System (Vector Laboratories) and 3, 3'diaminobenzidine.

#### Western Blot Analysis

We used 19 D1RKD (3-4 in each condition, 0, 7, 14, 21, and 28 days after starting Dox treatment and 7 days after cessation of Dox treatment for 14 days), 3 WT, and 1 D1RKO mice for western

blot analysis as described previously (Tran et al. 2008) with minor modifications. Briefly, after euthanasia by cervical dislocation, the striatum was dissected and homogenized in lysis buffer. Total lysates were resolved with 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Millipore). The membrane was blocked with 5% skim milk and incubated with anti-D1R (1:5000; Sigma) or anti-actin (as a protein loading control; 1:500; Sigma) antibodies, followed by incubation with secondary antibodies conjugated to horseradish peroxidase (Sigma). Signals were developed with an ECL detection kit (GE Healthcare). The density of the bands was determined with CS Analyzer software (Atto).

#### **Behavioral Analyses**

D1RKD and age-matched WT male mice (7–28 weeks old) were used for behavioral analyses. D1RKD and WT mice were randomly divided into Dox-treated and untreated groups. All behavioral data are presented as the mean  $\pm$  SD.

#### Spontaneous Motor Activity

We assessed spontaneous motor activity of 9 Dox-treated D1RKD, 4 untreated D1RKD, and 5 Dox-treated WT mice. The mice were individually housed in an 11.8 cm (L)  $\times$  20.8 cm (W)  $\times$  14.5 cm (H) home cage, and movements of each mouse were detected before, during, and after Dox treatment with a pyroelectric infrared sensor installed above the cage (O'hara) as reported previously (Nakamura et al. 2014). Movements were continuously counted in 10-min bins, and spontaneous motor activity per day was calculated as the cumulative number of movements in 24 h beginning at 8:00 AM. The bedding was replaced every 7 days, and motor activity was increased after bedding replacement because of exploratory behavior. Thus, data on these days were excluded when calculating the mean weekly spontaneous motor activity. Spontaneous motor activity was classified into 3 levels based on counts per 10 min: inactive ( $\leq$ 9), low (10–199), and high (≥200). We also assessed spontaneous motor activity of another 4 Dox-treated D1RKD and 4 Dox-treated WT mice until 10 days after cessation of Dox treatment for 14 days.

#### Rotarod Test

We used another 13 Dox-treated D1RKD, 5 untreated D1RKD, 8 Dox-treated WT, and 15 untreated WT mice for the rotarod test. Dox treatment was started 35 days before the test and continued during the test periods for Dox-treated groups. Mice were placed on a rotating rod (32 mm diameter, O'hara), which initially rotated at 4 rpm, and then was accelerated at a constant rate from 4 to 40 rpm over 4 min, with the final speed maintained for 1 min. The time spent on the rotarod was measured in 3 trials every day between 1:00 PM and 7:00 PM and averaged.

#### Electrophysiology

#### Surgery

We used 4 D1RKD (mouse W, K, T, and O, 20–50 weeks old, males) and 3 age-matched WT mice for the electrophysiological experiments. Under anesthesia with ketamine hydrochloride (100 mg/kg, i.p.) and xylazine hydrochloride (4–5 mg/kg, i.p.), a small U-frame polyacetal head holder was fixed to the exposed skull of the mouse with transparent acrylic resin (for details, see Chiken et al. 2008; Sano et al. 2013). After recovery from the first surgery, under light anesthesia with ketamine hydrochloride (30–50 mg/ kg, i.p.), a portion of the skull was removed to access the motor cortex, EPN, and GPe. Two pairs of bipolar stimulating electrodes were chronically implanted into the caudal forelimb and orofacial regions of the motor cortex (for details, see Chiken et al. 2008).

#### Recording of Neuronal Activity in the Awake State

After full recovery from the second surgery, neuronal recording was started (for details, see Chiken et al. 2008; Sano et al. 2013). The awake mouse was kept quiet in a stereotaxic apparatus with its head restrained painlessly using the U-frame head holder. A glass-coated Elgiloy-alloy microelectrode was inserted vertically into the EPN/GPe through the dura mater. Unit activity was isolated and converted to digital pulses using a window discriminator. Spontaneous discharges and responses to the cortical stimulation (200-µs duration, single pulse, 50-µA strength) through the electrodes implanted in the motor cortex were recorded in the EPN/GPe from the same mouse in 3 conditions: before, during, and after Dox treatment. We first recorded neuronal activity before Dox treatment ("before" condition) and then started Dox treatment. Recording of neuronal activity was resumed 5 days after starting Dox treatment and continued till 22 days after starting Dox treatment ("during Dox" condition) when D1R expression was greatly suppressed and spontaneous motor activity was distinctly decreased (Figs 1D, 2A). Finally, we stopped Dox treatment and resumed recording 15 days after cessation of Dox treatment ("after" condition) when D1R expression and spontaneous motor activity had fully returned to the level prior to Dox treatment (Figs 1D and 2C). During the recording session, we carefully monitored vigilance state of the mouse by visual inspection.

#### Histology

In the final experiment, several sites of neuronal recording were marked by passing cathodal DC current (20 µA for 30 s) through the recording electrodes. The mice were anesthetized deeply with sodium pentobarbital (120 mg/kg, i.p.) and perfused transcardially with 0.1 M PB (pH 7.3) followed by 10% formalin in 0.1 M PB, and then 0.1 M PB containing 10% sucrose. The brains were removed immediately and saturated with the same buffer containing 30% sucrose. They were cut into frontal 50-µm-thick sections on a freezing microtome. The sections were mounted onto gelatin-coated glass slides, stained with 0.7% neutral red, dehydrated, and coverslipped. The sections were observed under a light microscope, and the recording sites were reconstructed according to the lesions made by current injection and traces of electrode tracks. The sites of stimulation in the motor cortex were also examined histologically.

#### Data Analysis

Spontaneous discharge rates were calculated from continuous digitized recordings for 50 s. The following parameters characterizing firing patterns were calculated from the first 30 s of the same recordings: the coefficient of variation (CV) of interspike intervals (ISIs), the burst index (Hutchison et al. 1998; Sano et al. 2013), and the percentage of spikes in bursts detected by the Poisson surprise method (Legéndy and Salcman 1985; Chiken et al. 2008; Sano et al. 2013) (Poisson surprise value ( $-\log_{10} P \ge 2.0$ ; the minimum number of spikes during bursts was 3). Autocorrelograms (bin width of 0.5 ms) were constructed from continuous digitized recordings for 50 s.

Responses to cortical stimulation were examined by constructing peristimulus time histograms (PSTHs; bin width of 1 ms) for 100 stimulus trials. The mean value and SD of the discharge rate during the 100-ms period preceding the stimulation onset were calculated for each PSTH and considered as the



**Figure 2.** Spontaneous motor activity and rotarod performance during D1R suppression. (A) Spontaneous motor activity of Dox-treated D1RKD (D1RKD Dox (+)), untreated D1RKD (D1RKD Dox (-)), and Dox-treated WT (WT Dox (+)) mice in their home cages. Dox treatment was started on Day 0 and continued for 4 weeks for Dox-treated groups (gray bar). Daily changes in spontaneous motor activity during Dox treatment were observed. Bedding was replaced every 7 days (Days 0, 7, 14, and 21), and spontaneous motor activity was increased because of exploratory behavior. Thus, data on these days were excluded from further analyses. Colored lines and light-colored areas represent mean and  $\pm$  SD, and filled circles and whiskers represent mean weekly spontaneous motor activity and  $\pm$ SD. 'P<0.05; significantly different from before Dox treatment (Bonferroni test). */P*<0.05, significantly different from control (WT Dox (+) and D1RKD Dox (-) mice), which is indicated by the corresponding color (Bonferroni test). (B) Classifications of spontaneous motor activity of V Dox (+), D1RKD Dox (-), and D1RKD Dox (+) mice before (Days - 6 to -1) and during (Days 22-27) Dox treatment. Spontaneous motor activity was classified into 3 levels based on counts per 10 min: inactive ( $\leq$ 9), low (10-199), and high ( $\geq$ 200). \*, the percentage of inactive ( $\leq$ 9) time significantly increased ( $\chi^2$  test with Bonferroni correction, P = 0.008), and that of high-active ( $\geq$ 200) time significantly decreased (P = 0.0004). (C) Spontaneous motor activity of D1RKD Dox (+) mice after cessation of Dox treatment. The 2-week Dox treatment was stored was stored every 7 days (Days -14, -7, 0, and 7). (D) Rotarod performance of D1RKD Dox (+), D1RKD Dox (-), WT Dox (+), and untreated WT (WT Dox (-)) mice. Dox treatment was started 35 days before the rotarod test. <sup>†</sup>P < 0.01, significantly different from control, which is indicated by the corresponding color (Bonferroni test). The number of mice used for each experiment is indicated by n.

baseline discharge rate. Changes in neuronal activity in response to cortical stimulation were judged significant if the discharge rate during at least 2 consecutive bins (2 ms) reached a significance level of P < 0.05 (Nambu et al. 2000; Chiken et al. 2008; Tachibana et al. 2008; Sano et al. 2013). The latency of each response was defined as the time at which the first bin exceeded this level. The responses were judged to end when 2 consecutive bins fell below the significance level. The end point was determined as the time at which the last bin exceeded this level. The amplitude of each component of cortically evoked responses was defined as the number of spikes during the significant response minus that of the baseline discharge in the PSTH (i.e., the area of the response; positive and negative values indicate excitation and inhibition, respectively). If no significant changes were found, the amplitude was set to zero. For population PSTHs, the PSTH of each neuron with a significant response was averaged and filtered with a Gaussian filter ( $\sigma$  = 1.6 ms). All electrophysiological data are presented as the mean  $\pm$  SD.

#### Results

#### **Biochemical Characteristics of D1RKD Mice**

We developed D1RKD mice in which D1Rs were conditionally and reversibly regulated by Dox using the Tet-Off system (Fig. 1B, see also Materials and Methods). We first examined the distribution of D1Rs in D1RKD mice immunohistochemically (Fig. 1C) using D1R antibodies. The specificity of the antibodies was examined in WT C57BL/6J mice and D1RKO mice (Fig. 1C, WT and D1RKO). D1Rs were expressed at a high level in the striatum including the ventral striatum and at moderate levels in the cortex of D1RKD mice (Fig. 1C, D1RKD). The distribution of D1Rs was similar to that of WT mice (Fig. 1C, WT) and that reported previously (Fremeau et al. 1991; Weiner et al. 1991; Gaspar et al. 1995). We next quantitatively evaluated D1R expression in the striatum of D1RKD mice before, during, and after Dox treatment using western blot analysis (Fig. 1D). Before Dox treatment, D1R expression in the striatum was 27-fold higher than that in WT mice (Fig. 1D, Day 0 of D1RKD and WT). Dox treatment rapidly and completely suppressed D1R expression (Day 7, 27.1 ± 4.2%; Day 14, 3.1 ± 1.4%; Days 21 and 28, undetectable). After cessation of Dox treatment, D1R expression recovered to the level prior to Dox treatment over 7 days (Fig. 1D, After).

#### Motor Behaviors During D1R Suppression

Before Dox treatment, D1RKD mice showed normal behaviors and similar spontaneous motor activity to WT mice (Days -6 to -1 in Fig. 2A), despite the higher D1R expression in D1RKD mice. Dox treatment significantly decreased spontaneous motor activity in D1RKD mice from the first week (Days 1-6 of D1RKD Dox (+) in Fig. 2A) and monotonically decreased their activity during the second, third, and fourth weeks. Dox had little effect on WT mice (WT Dox (+)), and spontaneous motor activity in untreated D1RKD mice (D1RKD Dox (-)) was also unchanged (repeated-measures 2-way ANOVA, P = 0.0024 for genotype-day interaction; Bonferroni test, P = 0.013 for Days 1–6, P < 0.01 for Days 8–13, 15–20 and 22–27). Finally, the difference in the spontaneous motor activity of D1RKD Dox (+) mice and that of WT Dox (+) and D1RKD Dox (-) mice became significant in the fourth week (Days 22–27) (Bonferroni test, P < 0.01). Decreased spontaneous motor activity in D1RKD Dox (+) mice was due to an increase in inactive time and a decrease in highly active time (Fig. 2B;  $\chi^2$ test with Bonferroni correction, P = 0.008); such changes in active and inactive times were observed in neither WT Dox (+) nor D1RKD Dox (-) mice. After cessation of Dox treatment, spontaneous motor activity temporarily increased and then returned to the normal level over 7 days (Fig. 2C).

Motor ability was also impaired during Dox treatment as evidenced by the rotarod test. The time spent on the rotarod by D1RKD Dox (+) mice was significantly shorter than that for WT Dox (-), WT Dox (+), and D1RKD Dox (-) mice (Fig. 2D; repeated-measures 2-way ANOVA, P < 0.0001 for genotype–day interaction; Bonferroni test, P < 0.01).

# Spontaneous Activity of EPN and GPe Neurons During D1R Suppression

To assess the mechanism of the decreased motor activity in D1RKD mice following D1R suppression, we examined neuronal activity in awake conditions in the motor-related area of the EPN (Chiken et al. 2008), which is the target of D1R-expressing striatal neurons and the main output nucleus of the BG to the thalamus (Fig. 1A). We first recorded spontaneous activity of 127 and 89 EPN neurons before and during Dox treatment, respectively, in 4 awake D1RKD mice. EPN neurons continuously and irregularly fired at a high discharge rate (53.9 ± 14.8 Hz) before Dox treatment (Table 1, Fig. 3A1) as observed in WT mice (Chiken et al. 2008). Dox treatment did not change either the firing rate (54.2 ± 13.5 Hz; Table 1; 1-way ANOVA, P = 0.93) or pattern (Table 1, Fig. 3A2). The CV of ISIs, the burst index, and percentage of spikes in bursts, which characterize firing patterns, also did not change. These results indicate that D1R suppression had little influence on the spontaneous activity of EPN neurons. More than 15 days after the cessation of Dox treatment, we recorded 36 EPN neurons and found that the firing rates and percentage of spikes in bursts remained unchanged, whereas the CV of ISIs and burst index were increased (Table 1; 1-way ANOVA with Tukey's post hoc test, P < 0.001 for the CV of ISIs and burst index).

For comparison, we also examined the activity of GPe neurons in the same 4 D1RKD mice, because striato-GPe neurons are assumed to express D2Rs, not D1Rs (Fig. 1A). We found no significant differences in the firing rate or pattern among the 3 conditions (Table 1; 1-way ANOVA, P = 0.84 for the firing rate,

Table 1 Spontaneous firing rates and patterns of EPN and GPe neurons before, during, and after Dox treatment in D1RKD mice

	Before	During Dox (5–22 days after starting Dox treatment)	After
EPN			
No. of neurons	127	89	36
Firing rate (Hz)	$53.9 \pm 14.8$	54.2 ± 13.5	53.8 ± 19.0
CV of ISIs	$0.53 \pm 0.15^{*}$	$0.49 \pm 0.15^{\#}$	$0.65 \pm 0.19^{*,i}$
Burst index	$1.33 \pm 0.36^{*}$	$1.23 \pm 0.30^{\#}$	$1.91 \pm 1.19^{*,i}$
Spikes in bursts (%)	$0.82 \pm 2.02$	$0.69 \pm 1.27$	$1.16 \pm 1.70$
GPe			
No. of neurons	120	100	38
Firing rate (Hz)	$51.0 \pm 15.8$	51.1 ± 15.5	$52.9 \pm 16.4$
CV of ISIs	$0.63 \pm 0.21$	$0.68 \pm 0.24$	$0.69 \pm 0.15$
Burst index	$1.61 \pm 1.08$	$1.54 \pm 0.47$	$1.69 \pm 0.43$
Spikes in bursts (%)	$1.43 \pm 3.17$	$1.12 \pm 1.66$	$1.33 \pm 1.80$

 $^{*,\theta}{\rm P}$  < 0.01, significantly different from each other (1-way ANOVA with Tukey's post hoc test).



Figure 3. Spontaneous activity of EPN and GPe neurons during D1R suppression. Digitized spikes (top) and autocorrelograms (bottom) of spontaneous activity of EPN (A) and GPe (B) neurons before (1) and during (2) Dox treatment in D1RKD mice are shown.

P = 0.26 for the CV of ISIs, P = 0.69 for the burst index, P = 0.73 for the percentage of spikes in bursts; Fig. 3B).

#### Cortically Evoked Responses of EPN and GPe Neurons During D1R Suppression

After recording spontaneous activity of EPN neurons, we next examined the responses of these neurons to electrical stimulation of the forelimb and orofacial regions of the motor cortex (Chiken et al. 2008) (Fig. 4A), because cortical stimulation induces neuronal activity in the BG and mimics information processing during voluntary movements (Chiken et al. 2008; Tachibana et al. 2008; Sano et al. 2013). Before Dox treatment, 60% of EPN neurons (75/125 cells, Table 2) responded to stimulation of the motor cortex. The most common (64%) response was a triphasic response composed of early excitation, followed by inhibition and late excitation (ex-inh-ex) as observed in PSTHs (Fig. 4A1, left, see Supplementary Fig. 2A, Before), which is the typical response in WT mice (Chiken et al. 2008, see also Supplementary Fig. 3B). During Dox treatment, a similar percentage of neurons (72%, 63/88 cells, Table 2) responded to the motor cortical stimulation in the same area of the EPN (Fig. 5A); however, response patterns drastically changed. The inhibition was mostly lost (Fig. 4A2, left). The most common (65%) response was biphasic excitation consisting of early and late excitation, and the percentage of neurons exhibiting responses with inhibition, such as ex-inh-ex, ex-inh, inhex, and inh, was significantly decreased (before, 84%; during Dox, 24%;  $\chi^2$  test, P<0.0001, see Supplementary Fig. 2A). These changes were also clearly observed in population PSTHs. The inhibition disappeared during Dox treatment (Fig. 4A1, A2, right).

The disappearance of the inhibition was already observed in the first half (5-14 days) of Dox treatment (see Supplementary Fig. 3A). Population PSTHs constructed for each mouse (Fig. 4B) evidenced that the disappearance of the inhibition was commonly observed in all 4 mice. Quantitative analysis showed that the duration and amplitude of the inhibition were markedly diminished during Dox treatment (Table 2; 1-way ANOVA with Tukey's post hoc test, P < 0.001 for the duration and amplitude). On the other hand, the latency, duration, and amplitude of the early excitation remained unchanged. The latency of the late excitation was decreased, and its amplitude was increased during Dox treatment (P < 0.001 for the duration, P < 0.01 for the amplitude), probably because the diminution of inhibition may unmask the late excitation. More than 15 days after the cessation of Dox treatment, a similar percentage of neurons (73%, Table 2) responded to the motor cortical stimulation, and the most common (58%) pattern was again triphasic, and was similar to that observed before Dox treatment (Fig. 4A3, left, see Supplementary Fig. 2A, After). Such recovery was also evident in population PSTHs (Fig. 4A3, right) and quantitative analyses (Table 2).

We also examined the cortically evoked responses of GPe neurons in the same 4 D1RKD mice for comparison. The most common cortically evoked response was triphasic with early excitation, followed by inhibition and late excitation throughout the 3 conditions (Fig. 4C, left; 67% before, 67% during, and 61% after Dox treatment, see Supplementary Fig. 2B); this is the typical response in WT mice (Chiken et al. 2008). In addition, population PSTHs (Fig. 4C, right) and the amplitude and duration of each component (Table 2; 1-way ANOVA; early excitation, P = 0.51 for the duration, P = 0.42 for the amplitude; inhibition, P = 0.67 for



Figure 4. Cortically evoked responses of EPN and GPe neurons during D1R suppression. Cortically evoked responses of EPN (A) and GPe (C) neurons before (1), during (2), and after (3) Dox treatment in D1RKD mice. Raster and peristimulus time histograms (PSTHs; 100 trials; bin width, 1 ms) for typical examples (left) and population PSTHs (right, with Gaussian filter) are shown. Stimulation was delivered at time 0 (arrows). The mean firing rate and statistical levels of P < 0.05 (1-tailed t-test) are indicated in PSTHs by a black and white dashed line (mean) and black (upper limit) and white (lower limit) solid lines, respectively. The number of neurons used for population PSTHs is indicated by *n*, and the shaded areas represent ±SD. (B) Population PSTHs (with Gaussian filter) of cortically evoked EPN responses constructed for each mouse (mouse W, K, T, and O). Blue and red lines represent before and during Dox treatment, respectively.

the duration, P = 0.91 for the amplitude; late excitation, P = 0.72 for the duration, P = 0.83 for the amplitude) were similar throughout the 3 conditions.

Finally, we examined whether the effects of Dox to EPN neurons described above were specifically observed in D1RKD mice, but not in WT mice. We recorded neuronal activity in the motor-related area of the EPN in 3 age-matched WT mice before and during Dox treatment. In WT mice, Dox treatment did not change the cortically evoked response in the EPN as observed in PSTHs and population PSTHs (see Supplementary Fig. 3B).

#### Location of Recorded Neurons

Recording sites in the EPN (mouse W) and GPe (mouse K) of D1RKD mice are shown in frontal sections using different symbols based on cortically evoked response patterns (Fig. 5). EPN neurons that responded to stimulation of the motor cortex were distributed throughout the middle and lateral parts of the EPN as

observed in WT mice (Chiken et al. 2008). The most common response before Dox treatment was a triphasic response composed of early excitation, followed by inhibition and late excitation (Fig. 5A1). During Dox treatment, cortically evoked responses in the same area mostly changed to biphasic excitation consisting of early and late excitation (Fig. 5A2). GPe neurons that responded to motor cortical stimulation were distributed in the middle and lateral parts of the GPe as observed in WT mice (Chiken et al. 2008; Sano et al. 2013). The most common response was triphasic both before (Fig. 5B1) and during (Fig. 5B2) Dox treatment.

#### Discussion

The present study examined motor behaviors and neuronal activity of the BG in the presence and absence of D1Rs using novel D1RKD mice that we developed in which D1R expression can be conditionally and reversibly regulated by Dox treatment. Suppression of D1R expression by Dox treatment severely
	Before During Dox (5–22 days after starting Dox treatment)		After	
EPN				
No. of	75/125 (60%)	63/88 (72%)	24/33 (73%)	
responded				
neurons/No. of				
tested neurons				
Early excitation				
Latency (ms)	$3.7 \pm 1.0$	$3.5 \pm 0.9$	3.4 ± 0.8	
Duration (ms)	3.8 ± 2.5	4.5 ± 2.7	$3.8 \pm 2.6$	
(spikes)	43.1±30.2	58.1±54.0	64.7 ± 56.4	
Inhibition				
Latency (ms)	$11.0 \pm 2.5$	10.5 ± 2.5	10.3 ± 3.2	
Duration (ms)	$6.1 \pm 5.9^*$	0.8 ± 1.5*,#	9.1 ± 6.2 <sup>#</sup>	
Amplitude (spikes)	$-27.4 \pm 24.4^{*}$	-3.2 ± 7.7*,*	-39.3 ± 33.9*	
Late excitation				
Latency (ms)	$19.2 \pm 3.8^{*}$	$14.1 \pm 3.2^{*,\#}$	$21.0 \pm 5.4^{\#}$	
Duration (ms)	$7.7 \pm 5.9$	9.8±6.1	7.1±7.4	
Amplitude (spikes)	54.3 ± 54.2*	92.4 ± 79.3 <sup>*,#</sup>	51.2 ± 71.6 <sup>#</sup>	
GPe				
No. of	67/114 (59%)	45/82 (55%)	18/30 (60%)	
responded				
neurons/No. of				
tested neurons				
Early excitation				
Latency (ms)	$3.9 \pm 1.3$	$4.1 \pm 1.0$	$4.0 \pm 1.4$	
Duration (ms)	$5.4 \pm 2.7$	$6.0 \pm 3.5$	$5.9 \pm 1.9$	
Amplitude	80.5 ± 48.7	83.5 ± 40.7	96.3 ± 42.8	
(spikes)				
Inhibition	40.0.04	11.0 0.0	44.0.4.0	
Latency (ms)	$10.8 \pm 2.4$	$11.2 \pm 2.6$	$11.8 \pm 1.9$	
Duration (ms)	$6.1 \pm 5.4$	7.3±6.7	$6.6 \pm 10.6$	
Amplitude (spikes)	$-31.8 \pm 30.6$	$-32.3 \pm 24.9$	-36.0±/4./	
Late excitation				
Latency (ms)	$20.2 \pm 4.2$	19.9 ± 4.0	19.8 ± 3.8	
Duration (ms)	21.6 ± 36.0	17.2 ± 25.1	17.5 ± 16.5	
Amplitude (spikes)	196.1 ± 392.0	159.5 ± 292.7	162.5 ± 177.6	

**Table 2** Cortically evoked responses of EPN and GPe neurons before,during, and after Dox treatment in D1RKD mice

 $^{*,\ell}P<0.01,$  significantly different from each other (1-way ANOVA with Tukey's post hoc test).

impaired motor behaviors in the mice. Cortically evoked inhibition in the EPN, which is mediated by the cortico-striato-EPN direct pathway, was mostly lost during suppression of D1R expression, whereas spontaneous firing rates and patterns of EPN neurons remained unchanged. These results suggest that D1R-mediated dopaminergic transmission maintains the information flow through the cortico-striato-EPN direct pathway to appropriately release motor actions.

# Motor Behaviors and Spontaneous Activity of EPN and GPe Neurons

Based on the classical model of the BG, dopaminergic inputs exert excitatory effects on striatal direct pathway neurons through D1Rs (Albin et al. 1989; DeLong 1990; Gerfen et al.

1990). Thus, the loss of dopaminergic inputs through D1Rs would be expected to increase mean firing of EPN neurons and result in decreased motor activity. Increased mean firing rates were originally reported in the GPi (EPN in rodents) of primate models of PD (Miller and DeLong 1987; Filion and Tremblay 1991; Boraud et al. 1998; Heimer et al. 2002; Wichmann et al. 2002). However, recent studies have failed to detect the expected firing rate increase in the GPi (Wichmann et al. 1999; Raz et al. 2000; Tachibana et al. 2011). Instead, abnormal firing patterns, such as bursts and oscillations, were recorded in the BG of PD animals and patients (Raz et al. 2000; Brown et al. 2001; Tachibana et al. 2011), and synchronous activation may disable the ability of individual neurons to process and relay motor-related information, resulting in failure of appropriate movements (Bergman et al. 1998; Brown 2007). The present study revealed that spontaneous motor activity in the mice was decreased during suppression of D1R expression (Fig. 2) without any prominent effects on spontaneous firing rates in either the EPN or GPe (Table 1, Fig. 3). The results indicate that the motor deficits during the absence of D1R-mediated dopaminergic transmission cannot be explained simply by changes in spontaneous firing rates in the EPN.

#### Cortically Evoked Responses of EPN and GPe Neurons

The BG receive inputs from a wide area of the cerebral cortex (Mink 1996; Nambu et al. 2002). The information is processed through the cortico-STN-EPN hyperdirect, cortico-striato-EPN direct, and cortico-striato-GPe-STN-EPN indirect pathways and reaches the EPN, the output station of the BG (Fig. 1A). During voluntary movements, neuronal signals originating in the cortex are considered to be transmitted through these pathways and reach the EPN. Thus, evaluating how neuronal signals originating in the motor cortex are transmitted through the BG is essential for assessing the mechanism of abnormal motor behaviors. Cortical stimulation induces neuronal activity in the BG mimicking information processing during voluntary movements and providing important clues for understanding the changes in information processing through the BG (Chiken et al. 2008; Tachibana et al. 2008; Sano et al. 2013). Cortically evoked responses in the BG are dramatically altered in hyper- and hypokinetic movement disorders (Chiken et al. 2008; Kita and Kita 2011; Nishibayashi et al. 2011).

Before Dox treatment, cortical stimulation induced a triphasic response composed of early excitation, followed by inhibition and late excitation in the EPN of D1RKD mice (Fig. 4A1); this is the typical response in WT mice (Chiken et al. 2008, see Supplementary Fig. 3B). D1RKD mice also showed normal behaviors and similar spontaneous motor activity to WT mice despite a high level of D1R expression, suggesting compensatory mechanisms, such as desensitization of D1Rs (Staunton et al. 1982) and decrease of dopamine release. Transient increase of spontaneous motor activity after cessation of Dox treatment (Fig. 2C) also suggests involvement of compensatory mechanisms, such as sensitization of D1Rs and increase of dopamine release. During suppression of D1R expression, cortically evoked inhibition in the EPN was mostly lost (Fig. 4A2, Table 2, see Supplementary Fig. 2A). On the other hand, cortical stimulation induced a triphasic response composed of early excitation, followed by inhibition and late excitation in the GPe that was not changed during D1R suppression (Fig. 4C, Table 2, see Supplementary Fig. 2B).

Many studies have revealed that cortically evoked early excitation, inhibition, and late excitation in the EPN/GPi are mediated by the hyperdirect, direct, and indirect pathways, respectively (Maurice et al. 1999; Nambu et al. 2000, 2002; Tachibana et al.



Figure 5. Recording sites in the EPN (A) (Mouse W) and GPe (B) (Mouse K) of D1RKD mice before (1) and during (2) Dox treatment. Frontal sections are arranged rostrocaudally from left to right, and the distance from bregma to each section is indicated. Locations of recorded neurons are indicated by different symbols based on cortically evoked response patterns. ex, excitation; ic, internal capsule; inh, inhibition; ot, optic tract; Rt, reticular thalamic nucleus; Str, striatum.

2008) (Fig. 1A). Thus, the loss of inhibition in the EPN during D1R suppression indicates that information flow through the corticostriato-EPN direct pathway was strongly suppressed. Several possible mechanisms for this suppression can be considered. First, the excitability of striatal direct pathway neurons may be decreased during D1R suppression. The heads of dendritic spines of striatal projection neurons receive excitatory inputs from cortical neurons, with the neck of the spine receiving dopaminergic inputs through synapses and/or volume transmission (Hersch et al. 1995; Smith and Kieval 2000; Arbuthnott and Wickens 2007). This spatial arrangement allows dopamine to modulate incoming excitatory glutamatergic drive. D1Rs are coupled to G<sub>s/olf</sub>, which activates adenylyl cyclase and facilitates intrinsic excitability and glutamate receptor-mediated responses in striatal neurons (Hervé et al. 1995; Surmeier et al. 2007; Gerfen and Surmeier 2011). D1R activation increases synaptic efficacy in

cortico-striatal synapses through long-term potentiation (Calabresi et al. 2007; Gerfen and Surmeier 2011). A significant loss of dendritic spines on striatal projection neurons in PD has also been reported (Villalba and Smith 2010). Second, other extrastriatal mechanisms can also be considered. A previous study (Kliem et al. 2007) revealed that D1Rs are also expressed in striatal axon terminals in the GPi, and their activation increases GABA release from the striato-GPi axon terminals. Decreased GABA release from the striato-EPN (homologous to GPi) terminals may contribute to loss of cortically evoked inhibition in the EPN. The EPN also receives GABAergic inhibitory inputs from the GPe. However, considering the fact that both spontaneous firing and the cortically evoked response in the GPe remained unchanged during D1R suppression (Fig. 3B, Fig. 4C, Tables 1 and 2, see Supplementary Fig. 2B), the GPe cannot be responsible for the attenuated cortically evoked inhibition in the EPN.

The cortically evoked triphasic response in the GPe is mediated sequentially by the cortico-STN-GPe, cortico-striato-GPe, and cortico-striato-GPe-STN-GPe pathways (Maurice et al. 1999; Nambu et al. 2000, 2002; Kita et al. 2004) (Fig. 1A). The maintained triphasic response in the GPe in the absence of D1Rs (Fig. 4C, Table 2, see Supplementary Fig. 2B) suggests that the excitability of these components, including the cortex, striatal indirect pathway neurons, GPe, and STN, remains unchanged despite the fact that D1Rs are also expressed at moderate levels in the cortex (Fig. 1C; Fremeau et al. 1991; Weiner et al. 1991; Gaspar et al. 1995). In situ hybridization study using a D2R-specific riboprobe followed by immunohistochemistry with Cre-specific antibody indicated that D1Rs may also be aberrantly expressed in striato-GPe indirect pathway neurons in transgenic mice expressing a Cre recombinase under the control of the D1R regulatory elements (Lemberger et al. 2007). However, neither spontaneous discharges nor cortically evoked responses of GPe neurons changed during D1R suppression in our current electrophysiological study (Figs 3B and 4C, Tables 1 and 2, see Supplementary Fig. 2B), suggesting that they are less functional.

Recent studies have shown conflicting results regarding the classical direct and indirect pathways model. Striatal neurons projecting to the EPN also have axon collaterals to the GPe, indicating no clear anatomical separation of the direct and indirect pathways (Graybiel 2005; Lévesque and Parent 2005; Fujiyama et al. 2011). Some striatal projection neurons express both D1Rs and D2Rs (Surmeier et al. 1996). However, the present results support the original notion that striatal projection neurons can be functionally separated into 2 groups, that is, one with D1Rs projecting to the EPN (GPi) and the other with D2Rs projecting to the GPe (Albin et al. 1989; DeLong 1990; Gerfen et al. 1990; Hersch et al. 1995; Sano et al. 2013). The axon terminals of striatal neurons that express D1Rs in the GPe may be minor or less functional compared with those with D2Rs.

#### D1R-Mediated Dopaminergic Transmission Maintains the Information Flow Through the Direct Pathway to Release Motor Actions

During D1R suppression, cortically evoked inhibition in the EPN was mostly lost (Fig. 4A,B), and motor behaviors were severely impaired (Fig. 2). Upon D1R re-expression, both the inhibition and motor behaviors were restored. This observation can explain the mechanism of reduced motor activity during D1R suppression (see Supplementary Fig. 4). Under normal D1R expression (see Supplementary Fig. 4, left), signals through the cortico-striato-EPN direct pathway induce inhibition in the EPN. The phasic inhibition in the EPN increases thalamocortical activity by disinhibitory mechanism and releases motor actions (Albin et al. 1989; DeLong 1990; Gerfen et al. 1990; Mink 1996; Hikosaka et al. 2000; Nambu et al. 2000, 2002; Nambu 2007; Kravitz et al. 2010). During D1R suppression (see Supplementary Fig. 4, right), signals through the cortico-striato-EPN direct pathway are strongly suppressed and fail to induce inhibition in the EPN, resulting in the reduced motor activity. Moreover, this observation suggests that the loss of the phasic inhibition in the EPN through the direct pathway, but not the firing rate or pattern changes, is a fundamental phenomenon in reduced motor activity during D1R suppression. The reduction of spontaneous motor activity was observed even in the first week of Dox treatment (Fig. 2A) when the level of D1R expression was still higher than that in WT mice (Fig. 1D). The loss of cortically evoked inhibition in EPN neurons was already observed in the first half (5-14 days) of Dox treatment (see Supplementary Fig. 3A). These results suggest that relative decrease of D1R expression would be responsible for the changes in motor behavior and neuronal activity. Considering the fact that expression of D1Rs not only in the striatum but also outside the striatum was suppressed during Dox treatment in D1RKD mice (Fig. 1C), we cannot rule out the possibility that loss of extrastriatal D1Rs were also involved in their behavioral changes observed in the current study.

The present study revealed a crucial role for dopamine in maintaining the dynamics of the BG circuitry: D1R-mediated dopaminergic transmission maintains the information flow through the cortico-striato-EPN (GPi) direct pathway to appropriately release motor actions through disinhibitory mechanisms (see Supplementary Fig. 4). The present results also suggest that phasic activity changes in the EPN through the corticostriato-EPN direct pathway are fundamental to both normal functions of the BG and the pathophysiology of movement disorders. Dopamine deficiency impairs phasic activation of the cortico-striato-EPN direct pathway and release of motor actions, and would be involved in the bradykinesia seen in PD.

#### **Supplementary Material**

Supplementary material can be found at: http://www.cercor. oxfordjournals.org/.

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## A Modification cassette construct



tTA-pA, tetracycline transactivator and SV40 polyA A, Asc I; B, Bam HI; E, Eco RI; Nc, Nco I; S, Sau 3AI; Xb, Xba I; Xh, Xho I; Xh#, Xho I (artificial)

## B BAC4-tTA construct





# A D1RKD EPN



2 During Dox (15–22 days)





## **B** WT EPN







**Supplementary Figure 1.** Construction of BAC4-tTA and D1R-TRE. (*A*) Schematic representation of mouse D1R and the modification cassette D1Txx for bacterial artificial chromosome (BAC) modification using a two-step Rec A strategy. The 5' probe, tTA probe, A-arm and B-arm were used for Southern analysis. (*B*) Schematic representation of the BAC clone containing mouse D1R and the construct for generation of tTA-expressing transgenic (Tg) mice. BAC4-D1R was modified using homologous recombination with the pSV1-RecA-D1T shuttle vector (see Materials and Methods) to generate BAC4-tTA. (*C*) Schematic representation of the D1R-TRE construct for the generation of tetracycline operator-target Tg mice. The bidirectional tetracycline-responsive promoter leads to simultaneous expression of both D1R and lacZ depending on the action of tTA.

**Supplementary Figure 2.** Proportions of neurons in the entopeduncular nucleus (EPN, A) and external pallidum (GPe, B) classified according to the cortically evoked response patterns before, during, and after doxycycline (Dox) treatment in Dopamine D1 receptor knockdown (D1RKD) mice. ex, excitation; inh, inhibition. Response patterns can be grouped into 2 groups: responses with inhibition, such as ex-inh-ex, ex-inh, inh-ex and inh, and responses without inhibition, such as ex-ex and ex.

**Supplementary Figure 3.** (*A*) Cortically evoked responses of EPN neurons in D1RKD mice in the first half (5–14 days after starting Dox treatment, 1) and in the latter half (15–22 days after starting Dox treatment, 2) of Dox treatment. Population peristimulus time histograms (population PSTHs, with Gaussian filter) are shown. Stimulation was delivered at time 0 (arrows). The number of neurons is indicated by n, and the shaded areas represent  $\pm$  SD. (*B*) Cortically evoked responses of EPN neurons before (1) and during (2) Dox treatment in wild-type (WT) mice. Raster and PSTHs (100 trials; bin width, 1 ms) for typical examples (left) and population PSTHs are shown. The mean firing rate and statistical levels of p < 0.05 (one-tailed t-test) are indicated in PSTHs by a black and white dashed line (mean) and black (upper limit) and white (lower limit) solid lines, respectively.

**Supplementary Figure 4.** D1R-mediated dopaminergic transmission maintains the information flow through the cortico-striato-EPN (homologous to GPi) *direct* pathway to appropriately release motor actions. Under normal D1R expression (left), signals through the cortico-striato-EPN (GPi) *direct* pathway induce inhibition in the EPN (GPi) and release motor actions by disinhibiting the thalamus. During D1R suppression (right), the information flow through the cortico-striato-EPN (GPi) *direct* pathway is strongly suppressed and fails to induce inhibition in the EPN (GPi), resulting in the reduced motor activity. Open and shaded circles represent excitatory and inhibitory neurons, respectively.

# Expression pattern of immediate early genes in the cerebellum of D1R KO, D2R KO, and wild type mice under vestibular-controlled activity

Toru Nakamura<sup>1, 2, 3</sup>, Asako Sato<sup>4, 5</sup>, Takashi Kitsukawa<sup>1, 3</sup>, Toshikuni Sasaoka<sup>4, 5, 6</sup> and Tetsuo Yamamori<sup>1, 2, 7\*</sup>

<sup>1</sup> Division of Brain Biology, National Institute for Basic Biology, Okazaki, Japan, <sup>2</sup> Department of Basic Biology, Graduate University for Advanced Studies (SOKENDAI), Okazaki, Japan, <sup>3</sup> KOKORO-Biology Group, Laboratories for Integrated Biology, Graduate School of Frontier Biosciences, Osaka University, Suita, Japan, <sup>4</sup> Laboratory of Neurochemistry, National Institute for Basic Biology, Okazaki, Japan, <sup>5</sup> Department of Laboratory Animal Science, Kitasato University School of Medicine, Sagamihara, Japan, <sup>6</sup> Department of Comparative and Experimental Medicine, Brain Research Institute, Niigata University, Niigata, Japan, <sup>7</sup> Laboratory of Molecular Analysis for Higher Brain Function, RIKEN Brain Science Institute, Wako, Japan

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#### \*Correspondence:

Tetsuo Yamamori, RIKEN Brain Science Institute, Wako, Saitama 351-0198, Japan yamamori@nibb.ac.jp; tetsuo.yamamori@riken.jp

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We previously reported the different motor abilities of D1R knockout (KO), D2R KO and wild-type (WT) mice. To understand the interaction between the cerebellum and the striatal direct and indirect pathways, we examined the expression patterns of immediate early genes (IEG) in the cerebellum of these three genotypes of mice. In the WT naive mice, there was little IEG expression. However, we observed a robust expression of c-fos mRNA in the vermis and hemisphere after running rota-rod tasks. In the vermis, c-fos was expressed throughout the lobules except lobule 7, and also in crus 1 of the ansiform lobule (Crus1), copula of the pyramis (Cop) and most significantly in the flocculus in the hemisphere. jun-B was much less expressed but more preferentially expressed in Purkinje cells. In addition, we observed significant levels of *c-fos* and *jun-B* expressions after handling mice, and after the stationary rota-rod task in naive mice. Surprisingly, we observed significant expression of *c-fos* and *jun-B* even 30 min after single weighing. Nonetheless, certain additional c-fos and jun-B expressions were observed in three genotypes of the mice that experienced several sessions of motor tasks 24 h after stationary rota-rod task and on days 1 and 5 after rota-rod tasks, but no significant differences in expressions after the running rota-rod tasks were observed among the three genotypes. In addition, there may be some differences 24 h after the stationary rota-rod task between the naive mice and the mice that experienced several sessions of motor tasks.

Keywords: IEG, c-fos, jun-B, rota-rod, striatum

### Introduction

We have recently reported the distinct motor impairments of congenic dopamine D1 and D2 receptor KO mice in their performance of three different types of motor behavioral tasks, that is, spontaneous motor activity tasks in the home cage, rota-rod tasks, and our own invented Step-Wheel task (Kitsukawa et al., 2011). In the Step-Wheel task, mice run as they adjust their steps on

foothold pegs to drink water (Nakamura et al., 2014). Durieux et al. (2012) showed that the ablation of D1R medium spiny neurons (MSNs) in the dorsolateral striatum (DLS) of mice corresponding to the primate putamen decreases the learning curve in rota-rod tasks. They also showed that the ablation of D2R MSN in the dorsomedial striatum (DMS) of mice corresponding to the primate caudate decreases the initial learning curve of the task. These results of local ablation are generally consistent with those of the entire striatal ablation of D1R and D2R neurons (Durieux et al., 2012) and the results of our behavioral experiments using general D1R and D2R KO mice, suggesting that DLS and DMS play major roles in rota-rod tasks. However, there are also differences in the results between local ablation and general D1R KO and D2R KO mice (Nakamura et al., 2014). The ablation of D1R MSNs in either DMS or the entire striatum reduced locomotion in the open field, which behavior is the reverse of general D1R KO mice. The ablation of D2R MSNs in either DMS or the entire striatum does not affect the late-phase performance (Durieux et al., 2012), whereas the general D2R KO mice showed poorer leaning than WT mice in the Step-Wheel tasks (Nakamura et al., 2014). These differences between local ablation of DRs and general KO suggest that the involvement and interaction between the striatum and other brain regions.

To explore this possibility further, in this study we employed the immediate early gene (IEG) mapping technique, with which the neuronal activity in the cerebellum is monitored on the basis of IEG expressions. Among eight IEGs we examined, *cfos* and *jun-B* showed marked expression and specific induction, respectively, in the cerebellum immediately after the running rota-rod tasks. Interestingly, by examining *c*-*fos* and *jun-B* expressions in the vermis, Crus1, Cop and the flocculus in the cerebellar hemisphere, we found significant expressions of *c*-*fos* and *jun-B* 24 h after the stationary rota-rod tasks, during which time the mice did not perform any motor tasks but were allowed to move freely. We consider that this may be consistent with the idea that motor sequence learning and adaptation progress with time, through "consolidation" and "automatization" (Doyon et al., 2009).

We therefore examined in detail the expression patterns of c-fos and jun-B in the congenic D1R KO, D2R KO, and WT mice after the stationary and running rota-rod tasks in order to determine how cerebellar activities related to the D1R- and D2R-dependent pathways. Although we have not confirmed the alteration of *c-fos* expression patterns in the cerebellar flocculus on days 1 and 5 after the rotating rota-rod tasks among the three genotypes of mice, D1R KO, D2R KO, and WT mice, we found significant expressions of *c-fos* and *jun-B* not only immediately (30 min) after the mice ran on the rota-rod but also after handling them, and 30 min and unexpectedly even 24 h after the stationary rota-rod task. This observation led us to examine a series of examination for *c-fos* and *jun-B* expression patterns. We found that vestibular-controlled activities such as handling mice and weighing caused significant *c-fos* and *jun-B* expressions in cerebellar flocculus. However, there may be still some differences in expressions 24 h after the stationary rota-rod task between the naive and trained mice. Our results indicate that the analysis of *c-fos* and *jun-B* expression patterns is a useful tool for studying cerebellar activity, which may play a role complementary to imaging studies such as functional MRI, because of the fine resolution at the cellular level, in understanding the sequential events of motor learning in the cerebellum.

#### Materials and Methods

The expression of eight IEGs was first examined in the cerebellum of naive WT mice, that is, mice that did not experience any motor tasks but were allowed to move freely. The IEG expression patterns were examined in the mice that performed a rota-rod task (Experiment 1). Next, we compared *c-fos* and *jun-B* mRNA expression levels, in the cerebellar hemisphere, especially the flocculus among the three genotypes of mice: D1R KO, D2R KO, and WT mice (Experiment 2). Additionally, we examined the expressions patterns of *c-fos* and *jun-B* in the mice 30 min and 24 h after the stationary rota-rod tasks and 1-week handling.

For Experiment 1, adult male mice (8–9 week old, n = 5, C57BL/6J, Charles river laboratories Japan Inc.) were purchased. For Experiment 2, mice lacking either D1R (n = 7) or D2R (n = 9) were generated in accordance with the protocol previously published (Yamaguchi et al., 1996; Tran et al., 2008) and backcrossed for up to 10 generations with C57BL/6J (CLEA Japan Inc.) mice. Their genotypes were determined by PCR analysis of genomic DNA extracted from the tail of each mouse. As a control, C57BL/6J WT mice were purchased. All mice were housed individually in a plastic cage under 12 h light/dark cycle (9:00-21:00) and given food (Rodent Diet CA-1, CE-2, CLEA Japan Inc.) and water ad libitum. To encourage food intake to maintain their health (Drago et al., 1994; McNamara et al., 2003), D1R KO mice were additionally given palatable food (Rodent Diet B-F, CLEA Japan Inc.) on the floor of their cages. All the experiments were performed in accordance with the guidelines of the National Institutes of Health and the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan, and were approved by the Institutional Animal Care and Use Committees of the National Institutes of Natural Sciences, Kitasato University School of Medicine, and Frontier Biosciences of Osaka University. We made all efforts to minimize the number of animals used and the incidence or severity of distress experienced by the animals.

#### Motor Behavior Tasks

In Experiment 1, WT naive mice were initially group housed for 1 week until they were segregated and individually housed during motor task training and testing as described in the Results. In Experiment 2, the same mice that experienced series of two types of motor tasks (rota-rod and Step-Wheel tasks) over a period of 4 months as described previously were used (Nakamura et al., 2014, detailed history is shown in **Figure 3** and Supplementary chart 1). In these series of experiments, the mice were divided into two groups for the rota-rod tasks: fast-slow (15–5 rpm) and slow-fast (5–15 rpm) groups (Nakamura et al., 2014). The mice were sacrificed at 10 months of age. We examined the expression patterns of *c-fos* and *jun-B* in the WT mice that did

not previously experience any motor task at  $30 \min (n = 3)$ and 24 h (n = 3) after the stationary (0 rpm) rota-rod task. The rota-rod apparatus (MK-660A, Muromachi Kikai Co., Ltd.) was used. The rod diameter was 3 cm and the rotation speed was 10 rpm (Experiment 1) or 5 rpm (Experiment 2). In the running rota-rod tasks, a trial was considered when any one of the following three events occurred: The mouse (1) fell, (2) remained on the rod up to 60s (Experiment 1) or 120s (Experiment 2), or (3) clung to the rod for two complete turns, in which the mouse was considered to have fallen. The duration (retention times) that the mouse remained on the rod was recorded as the score. Five trials (Experiment 1) or three trials (Experiment 2) per day were performed and the interval between the trials was set at 30-60 s, during which time, the mice were placed in their home cages. Presessions consisted of trials for 1 day (Experiment 1) or 3 days (Experiment 2), during which the mice were placed on a stationary rod for habituation to the apparatus. Following the presessions, the running experiments were started. In Experiment 1, the mice were sacrificed 30 min after the last trial on day 1 (n = 2). In Experiment 2, the mice were divided into 3 groups: Pre (WT, n = 2; D1R KO, n = 2; D2R KO, n = 3), day 1 (WT, n = 3; D1R KO, n = 3; D2R KO, n = 3), and day 5 (WT, n = 3; D1R KO, n = 2; D2R KO, n = 3) groups. The mice of the Pre group were sacrificed on day 1, that is, the next day after the presessions (24 h after riding on the stationary rod). For the days 1 and 5 groups, the mice were sacrificed 30 min after the last trial. The rota-rod tasks were performed during the light phase (13:00-19:00). For this series of experiments, we weighed 30 min before the decapitation except for naive mice in which no weighing was done at least for a week before sacrifice.

#### Handling

To investigate the effects of handling, WT mice (n = 2) that did not experience any motor tasks were handled for 1 week. The mice were placed on the palm of an experimenter for about 3 min per day. On the last day, the mice were decapitated 30 min after they were handled. On the other hand, the naive mice (n = 2) were fed *ad libitum*, and exposed to external factors as minimally as possible including experimenters and immediately decapitated.

#### In Situ Hybridization (ISH)

In situ hybridization (ISH) was performed as described previously (Schaeren-Wiemers and Gerfin-Moser, 1993; Liang et al., 2000; Komatsu et al., 2005) with some modifications. The mice were quickly decapitated. The brain was removed, embedded in O.C.T. compound (Sakura) and quickly frozen in isopentane cooled with liquid nitrogen. Sections ( $10 \mu m$  thick) were cut on a cryostat (CM3050, Leica), thaw-mounted on slides (MAS coated glass slides, Matsunami), and air-dried. The sections were fixed in 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB, pH 7.3) for 15 min, washed three times in PBS, pH 7.4, acetylated for 10 min in 0.25% acetic anhydride in 0.1 M triethanolamine-HCl, pH 8.0, and washed three times with PBS. Prehybridization was performed using hybridization buffer [50% formamide,  $5 \times SSC$  ( $20 \times SSC$  in 3 M NaCl, 0.3 M sodium citrate, pH 7.0),  $5 \times$  Denhardt's,  $250 \mu$ g/ml yeast tRNA,

and 500 µg/ml salmon sperm DNA] for 30 min. DIG-labeled cRNA probes were denatured for 2 min at 82°C and chilled on ice. The sections were covered with hybridization buffer containing  $1 \,\mu$ g/ml of the cRNA probes, added dropwise, and coverslipped. The information of each probes used in this study is shown in Supplementary Table 1. The slides were incubated overnight (14-18 h) at 72°C in a humidified chamber (50% formaldehyde, 5  $\times$ SSC), washed three times with  $0.2 \times SSC$  at  $72^{\circ}C$ , and then rinsed with TBS, pH 7.5 (100 mM Tris-HCl, pH 7.5, 150 mM NaCl). To detect the hybridized probes, the sections were blocked with  $1 \times$ blocking solution (Roche) in TBS, pH 7.5, for 30 min, and then incubated in the blocking solution with an alkaline phosphatase (AP)-conjugated anti-DIG antibody (1:1000 dilution, Roche) overnight at 4°C. The sections were rinsed three times with TBS, pH 7.5, and equilibrated in TBS, pH 9.5 (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 50 mM MgCl<sub>2</sub>). Enzymatic activity was visualized by staining with 0.2 mM 5-bromo-4-chloro-3indolyl-phosphate, 0.2 mM nitroblue tetrazolium (NBT/BCIP) in TBS, pH 9.5, in the dark, until the signal reached a satisfactory intensity. After a brief wash in PBS and distilled water, the sections were dehydrated in a series of gradually increasing concentrations of ethanol solutions. After immersion in xylene, the sections were mounted in Entellan, a new rapid mounting medium (Merck).

#### Results

#### **IEG Expression Patterns in the Cerebellum**

We examined the IEG expression patterns in the cerebellum. In our previous study, we examined the expression patterns of IEGs including the fos and jun families in cerebellar slices without and with treatment with α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate (AMPA) and/or 8-bromo-cGMP (8-BrcGMP) (Nakazawa et al., 1993). That study showed that untreated slices express low levels of IEGs but slices treated with AMPA and 8-Br-cGMP express high levels of c-fos, jun-B and zif-268 (NGFI-A). Following that study, we first examined IEG expression patterns in the cerebellum. Naive mice that were moving freely but doing no specific tasks were sacrificed, and the IEG expression patterns of the fos family (c-fos, fos-B), jun family (jun-B, c-jun, and jun-D), and other IEGs of zif-268 (egr 1, NGFI-A), krox 20 (egr 2, NGFI-B), and arc were examined immediately after the sacrifice (see Materials and Methods). Consistent with the previous results, we observed little expression of these eight IEGs in the cerebellum of naive mice (Figures 1, 2). We next examined the expression patterns of these IEGs immediately after the mice performed five trials of running rota-rod tasks (Figure 3A). The mice were sacrificed 30 min after the last trial and the IEG expression patterns in the cerebellum were examined (Figures 4, 5). Among the members of the fos and jun families, c-fos was expressed in almost all the lobules of the vermis (except lobule 7), and Crus1, Cop and most significantly in the flocculus in the hemisphere. It is strongly expressed in the granular and Purkinje layers, and some intense signals were observed in the molecular layer. jun-B was also expressed in the same regions as *c-fos* was expressed but more preferentially in Purkinje cells and some sparsely scattered intense signals

	Vermis	Hemisphere	Flocculus
c-fos	P+++A V		200µm
fos-B	1000-		f man
	Back	AND AND	
jun-B	095		
	Bala		
c-jun	09	10 27	
	3062	RU	
jun-D	11	87 4	and day
FIGURE 1   Expression pattern	s of eight IEGs in the carabally	n showing floca life	right namely. Each row shows expression
of naive mice. Vermis (left particular and the magnified image within	anel), hemisphere (middle panel), the box in the middle panel	patterns of eight lf c-jun, jun-D).	EGs (from top to bottom: <i>c-fos, fos-B, jun-B,</i>

of *jun-B* were observed in the molecular layer. We observed some weak expression of *fos-B* in the flocculus as well. In the cerebellar slices, although we previously observed significant *zif-268* expression (Nakazawa et al., 1993), here we observed only

a generally moderate expression of *zif-268* under the present condition. *krox 20* was also expressed to some extent in the flocculus. Note that the adjacent external layer of the inferior colliculus expressed *arc* and *zif-268* at certain levels, which may



be used as internal controls for evaluating the IEG expression levels in the cerebellum. Overall, *c-fos* and *jun-B* among the IEGs examined in the flocculus showed most prominent expressions immediately (30 min) after the rota-rod running tasks. Therefore, hereinafter, we focused on the expression patterns of *c-fos* and *jun-B* in the flocculus.

#### *c-fos* and *jun-B* expression Patterns in Stationary Rota-Rod Task in Naive Mice

As we mentioned in Introduction, we were interested in the functional relationship between the cerebellum and the striatum and explored a model experimental system. Dopamine receptors are distributed in certain brain regions (Gingrich and Caron, 1993). However, our previous studies demonstrated that D1R KO and D2R KO mice showed distinguished motor impairments (Nakamura et al., 2014). In the running rota-rod tasks, the impairments were largely similar to those resulting from local ablation of D1R neurons and D2R neurons in DLS and DMS (Durieux et al., 2012), respectively. These results suggest that when using D1R KO and D2R KO mice in the rota-rod tasks, the main target is the striatum. We therefore used D1R KO and D2R KO mice to examine the cerebellar flocculus

IEG (*c-fos* and *jun-B*) expression patterns and the relationship between the striatum and the cerebellum in relation to motor learning.

We first examined the expression patterns of *c-fos* and *jun-*B in the stationary rota-rod task. In this task, a mouse was placed on a non-rotating bar with a thickness about half the length of the mouse's body. This task was not too difficult for mice to perform, and not only WT mice but also D1R KO and D2R KO mice were able to stay for the time examined (Supplementary Figure 2). Nonetheless, this task requires mice to balance on the bar and therefore activates related brain regions. In parallel, we first examined the *c-fos* and *jun-B* expression patterns in the cerebellum of WT mice after they were handled for 1 week (Figure 6). Herein, from Figures 6-10 for all the ISH data, we examined 6-13 sections of the cerebellums of individual mice under each motor condition as described for each figure. We examined two to five sections of the vermis and four to nine sections of the hemisphere in each mouse, and here used the sections that showed the highest signal intensity of the IEG expression in the flocculus among the sections of the cerebellum under each condition. We were surprised to find high expression levels of *c-fos* and *jun-B* in the cerebellum immediately



(30 min) after handling (**Figure 6**). In the hemisphere, we found moderately high expression levels in Crus1, and Cop and high expression levels in the flocculus. The expressions of *c-fos* and *jun-B* were probably induced by the attempts of mice to maintain their balance on the palm of the hand during the handling. The expression patterns of *c-fos* and *jun-B* appeared to be overall similar between the conditions (handling, stationary and running rota-rod), although there may have been some differences. We also found the expression of these genes not only immediately (30 min) after but also 24 h after the stationary rota-rod task for WT naive mice (**Figures 3D**, **7A**,**B**) and for the same duration in the D1R KO, D2R KO, WT trained mice (**Figures 3B,C**, **8**, three sessions as the trained mice). The expressions of *c-fos* and *jun-B* in the flocculus even 24 h after the stationary rota-rod task was unexpected because IEG expressions would not last for such a

long time. We weighed the mice 30 min prior to increase their sacrifice 24 h after the stationary rota-rod task. The weighing involved placing the mice on the scales of a balance, which may have activated the vestibular systems. It was indeed the case because we observed significant expression of *c-fos* and *jun-B* 30 min after only single weighing (Supplementary Figure 3A). We also examined the expression patterns of these two IEGs 24 h after the stationary rota-rod task in the mice that experienced several sessions of the wheel running and rota-rod tasks (rotating rota-rod and Step-Wheel tasks, see Materials and Methods for details, **Figure 8**). In the naive mice, which only experienced the stationary rota-rod task, the expression levels of *c-fos* and *jun-B* were reduced to some extent but were still significant. In the mice that experienced several sessions of motor tasks, the expressions, in particular that of *jun-B*, appeared to be more sustained



FIGURE 4 | Expression patterns of eight IEGs in the cerebellum of mice that performed rota-rod tasks. IEG expression patterns in the mice that performed three trials of the rota-rod tasks (see Materials and Methods). The figures are lined up in the same order as in Figure 1.

than those in the naive mice (**Figures 7**, **8**). We also noticed that the expression pattern of *c-fos* and *jun-B* may be different between those of 30 min after only weighing in naive mice and 24 h after stationary rota-rod in trained mice (Supplementary Figures 4, 5).

# *c-fos* and *jun-B* Expression Patterns in Stationary and Rotating Rota-Rod-Tasks In Trained Mice

Doyon et al. (2009) proposed that the basal ganglia are functionally related to motor learning. They propose that motor sequence learning and adaptation progress with time through



the "consolidation" and "automatization" processes. During the course of these processes, the basal ganglia, cerebellum, hippocampus and cerebral cortex interact with each other, and, with time, the sites of plasticity are shifted to the cerebral cortex and striatum for motor sequence learning and the cerebral cortex and cerebellum for motor adaptation. We thus examined the cerebellar IEG expression patterns 24 h after the stationary rota-rod task in the mice that experienced several motor tasks (rotating rota-rod and Step-Wheel tasks, see Materials and Methods for details) in order to determine whether there are any differences in cerebellar activity among the three different genotypes of mice immediately (30 min) after they performed running rota-rod tasks on days 1 and 5 (**Figures 9, 10**). **Figures 9**, **10** show the expression patterns of *c-fos* 24 h after the stationary rota-rod task in the three genotypes of mice after the rota-rod tasks on days 1 and 5, respectively. We observed significant expressions of *c-fos* in the hemisphere even 24 h after the stationary rota-rod task. Among the regions in the hemisphere, the expression in the flocculus was most significant. The *c-fos* expression is generally similar between days of 1 and 5. **Figures 9**, **10** show cerebellar *jun-B* expression patterns in WT mice. *jun-B* was expressed in the cerebellar vermis, and Crus1 and Cop of cerebellar hemisphere. Although *jun-B* was expressed to some extent in the molecular layer and at a lower level in the granular layers, the expression was most prominent in Purkinje cells. *jun-B* expression in the Purkinje cells in the



30 min, (B) 24 h after the stationary rod task (see Materials and Methods), or (C) 30 min after single weighing. Right panels show magnified images of D1R KO, D2R KO, and WT mice expressed c-fos in the floccular signals. cerebellar hemisphere, particularly in flocculus 24 h after the

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and 5.

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#### FIGURE 8 | Continued

**(B)** D1R KO, and **(C)** D2R KO mice were trained to perform a series of rota-rod and Step-Wheel tasks (see Materials and Methods). These mice were sacrificed 24 h after the stationary rota-rod task and designated as the Pre group for the running rota-rod tasks (see Materials and Methods). The left panels show the sections from the hemisphere that showed the highest signal intensity among the sections examined in each row. The right panels show the magnified images of the flocculus enclosed in a box in the first row of the left panel.

stationary rota-rod tasks and on days of 1 and 5 after the rotating rota-rod tasks. The expression levels of *c-fos* and *jun-B* 24 h after the stationary rota-rod task in D1R KO mice appeared lower than those in D2R KO and WT mice, but since we failed in the staining of ISH in one of D1R KO mice and we were unable to make a conclusion on the significance of this finding. The expression of *c-fos* in the granular layer of the flocculus of D2R KO mice might be more enhanced on day 1 after the running rota-rod tasks than under the other conditions (**Figures 8–10**). *jun-B* expression in D2R KO mice was also enhanced 24 h after stationary rota-rod and on days 1 and 5, and may be more enhanced on day 1. These observation, however, were not statistically confirmed any further owing to the lack of a sufficient number of mice of these three genotypes available for series of motor tasks.

#### Discussion

We examined cerebellar expression patterns of eight different IEGs in D1R KO, D2R KO, and WT mice following stationary and rotating rota-rod tasks. *c-fos* expression was enhanced in all the lobules of the vermis except lobule 7 and in Crus1, Cop and the flocculus in the hemisphere, whereas *jun-B* expression was only enhanced in some of the Purkinje cells in these regions. Furthermore, in this study we surprisingly found that *c-fos* and *jun-B* in the cerebellar vermis and flocculus were significantly expressed in mice after handling and 24 h after the stationary rota-rod task. We also found robust expressions of *c-fos* and *jun-B* after the running rota-rod tasks on days 1 and 5 in D1R KO, D2R KO, and WT mice.

#### **IEG Expression Patterns in the Cerebellum**

We examined the IEG expression patterns in the cerebellum. Among the eight IEGs we examined, *c-fos* was significantly expressed in all lobules of the cerebellar vermis except lobule 7 and in Crus1, Cop and the flocculus of the cerebellar hemisphere. *jun-B* was preferentially expressed in the Purkinje cells in these regions. *arc* is known to be induced by various types of neural activities including visual stimulation (e.g., Nakagami et al., 2013); there is virtually no induction in the cerebellum (**Figure 5**). *zif-268* is also known to be induced upon various neural activation, but was little induced in the cerebellum (**Figure 5**). One plausible reason why *arc* and *zif-268* are not induced in the cerebellum is that cerebellar Purkinje cells do not express functional NMDA receptors (Perkel et al., 1990; Llano et al., 1991). For example, *arc* transcription is regulated



by NMDA- or voltage-gated calcium channel (VGCC)- mediated membrane depolarization, cAMP-dependent pathways and brain-derived neurotrophic factor (BDNF)-mediated pathways (Zheng et al., 2009). However, the exact mechanisms underlying non-expression of arc in the cerebellum need to be elucidated in future studies.

*c-fos* mapping showed the regions activated immediately after the running rota-rod tasks. In the vermis, *c-fos* was moderately induced in all the lobules except lobule 7 throughout the cerebellar vermis. In the hemisphere, significant expressions of *cfos* were observed in Crus1, Cop, and flocculus. This observation indicates that the cerebellar vermis plays a central role in visually guided movements (Edge et al., 2003) and receives limb and visual inputs (see Edge et al., 2003 for reference). Crus1 also receives visuomotor inputs (Edge et al., 2003; Cerminara et al., 2005). Hindlimb stimulation primary activates the medial aspect of Cop (Santori et al., 1986). Our *c-fos* mapping results in this report well match those of the previous studies that examined the cerebellar areas activated by visuomotor inputs. One new observation in *c-fos* mapping may be the neuronal activation in the flocculus in the cerebellar hemisphere after the stationary and running rota-rod tasks. This is probably because the rota-rod tasks required mice to maintain their balance, which likely activated eye movements and the vestibular systems. The vestibular-ocular reflex presumably activates the flocculus (Ito, 1982).



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# *c-fos* and *jun-B* Expression Patterns after Stationary Rota-Rod Task

We first examined the *c-fos* and *jun-B* expression patterns in the cerebellum immediately and 24 h after the mice performed the stationary rota-rod task in which mice were required to stay on the non-rotating (stationary) rota-rod. This is not a very difficult task for mice to perform, and both D1R KO and D2R KO mice were able to stay as long as WT mice did (Supplementary Figure 2). However, to our surprise, even 24h after performing the stationary rota-rod task, we observed significant *c-fos* and *jun-B* expressions in the cerebellum. It was puzzling why the cerebellum was activated 24 h after the task, during which time the mice did not perform any specific tasks. One possible explanation for this would be as follows: there were other brain regions that were activated, which in turn activated the cerebellum. Interaction among the cerebellum, basal ganglia and cerebral cortex has been proposed (Doya, 2000; Hikosaka et al., 2002; Doyon et al., 2009; Bostan et al., 2013). It is therefore possible that the cerebellum was activated by other brain areas such as the basal ganglia. We therefore examined the *c-fos* and *jun-B* expression patterns in D1R KO and D2R KO mice after the running rota-rod tasks. We weighed the mice 30 min before sacrifice after 24 h after stationary rota-rod tasks. The weighing involved placing the mice on the scales of a balance, which may have activated the vestibular systems. It was indeed the case as shown in Supplementary Figure 3. However, the enhancement of *c-fos* and *jun-B* 24 h after the stationary rota-rod task in the mice that experienced several sessions of the motor tasks seems to be higher than that of 24 h after the stationary rota-rod for naive mice (Figures 7, 8). We also noticed the different IEG expression pattern between after only single weighing in naive mice and 24 h after stationary rotarod in trained mice although both groups of mice were sacrificed 30 min after the last weighing (Supplementary Figures 4, 5). This difference may reflect the shift of cerebellar activation by forming internal model within the cerebellum (Bursztyn et al., 2006).

The expression of *c-fos* did not appear to be altered between days 1 and 5 after the running rota-rod tasks in D1R KO, D2R KO, and WT mice. However, there may be some difference in

*jun-B* expression under our conditions. Durieux et al. (2012) and Nakamura et al. (2014) demonstrated that D1R KO mice and, mice with complete ablation of D1R neurons in the striatum showed decreased learning curve and performance after learning of the rota-rod tasks, whereas mice with ablated D2R neurons in the DMS and the entire striatum showed decreased initial performance of the rota-rod tasks. It has been reported that cerebellar activity reflects an acquired internal model (Imamizu et al., 2000). At the time when the impairment of striatal functions is severe, the cerebellar region works to compensate for the impairment and will presumably be most activated.

The expression of IEG and the downstream regulated genes in the striatum of D1R KO, D2R KO mice has been reported upon pharmacological stimulation (Drago et al., 1996; Aoyama et al., 2000; Welter et al., 2007). In D1R KO mice cocaine did not induce c-fos and zif-268 expressions in the striatum (Drago et al., 1996). In D2R KO mice cocaine did not induce c-fos but induce zif-268 in the striatum (Welter et al., 2007). In the striatum D1R is coexpressed with substance P (SP) and D2R is coexpressed with encephalin (ENK). In D1R KO mice SP expression was reduced and ENK expression was unaltered. Cocaine induced SP but not ENK in the striatum of D1R KO mice. In D2 KO mice ENK expression was increased and SP expression was decreased (Aoyama et al., 2000). In D1R KO mice cocaine induced SP and but did not affect ENK expression (Drago et al., 1996). In D2 KO mice cocaine induced SP expression. Therefore, These previous studies indicated that by in large the direct (D1R) and indirect pathways (D2R) are affected as expected as we write in the Introduction in D1R KO and D2R KO mice in terms of the IEG expression. Compared to these previously reported results in the striatum where the D1R and D2R play direct roles in the regulation of IEGs, in the cerebellum there is little or only indirect effect in the IEG expression, if any, in D1R and D2R KO mice as we showed in this paper.

We have been using chemical methods for detecting ISH because of their high spatial resolution. However, for quantitative evaluation, chemical detection methods are not as quantitative

TABLE 1   <i>c-fos</i> and <i>jun-B</i> expressions in cerebellar flocculus.									
	WT		D1R KO		D2R KO				
	c-fos	jun-B	c-fos	jun-B	c-fos	jun-B			
Naïve	_	_	NT	NT	NT	NT			
Handling (for 1 week)	++	++	NT	NT	NT	NT			
Stationary RR 30 min	+ + +	++	NT	NT	NT	NT			
Stationary RR 24 h after (for naive mice)	++	+	NT	NT	NT	NT			
Stationary RR 24 h (for trained mice)	+ + +	++	+ + *	+*	+ + +	++			
Running RR Day 1	+ + +	++	+ + +	$+ + \sim + + + **$	+ + +	+++			
Running RR Day 5	+ + +	++	+ + +	++	+ + +	++			
Weighing	$+ \sim + +^{***}$	+***	NT	NT	NT	NT			

Summary of the expression levels of c-fos and jun-B in the cerebellar flocculus are represented; –, background level; +, weak positive; ++, positive; ++, highly positive signal. \*Only one mouse and somewhat weaker staining than other samples. \*Some variations between two mice. \*\*Uneven expression within floccullus. NT: not tested. Note that all animals were sacrificed 30 min after final task except for the naive mice that were sacrificed without weighing. The evaluation was done subjectively with agreement of by two of authors (Toru Nakamura and Tetsuo Yamamori). as radioisotope detection methods, in which the amount of radiation is strictly proportional to that of radioactive decay. Various conditions could affect chemical reactions; therefore, exact quantitation is difficult without an internal control. In this regard, our visual evaluation of *c-fos* expression in the flocculus on days 1 and 5 after the running rota-rod tasks in D1R KO and D2R KO mice should be more reliable because it enabled us to compare the expression with that in other lobules of the same section of the cerebellar hemisphere as an internal control.

It should be noted that *jun-B* was preferentially expressed in Purkinje cells after the rota-rod tasks. This expression pattern is quite in contrast to that of *c-fos*, which was particularly highly expressed in the granular layer and other layers as well. We previously reported that *jun-B* is selectively induced by conjunctive stimulation of climbing fibers and AMPA in the cerebellum, which mimics cerebellar LTD (Yamamori et al., 1995). *jun-B* expression level in the mice 24 h after the stationary rota-rod task may be higher in the mice that experienced several motor tasks than in the naive mice. However, these apparent differences should be examined by statistical analysis with a larger number of mice in future studies.

#### Implications of Studies of *c-fos* and *jun-B* Expression Patterns in the Cerebellum in D1R KO and D2R KO Mice

In this study, we applied techniques of IEG mapping in the cerebellum to detect the altered cerebellar neuronal activity after the stationary and running rota-rod tasks. We summarized the expression patterns of *c-fos* and *jun-B* in the flocculus in the cerebellum in **Table 1**. We consider that there are three main points in our studies reported in this paper. First, we found several different levels of expressions of *c-fos* and *jun-B* in the cerebellum in various conditions of weighing and handling,

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30 min and 24 h after the stationary rota-rod task, and 30 min after running rota-rod on days 1 and 5. There may be some enhancement of *c-fos* and *jun-B* 24 h after the stationary rota-rod in the trained mice even considering the weighing effect for the expressions of *c-fos* and *jun-B*. Second, to further examine the interaction between the cerebellum and the striatum, we examined the *c-fos* and *jun-B* expressions in the cerebellum in D1R KO, D2R KO, and WT mice after the stationary and running rota-rod tasks. We did not observe significant differences among the three genotypes although this does not still exclude the possibility that there are some differences among them. To clarify whether there are indeed differences among the three genotypes, a larger of number of mice need to be examined and statistical analysis should be conducted. Third, our results demonstrated the particular usefulness of *c-fos* and *jun-B* for examining expression patterns in the cerebellum, because other IEGs are little expressed in the cerebellum or not selectively expressed in Purkinje cells.

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#### Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fcell. 2015.00038/abstract

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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## ARTICLE

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# G-CSF supports long-term muscle regeneration in mouse models of muscular dystrophy

Nozomi Hayashiji<sup>1</sup>, Shinsuke Yuasa<sup>1</sup>, Yuko Miyagoe-Suzuki<sup>2</sup>, Mie Hara<sup>1</sup>, Naoki Ito<sup>2</sup>, Hisayuki Hashimoto<sup>1</sup>, Dai Kusumoto<sup>1</sup>, Tomohisa Seki<sup>1</sup>, Shugo Tohyama<sup>1</sup>, Masaki Kodaira<sup>1</sup>, Akira Kunitomi<sup>1</sup>, Shin Kashimura<sup>1</sup>, Makoto Takei<sup>1</sup>, Yuki Saito<sup>1</sup>, Shinichiro Okata<sup>1</sup>, Toru Egashira<sup>1</sup>, Jin Endo<sup>1</sup>, Toshikuni Sasaoka<sup>3</sup>, Shin'ichi Takeda<sup>2</sup> & Keiichi Fukuda<sup>1</sup>

Duchenne muscular dystrophy (DMD) is a chronic and life-threatening disease that is initially supported by muscle regeneration but eventually shows satellite cell exhaustion and muscular dysfunction. The life-long maintenance of skeletal muscle homoeostasis requires the satellite stem cell pool to be preserved. Asymmetric cell division plays a pivotal role in the maintenance of the satellite cell pool. Here we show that granulocyte colony-stimulating factor receptor (G-CSFR) is asymmetrically expressed in activated satellite cells. G-CSF positively affects the satellite cell population during multiple stages of differentiation in *ex vivo* cultured fibres. G-CSF could be important in developing an effective therapy for DMD based on its potential to modulate the supply of multiple stages of regenerated myocytes. This study shows that the G-CSF-G-CSFR axis is fundamentally important for long-term muscle regeneration, functional maintenance and lifespan extension in mouse models of DMD with varying severities.

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<sup>&</sup>lt;sup>1</sup> Department of Cardiology, Keio University School of Medicine, 35-Shinanomachi Shinjuku-ku, Tokyo 160-8582, Japan. <sup>2</sup> Department of Molecular Therapy, National Institute of Neuroscience, National Center of Neurology and Psychiatry, Tokyo 187-8502, Japan. <sup>3</sup> Department of Comparative and Experimental Medicine, Brain Research Institute, Niigata University, Niigata 951-8585, Japan. Correspondence and requests for materials should be addressed to S.Y. (email: yuasa@a8.keio.jp).

dult skeletal muscle has its own stem cell population known as satellite cells (SCs). After muscle injury, quiescent SCs are activated and then proliferate and differentiate into mature skeletal muscle to ensure that muscle function is recovered. SCs are at the top of the hierarchy of differentiating stages in adult myocytes, followed by myoblasts, immature myocytes and matured myocytes. Thus, maintaining the homoeostasis of skeletal muscle function over the long term requires a well-preserved population of SCs. However, SCs are still heterogenic, and among the distinct differentiation stages, the satellite stem cell population must be maintained to preserve their stemness, that is, long-term self-renewal and differentiation abilities. Accumulating data indicate that SCs maintain their population by asymmetric cell division<sup>1-4</sup>. The different differentiation stages of SCs are distinguishable by the expression of specific markers such as PAX7 and MYOD; PAX7 + /MYOD - cells are quiescent, PAX7 + /MYOD + cells are activated and PAX7 - /MYOD + cells are differentiating. Interestingly, some activated SCs exhibit asymmetric MYOD distribution within the cells, and each daughter cell is either PAX7 + /MYOD + or PAX7 + /MYOD -, the latter of which retains the quiescent state<sup>5</sup>. Such evidence indicated that asymmetric protein distribution produces asymmetric cell division, which has a critical role in maintaining the SC population. In our previous screen for myocyte differentiationpromoting factors<sup>6,7</sup>, we noted markedly elevated expression of granulocyte colony-stimulating factor receptor (G-CSFR, encoded by csf3r) in the developing somite<sup>8</sup>. Furthermore, G-CSFR was transiently expressed in regenerating myocytes of adult injured skeletal muscle, and extrinsic G-CSF supported short-term muscle regeneration in cardiotoxin-induced skeletal muscle injury<sup>9</sup> and crush injury<sup>10</sup>. However, although we found high G-CSFR expression in regenerating immature myocytes, whether G-CSFR would be expressed in SCs and whether G-CSF signalling would affect the SC population in skeletal muscle remain unclear.

Duchenne muscular dystrophy (DMD) is one of the most common inheritable muscle diseases in humans<sup>11,12</sup>. It is an X-linked progressive disease that affects approximately one in 3,500 male live births<sup>13</sup>. DMD is caused by a mutation in the gene encoding dystrophin, which links the internal muscle cytoskeleton to the extracellular matrix, enabling the lateral transmission of force from within muscle cells to the surrounding matrix. Mutations in the *dystrophin* gene lead to dystrophin deficiency at the myofibre membrane and progressive muscle fibre degeneration  $^{14,15}$ . The absence of dystrophin increases the fragility of the sarcolemma, which is susceptible to even mild stress<sup>16</sup>. Subsequent injury results in myofibre necrosis, followed by repetitive degeneration and regeneration supported by innate SCs. In DMD, muscle regeneration initially supports the mutation-driven functional impairment; however, such regeneration is eventually attenuated due to stem cell exhaustion<sup>17</sup>. Therapy that induces effective long-term skeletal muscle regeneration is, therefore, crucially needed for DMD.

Long-term muscle regenerative therapy for conditions such as DMD relies on increasing the pool of functional myocytes and maintaining the pool of SCs. We showed that G-CSFR is asymmetrically distributed in activated SCs, and that G-CSF increases the population of SCs via the G-CSF-G-CSFR axis in *ex vivo* cultured myofibres. On the basis of this, we hypothesized that G-CSF could be important in developing an effective therapy for DMD based on its potential to modulate the supply of multiple stages of regenerated myocytes. It is surprising that haploinsufficiency of G-CSFR leads to early lethality in DMD model mice, indicating that dose reduction of the G-CSF/G-CSFR

system could not support the balance between degeneration and regeneration. We also showed that G-CSF administration in mice with severe DMD markedly improves the phenotype, including muscle pathology, functioning and lifespan. Together, our data suggested that the G-CSF-G-CSFR axis is fundamentally important for long-term muscle regeneration, functional maintenance and lifespan extension in DMD mouse models with varying severities.

#### Results

G-CSFR is expressed in activated SCs. To explore the precise expression pattern of G-CSFR in skeletal muscle, we examined the SCs on isolated myofibres from the extensor digitorum longus (EDL) muscles of wild-type mice. This model preserves the physiological behaviour of SCs through activation, proliferation and differentiation on myofibres. In the isolated myofibres, PAX7 + quiescent SCs showed no G-CSFR expression by immunostaining at the time of myofibre isolation (Fig. 1a). G-CSFR expression was observed in the PAX7 + cells after 6 h (Fig. 1a,c), when MYOD expression also appeared (Fig. 1b; Supplementary Fig. 1a). G-CSFR expression was still apparent at 48 h in the MYOGENIN + SCs, which are committed to differentiation (Supplementary Fig. 1b,c)<sup>18</sup>. By 24 and 48 h, the number of G-CSFR-expressing cells increased to ~94% of PAX7 + cells (Fig. 1c).

Asymmetrically expressed G-CSFR in activated SCs. Unexpectedly, we found that G-CSFR is asymmetrically distributed within migrating SCs obtained from isolated myofibres at day 5 (Fig. 2a). This asymmetric distribution of G-CSFR was apparent in  $\sim$  20% of PAX7 + SCs at 72 h (Fig. 2b). Syndecan-4 (SDC4) was expressed in the activated SCs until mature myocyte fusion occurred; thus, paired SDC4 + cells were characterized as the daughter cells of SC division<sup>19,20</sup>. The majority of paired SDC4 + cells showed equivalent expression of G-CSFR (94.4%; Fig. 2c), but in the remaining 5.6% of paired SDC4 + cells, G-CSFR was detectable in only one daughter cell (Fig. 2d). Similarly, single SDC4 + SCs either express G-CSFR or not (Fig. 2e,f). In  $\sim 5\%$  of paired PAX7 + /SDC4 + cells, one daughter cell highly expressed G-CSFR; in the other daughter cell, G-CSFR expression was not observed (Fig. 2g). In addition, in  $\sim 5\%$  of paired PAX7 + cells, one G-CSFR + daughter cell highly expressed MYOD, whereas no MYOD expression observed in the other (Fig. 2h). These data suggested that G-CSFR is expressed from the activation of SCs through asymmetrical distribution during the first divisions but is not expressed in the self-renewing PAX7 + /MYOD - SCs (Supplementary Fig. 2).

G-CSF increases SCs via the G-CSF-G-CSFR axis. To elucidate whether G-CSFR signalling affects the populations of SCs, we examined the PAX7 + cell population in cultured myofibres from wild-type and G-CSFR-mutated ( $csf3r^{-/-}$ ) mice. At 0 and 24 h, there was no significant difference in the number of PAX7 + cellsbetween wild-type and  $csf3r^{-/-}$  mice; however, at 48 h, the csf3r - / - mice showed a significantly smaller number of PAX7 + cells than that of wild-type mice (Fig. 3a,b). At 24 h, most PAX7 + cells express MYOD, are activated and thereafter proliferate and commit to differentiation<sup>21–23</sup>. In the csf3r - / mice, the number of MYOD + /PAX7 + cells was significantly decreased in PAX7 + cells activated for 24 h (Fig. 3c,d), suggesting that the genetic ablation of G-CSFR signalling negatively affects the population of activated SCs. We then examined the direct role of G-CSF on SCs from wild-type mice. At 72 h, the subpopulation of activated SCs had lost MYOD expression in vivo and on cultured myofibres<sup>21-23</sup>. Surprisingly,

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**Figure 1 | G-CSFR is expressed in activated satellite cells. (a)** Immunostaining of satellite cells on myofibres isolated from EDL muscles for PAX7 (red) and G-CSFR (green), including nuclei stained with DAPI (blue) and bright-field images, at 0 h (T=0), 6 h (T=6) and 12 h (T=12). Scale bar, 10 µm. (b) Immunostaining of satellite cells on myofibres for MYOD (red) and G-CSFR (green) at 6 h (T=6), 24 h (T=24) and 48 h (T=48). Scale bar, 10 µm. (c) The G-CSFR +/PAX7 + cell ratio at 0, 6, 12, 24 and 48 h (T=0: n=53; T=6: n=73; T=12: n=45; T=24: n=41; T=48: n=297; each myofibre: n=20-30). All myofibres were obtained from ~10-week-old wild-type mice. Data are shown as mean ± s.d. DAPI, 4',6-diamidino-2-phenylindole dihydrochloride.

G-CSF significantly increased the population of SCs throughout the differentiation stages, including self-renewal SCs, as shown by the PAX7 + /MYOD – cells at 72 h in wild-type mice; in addition, csf3r - / - mice showed significantly decreased numbers of SCs throughout the differentiation stages (Fig. 3e,f; Supplementary Table 1). To elucidate the effect of G-CSF on the

population of PAX7 + cells *in vivo*, we examined SC behaviour in a cardiotoxin injury model (Fig. 3g). In the csf3r - / - mice, the population of PAX7 + -expressing cells was significantly decreased at 14 days after cardiotoxin injection (Fig. 3h,i), suggesting that genetic G-CSFR signal ablation negatively affects the population of PAX7 + cells *in vivo*.

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**Figure 2 | G-CSFR is asymmetrically expressed in activated satellite cells. (a)** Immunostaining of satellite cells for MYOD (red), G-CSFR (green) and DAPI (blue) at day 5. Scale bar, 20  $\mu$ m. (b) Immunostaining of satellite cells for G-CSFR (purple), PAX7 (green) and DAPI (blue) at day 3. Scale bar, 5  $\mu$ m. (c) Immunostaining of dividing satellite cells on myofibres for G-CSFR (green), SDC4 (red) and DAPI (blue), as well as bright-field (BF) images, at 48 h (satellite cells with symmetric G-CSFR expression: n = 51; satellite cells with asymmetric expression: n = 3). Scale bar, 10  $\mu$ m. (d) Immunostaining of satellite cells on myofibres for G-CSFR (green), SDC4 (red) and DAPI (blue), as well as BF images, at 48 h. Scale bar, 10  $\mu$ m. (e,f) Immunostaining of satellite cells on myofibres for SDC4 (red), G-CSFR (green) and DAPI (blue), as well as BF images, at 48 h. Scale bar, 10  $\mu$ m. (g) Immunostaining of cultured dividing satellite cells for G-CSFR (green) and DAPI (blue), as well as BF images, at 48 h. Scale bar, 10  $\mu$ m. (g) Immunostaining of cultured dividing satellite cells for G-CSFR (purple), PAX7 (red), SDC4 (green) and DAPI (blue). Scale bar, 5  $\mu$ m. (h) Immunostaining of cultured dividing satellite cells for G-CSFR (purple), PAX7 (red), MYOD (green) and DAPI (blue) at day 3 (among 273 dividing satellite cells, dividing satellite cells with symmetric G-CSFR expression: n = 5; dividing satellite cells with asymmetric expression: n = 268). Scale bar, 5  $\mu$ m. All myofibres were obtained from ~10-week-old wild-type mice. DAPI, 4',6-diamidino-2-phenylindole dihydrochloride.



**Figure 3** | **G-CSF increases satellite cells in multiple differentiation stages.** (a) Immunostaining of satellite cells on myofibres of ~10-week-old WT and csf3r - / - mice for PAX7 (red) and DAPI (blue) at 48 h. Scale bar, 50 µm. (b) Time course of the number of satellite cells (PAX7 +) at 0, 24 and 48 h per myofibre of WT and csf3r - / - mice. Data are shown as mean ± s.d. \*\**P* < 0.01; equal variance Student's *t*-test. Each fibre number: n = 20-29. (c) Immunostaining of satellite cells on myofibres of WT and csf3r - / - mice for PAX7 (red), MYOD (green) and DAPI (blue) at 24 h. Scale bar, 50 µm. (d) The percentage of MYOD + / PAX7 + satellite cells at 24 h. Data are shown as mean ± s.d. \*\**P* < 0.01; unequal variance Student's *t*-test. Each fibre number: n = 29. (e) Immunostaining of satellite cells on myofibres of WT mice administered control and G-CSF and csf3r - / - mice after being cultured for 72 h for PAX7 (red), MYOD (green) and DAPI (blue), as well as bright-field images. Scale bar, 50 µm. (f) Quantification of the number of PAX7 - /MYOD + (green), PAX7 + /MYOD + (red) or PAX7 + /MYOD - (blue) satellite cells per myofibre. Data are shown as mean ± s.d. One-way analysis of variance with Bonferroni's *post hoc* test. Quantification information in Supplementary Table 1. (g) Time course of cardiotoxin (CTX) injection and examination for 8-week-old WT and csf3r - / - mice. (h) Immunostaining of cross-sections of TA muscle of WT and csf3r - / - mice at 14 days after CTX injection for laminin, PAX7 and DAPI. Upper panel: merged with laminin (green), PAX7 (red) and DAPI (blue). Lower panel: merged with laminin (green) and DAPI (blue). Lower panel: merged with laminin (green) and DAPI (white). Arrows indicate PAX7 + /DAPI + cells. Scale bar, 20 µm. (i) Number of PAX7 + /DAPI + cells per 100 myocytes of WT and csf3r - / - mice. Data are shown as mean ± s.d. \*\**P* < 0.01; unequal variance Student's *t*-test. n = 5-6 per group. DAPI, 4',6-diamidino-2-phenylindole dihydrochloride; WT, wild

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**Figure 4 | G-CSF increases satellite cells through the G-CSF-G-CSFR-JNK axis. (a)** Immunostaining of satellite cells for G-CSFR (purple), PAX7 (red), pJNK (green) and DAPI (blue) at day 3. Scale bar, 5  $\mu$ m. (b) Immunostaining of dividing satellite cells for G-CSFR (purple), PAX7 (red), pJNK (green) and DAPI (blue) at day 3. Scale bar, 5  $\mu$ m. (c) Immunostaining of satellite cells on myofibres of WT mice for PAX7 (red), MYOD (green) and DAPI (blue), as well as bright-field images, following JNK inhibitor administration with or without G-CSF at 72 h. Scale bar, 50  $\mu$ m. (d) Quantification of the number of PAX7 – /MYOD + (green), PAX7 + /MYOD + (red) or PAX7 + /MYOD – (blue) satellite cells per myofibre. Data are shown as mean ± s.d. One-way analysis of variance with Bonferroni's *post hoc* test. Quantification information in Supplementary Table 1. DAPI, 4',6-diamidino-2-phenylindole dihydrochloride.

We previously showed that G-CSF apparently activates JNK and p38 signalling in cultured myoblasts9. Therefore, we examined whether these pathways are active in activated SCs. Immunostaining for pJNK and pp38 revealed that both JNK and p38 are activated in G-CSFR+ SCs (Fig. 4a; Supplementary Fig. 3a). The p38 pathway is required for SC activation and MYOD induction following proliferation<sup>24</sup>; herein, p38 inhibition in single myofibres decreased the population of PAX7 - /MYOD + SCs with or without G-CSF (Supplementary Fig. 3b,c; Supplementary Table 1). Although JNK signalling is involved in myoblast differentiation<sup>25</sup>, its role in SC behaviour remains unclear. In paired PAX7 + cells, the G-CSFR-expressing daughter cells highly expressed activated JNK, whereas the other daughter cells without G-CSFR expression did not (Fig. 4b). JNK inhibition also strongly reduced the number of PAX7+/ MYOD – and PAX7 + /MYOD + cells in single myofibres (Fig. 4c,d; Supplementary Table 1). These data indicated that G-CSF increases both activated and self-renewal SCs through the G-CSF-G-CSFR-JNK axis, suggesting that G-CSF could increase long-term regeneration in chronic skeletal muscle injury through maintenance of the SC pool.

**G-CSF increases muscle regeneration in** *mdx* **mice**. Patients with DMD show progressive physical impairment leading to the death by 20–30 years of age, whereas a mouse model of DMD (*mdx* mice) harbouring a *dystrophin* mutation has minimal physical impairment and a normal lifespan<sup>26</sup>. Indeed, *mdx* mice appear grossly normal (Supplementary Fig. 4a). However, pathological analysis revealed apparent muscle degeneration and regeneration in these *mdx* mice from 3 to 5 weeks of age

(Supplementary Fig. 4b). Myocyte numbers in the tibialis anterior (TA) muscle were decreased at 3 weeks of age, but were thereafter recovered (Supplementary Fig. 4c). Generally, healthy myocytes have peripherally located nuclei, and regenerating and regenerated myocytes have central nuclei. The mdx mice myocytes showed central nuclei from 4 weeks of age and increased in number up to 12 weeks of age (Supplementary Fig. 4d). These data suggested initial injury to the muscle fibres with subsequent regeneration to an almost normal state in the mdx mice. The histological observation of TA muscle showed strong G-CSFR expression in regenerating myocytes (Supplementary Fig. 4e), implicating the possible role of G-CSF in the muscle regeneration process. We then examined whether G-CSF administration would improve skeletal muscle regeneration in *mdx* mice. We examined the gross appearance and functional differences following daily intraperitoneal (i.p.) G-CSF injections from 3 to 5 weeks of age (Fig. 5a). Immunostaining for laminin showed the typically fine cellular architecture of TA muscle (Fig. 5b). The number of myocytes with central nuclei significantly increased with G-CSF administration at 5 weeks of age (Fig. 5c). The BF-45 antibody recognizes myosin heavy chain present during embryonic and neonatal skeletal muscle development and in newly formed adult regenerating myocytes<sup>27</sup>. Immunostaining for BF-45 in 4-weekold mice treated with G-CSF revealed significantly increased numbers of regenerating myocytes (Fig. 5d,e). Measuring the individual cross-sectional area of the myocytes further revealed a significantly increased proportion of small myocytes, indicating a substantial amount of newly formed regenerated myocytes at 5 weeks of age (Fig. 5f). Given that muscle size is a predictive value for muscle function, the demonstrated increase in the TA muscle

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**Figure 5 | G-CSF increases skeletal muscle regeneration in** *mdx* **mice.** (a) Time course of G-CSF administration and examination in *mdx* mice. (b) Immunostaining of cross-sections of TA muscle for laminin at 5 weeks of age *mdx* mice. Scale bar, 200  $\mu$ m (upper panel); 100  $\mu$ m (lower panel). (c) The percentages of myocytes with central nuclei in cross-sections of TA muscle at 5 weeks of age. Data are shown as mean ± s.d. \**P*<0.05; equal variance Student's *t*-test. *n* = 8 per group. (d) Immunostaining of cross-sections of TA muscle for BF-45 (red), laminin (green) and DAPI (blue) at 4 weeks of age. Scale bar, 100  $\mu$ m. (e) The percentages of BF-45-positive myocytes in cross-sections of TA muscle at 4 weeks of age. Data are shown as mean ± s.d. \**P*<0.01; unequal variance Student's *t*-test. *n* = 5 per group. (f) The individual fibre cross-sectional area of individual myocytes in cross-sections of TA muscles at 5 weeks of age. Data are shown as mean ± s.d. \**P*<0.01; unequal variance Student's *t*-test. *n* = 6 per group. (g) Muscle perimeters of cross-sections of TA muscles at 5 weeks of age. Data are shown as mean. *n* = 6 per group. (g) Muscle perimeters of cross-sections of TA muscles at 5 weeks of age. Data are shown as mean. *n* = 6 per group. (g) Muscle perimeters of cross-sections of TA muscles at 5 weeks of age. Data are shown as mean. *n* = 6 per group. (g) Muscle perimeters of cross-sections of TA muscles at 5 weeks of age. Data are shown as mean. *n* = 6 per group. (g) Muscle perimeters of cross-sections of TA muscles at 5 weeks of age. *n* = 9 per group. (i) Tetanic force examination of *ex vivo* EDL muscle tension at 5 weeks of age. Data are shown as mean ± s.d. No significant difference; unequal variance Student's *t*-test; *n* = 6 per group. (j) Specific forces, which are the ratios of tetanic force to muscle size, measured at 5 weeks of age. Data are shown as mean ± s.d. \**P*<0.05; unequal variance Student's *t*-test. *n* = 5 per group. DAPI, 4',6-diamidino-2-phenylindole dihydrochloride

perimeter in G-CSF-treated mice at 5 weeks of age suggested enhanced muscle function recovery (Fig. 5g). G-CSF also significantly improved exercise capacity in the mice by 5 weeks of age based on *in vivo* exercise tolerance testing (Fig. 5h). In a pure muscle function test, *ex vivo* tetanic force measurements and specific force examinations showed significantly improved muscle tension in the treated mice at 5 weeks of age (Fig. 5i,j).

Next, we examined whether G-CSF treatment would accelerate the regeneration process following cardiotoxin injury in mdxmice (Fig. 6a). The number of myocytes with central nuclei significantly increased by G-CSF administration in cardiotoxininjured mdx mice (Fig. 6b,c). The number of BF-45 + myocytes was also significantly increased by G-CSF treatment (Fig. 6d,e). The TA muscle perimeter was also increased by G-CSF treatment in cardiotoxin-injured mdx mice (Fig. 6f). These data suggested that G-CSF significantly improves muscle function through myocyte regeneration in mdx mice.

Haploinsufficiency of G-CSFR induces lethality in *mdx* mice. To clarify the roles of G-CSF in DMD, we next used  $csf3r^{-/-}$  mice, which show a slight haematological disorder and a normal lifespan<sup>28,29</sup>. We hypothesized that breeding G-CSFR homozygote-knockout *mdx* mice ( $mdx/csf3r^{-/-}$ ) would produce

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mice with severely impaired muscle regeneration and function. Surprisingly, G-CSFR heterozygote mdx  $(mdx/csf3r^{+/-})$  mice showed early partial lethality after birth and late partial lethality

at 3–5 weeks of age (Fig. 7a). These striking data indicated the importance of proper G-CSF signalling in DMD. The mdx,  $csf3r^{+/-}$  and  $mdx/csf3r^{+/-}$  mice were of normal gross



**Figure 6 | G-CSF increases muscle regeneration in** *mdx* **mice after CTX injury.** (a) Time course of CTX and G-CSF administration, and examination in 8-week-old *mdx* mice. (b) Haematoxylin and eosin staining of cross-sections of TA muscle at day 3 after CTX injection. Scale bar, 100  $\mu$ m. (c) The percentages of central nuclear myocytes in cross-sections of TA muscle at day 3 after CTX injection. Data are shown as mean ± s.d. \**P*<0.05; unequal variance Student's t-test. *n* = 4 per group. (d) Immunostaining of cross-sections of TA muscle for BF-45 (red), laminin (green) and DAPI (blue) at day 3 after CTX injection. Scale bar, 100  $\mu$ m. (e) The percentages of BF-45-positive myocytes in cross-sections of TA muscle at day 3 after CTX injection. Scale bar, 100  $\mu$ m. (e) The percentages of BF-45-positive myocytes in cross-sections of TA muscle at day 3 after CTX injection. Data are shown as mean ± s.d. \**P*<0.05; unequal variance Student's t-test. *n* = 5 per group. (f) Muscle perimeters of cross-sections of TA muscle at day 3 after CTX injection. Data are shown as box plot. Top, maximum; bottom, minimum. \**P*<0.05; Student's t-test. *n* = 4-6 per group. CTX, cardiotoxin; DAPI, 4',6-diamidino-2-phenylindole dihydrochloride; NS, not significant.

**Figure 7 | G-CSF signalling is essential for the survival in** *mdx* mice. (a) Kaplan-Meier survival curve of  $csf3r^{+/-}$  (red, n = 50), *mdx* (yellow, n = 50) and  $mdx/csf3r^{+/-}$  (blue, n = 53) mice. (b) Gross appearance of *mdx*,  $csf3r^{+/-}$ ,  $mdx/csf3r^{+/-}$  and WT mice at post-natal day 1. (c) Body weights of mdx (n = 7),  $csf3r^{+/-}$  (n = 9),  $mdx/csf3r^{+/-}$  (n = 11) and WT (n = 15) mice at post-natal day 1. Data are shown as box plot. Top, maximum; bottom, minimum. \*\*P < 0.01; unequal variance Student's t-test. (d) Evans blue staining of the diaphragm of mdx,  $csf3r^{+/-}$ ,  $mdx/csf3r^{+/-}$  and WT mice at post-natal day 1. (e) Haematoxylin and eosin staining of the diaphragm of mdx,  $csf3r^{+/-}$ ,  $mdx/csf3r^{+/-}$  and WT mice at post-natal day 1. Scale bar, 50 µm. (f) Diaphragm thickness of mdx,  $csf3r^{+/-}$ ,  $mdx/csf3r^{+/-}$  and WT mice at post-natal day 1. Scale bar, 50 µm. (f) Diaphragm thickness of mdx,  $csf3r^{+/-}$ ,  $mdx/csf3r^{+/-}$  and WT mice. Scale bar, 50 µm. (f) Diaphragm thickness of mdx,  $csf3r^{+/-}$ ,  $mdx/csf3r^{+/-}$  and WT mice. Scale bar, 50 µm. (f) Diaphragm thickness of mdx,  $csf3r^{+/-}$  and WT mice. Scale bar, 50 µm. (h) Number of PAX7 (red), laminin (green) and DAPI (blue) at post-natal day 1 of mdx,  $csf3r^{+/-}$ ,  $mdx/csf3r^{+/-}$  and WT mice. Data are shown as mean  $\pm$  s.d. \*\*P < 0.01; Student's t-test; n = 5 per group. (g) Immunostaining of cross-sections of the diaphragm for PAX7 (red), laminin (green) and DAPI (blue) at post-natal day 1 of mdx,  $csf3r^{+/-}$ ,  $mdx/csf3r^{+/-}$  and WT mice. Data are shown as mean  $\pm$  s.d. \*\*P < 0.01; Student's t-test; n = 5 per group. (i) Whole-body plethysmography to examine the respiratory function of individual mice. (j) Breath per min (BPM) in 8-week-old mdx and  $mdx/csf3r^{+/-}$  mice. Data are shown as mean  $\pm$  s.d. \*P < 0.05, \*\*P < 0.01; unequal variance Student's t-test; n = 7 per group. (k) Tidal volume of 8-week-old mdx and  $mdx/csf3r^{+/-}$  mice. Data are shown as mea

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appearance at post-natal day (P) P1 (Fig. 7b); however,  $mdx/csf3r^{+/-}$  mice had significantly reduced body weights compared with mdx littermates (Fig. 7c). To elucidate the cause of early lethality, we examined the mice for gross appearance of the diaphragm and degenerative lesions by Evans blue staining. Lesions were largely absent from the mdx,  $csf3r^{+/-}$  and wild-type mice, whereas the  $mdx/csf3r^{+/-}$  mice showed

obvious degenerative lesions (Fig. 7d,e). Diaphragm thickness was also significantly decreased in  $mdx/csf3r^{+/-}$  mice compared with mdx littermates (Fig. 7f). The population of PAX7 + cells in diaphragm was also significantly decreased in  $mdx/csf3r^{+/-}$  mice (Fig. 7g,h). Whole-body plethysmography indicated that  $mdx/csf3r^{+/-}$  mice showed mildly impaired respiratory function (Fig. 7i–k). In addition, the surviving



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**Figure 8 | Haploinsufficiency of G-CSFR reduces muscle regeneration in** *mdx* **mice.** (a) Gross appearance analyses showing body size differences among *mdx*, *mdx/csf3r<sup>+/-</sup>* and *mdx/csf3r<sup>-/-</sup>* mice at post-natal day 7 (left panel) and between *mdx/csf3r<sup>+/-</sup>* and *mdx/csf3r<sup>-/-</sup>* mice at post-natal day 13 (right panel). (b) Haematoxylin and eosin staining of cross-sections of TA muscle from *mdx/csf3r<sup>+/-</sup>* mice from 2 to 8 weeks of age are shown in the lower (upper panel) and higher (lower panel) magnified images. Scale bar, 100 µm (upper panel); 50 µm (lower panel). (c) The total numbers of myocytes in cross-sections of TA muscle at 8 weeks. Data are shown as mean ± s.d. \*\**P*<0.01; unequal variance Student's t-test; *n* = 5 per group. (d) Perimeters of cross-sections of TA muscle at 8 weeks of age. Data are shown as mean ± s.d. \**P*<0.05; unequal variance Student's t-test; *n* = 5 per group. (e) Tetanic force examination of *ex vivo* EDL muscle tension at 8 weeks of age. Data are shown as mean ± s.d. \**P*<0.05; unequal variance Student's t-test; *n* = 6 per group. (g) Immunostaining of cross-sections of TA muscle from *mdx* and *mdx/csf3r<sup>+/-</sup>* mice at 5 and 8 weeks of age for laminin, PAX7 and DAPI. Upper panel: merged with laminin (green), PAX7 (red) and DAPI (blue). Lower panel: merged with laminin (green) and DAPI (white). Arrows indicate PAX7 +/DAPI + cells. Scale bar, 20 µm. (h) Number of PAX7 +/DAPI + cells per 100 myocytes of *mdx* and *mdx/csf3r<sup>+/-</sup>* mice at 5 and 8 weeks of age. Data are shown as mean ± s.d. \**P*<0.05; *\*P*<0.01; unequal variance Student's t-test; *n* = 5 per group. (h) Number of PAX7 +/DAPI + cells per 100 myocytes of *mdx* and *mdx/csf3r<sup>+/-</sup>* mice at 5 and 8 weeks of age. Data are shown as mean ± s.d. \**P*<0.05; *\*P*<0.01; unequal variance Student's t-test; *n* = 5 per group. (h) Number of PAX7 +/DAPI + cells per 100 myocytes of *mdx* and *mdx/csf3r<sup>+/-</sup>* mice at 5 and 8 weeks of age. Data are shown as mean ± s.d. \**P*<0.05; *\*P*<0.01; unequal variance Student's t-t

 $mdx/csf3r^{+/-}$  mice showed consecutive body weight loss compared with  $csf3r^{+/-}$  mice (Fig. 7i). Because most  $mdx/csf3r^{+/-}$  mice with a strong phenotype died before 5 weeks of age and relative healthy  $mdx/csf3r^{+/-}$  mice survive, the differences in body weight and respiratory function would be decreased thereafter.

In *mdx* mice, skeletal muscle degeneration and regeneration is prominent at 3–5 weeks of age. We therefore proposed that  $mdx/csf3r^{+/-}$  mice might show deteriorating muscle regeneration and function around that age. Gross examination revealed a smaller body size in  $mdx/csf3r^{+/-}$  and  $mdx/csf3r^{-/-}$  mice at P7, and apparently smaller body size in  $mdx/csf3r^{-/-}$  mice at

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P13 (Fig. 8a).  $Mdx/csf3r^{+/-}$  mice that survived the initial early lethality prevalence showed significantly increased degeneration with decreased numbers of regenerating myocytes at ~4 weeks of age that remained at ~8 weeks of age (Fig. 8b). The myocyte number and TA muscle perimeter were also significantly decreased in  $mdx/csf3r^{+/-}$  mice at 8 weeks of age (Fig. 8c,d). *Ex vivo* muscle tension examination also indicated significantly decreased tetanic and specific forces in the  $mdx/csf3r^{+/-}$  mice compared with mdx mice (Fig. 8e,f). The population of PAX7 + cells was also significantly decreased in  $mdx/csf3r^{+/-}$  mice (Fig. 8g,h). These results confirmed that G-CSF signal is crucial for skeletal muscle regeneration in mdx mice.

**G-CSF supports the survival of severe DMD model mice**. The physically normal appearance of mdx mice could reflect a redundant function of the *utrophin* gene, a *dystrophin* paralogue. The  $mdx/utrn^{-/-}$  mice that express neither *dystrophin* nor *utrophin* manifest severe dystrophy resembling human DMD<sup>30,31</sup>. These mice showed an apparently small body size compared with the mdx mice (Supplementary Fig. 5a), with prominent skeletal muscle degeneration that remained to a late stage of post-natal growth (Supplementary Fig. 5b). Total myocyte number in TA muscle was also decreased from 2 to 5 weeks of age in these  $mdx/utrn^{-/-}$  mice, and recovered thereafter (Supplementary Fig. 5c), and central nuclear myocytes



**Figure 9 | G-CSF improves the survival of mice with severe muscular dystrophy. (a)** Time course of G-CSF administration. (b) Haematoxylin and eosin staining of cross-sections of TA muscle from  $mdx/utm^{-/-}$  mice at 5 weeks of age (left: control; right: G-CSF administration). Scale bar, 200 µm (upper panel); 50 µm (lower panel). (c) Perimeters of cross-sections of TA muscle at 5 weeks of age. Data are shown as box plot. Top, maximum; bottom, minimum. \*\**P*<0.01; unequal variance Student's *t*-test; *n* = 6 per group. (d) Ratios of moist muscle weight to body weight at 5 weeks of age. Data are shown as mean ± s.d. \**P*<0.05; equal variance Student's *t*-test; *n* = 9 per group. (e) Immunostaining of cross-sections of TA muscle for BF-45 (red), laminin (green) and DAPI (blue) at 4 weeks of age. Scale bar, 50 µm. (f) The percentages of BF-45-positive myocytes in cross-sections of TA muscle at 4 weeks of age. Data are shown as mean ± s.d. \*\**P*<0.01; equal variance Student's *t*-test; *n* = 6 per group. (g) The individual fibre cross-sectional areas of the myocytes in TA muscle at 5 weeks of age. Data are shown as mean ± s.d. \*\**P*<0.01; unequal variance Student's *t*-test; *n* = 5 per group. (g) The individual fibre cross-section at 5 weeks of age is presented. Data are shown as mean ± s.d. \*\**P*<0.01; unequal variance Student's *t*-test; *n* = 5 per group. (i) Specific force results, represented by tetanic force per muscle size ratios. Data are shown as mean ± s.d. \**P*<0.05; unequal variance Student's *t*-test; *n* = 5 per group. (j) Kaplan-Meier survival curve of  $mdx/utm^{-/-}$  mice without G-CSF (*n*=13), with G-CSF (*n*=10) or with G-CSF administration starting at day 100 (*n*=9). DAPI, 4',6-diamidino-2-phenylindole dihydrochloride.

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were apparent after 3 weeks (Supplementary Fig. 5d). G-CSFR was also expressed in regenerating myocytes in the  $mdx/utrn^{-/-}$  mice (Supplementary Fig. 5e).

To elucidate whether G-CSF improves skeletal muscle regeneration in these mice, we i.p. injected G-CSF using several schedules (Fig. 9a). After daily G-CSF injection from 3 to 5 weeks of age, muscle size was significantly increased in the G-CSFtreated animals at 5 weeks of age (Fig. 9b,c). G-CSF administration also significantly increased the moist muscle weight ratio of the gastrocnemius and quadriceps muscles in  $mdx/utrn^{-/-}$  mice (Fig. 9d), as well as the numbers of regenerated myocytes and total myocytes in TA muscle (Supplementary Fig. 6a,b; Fig. 9g). The number of BF-45 + myocytes was significantly increased by G-CSF treatment (Fig. 9e,f). The population of PAX7 + cell was also significantly increased by G-CSF treatment (Supplementary Fig. 6c,d). Ex vivo muscle functional analysis showed that tetanic and specific forces were significantly increased by G-CSF administration (Fig. 9h,i). These data suggested that G-CSF improves skeletal muscle regeneration and muscle function in  $mdx/utrn^{-/-}$  mice. The  $mdx/utrn^{-/-}$  mice died at ~3 to 5 weeks of age and after

100 days of age, resulting in 100% lethality by 180 days (Fig. 9j, blue line). Finally, we showed that long-term G-CSF treatment could notably improve the survival of  $mdx/utrn^{-/-}$  mice, with G-CSF injected daily from 3 to 5 weeks of age and twice a week after 10 weeks of age (Fig. 9j, red line). It is clinically important to treat developed DMD; hence, the G-CSF was also injected from 10 weeks of age. G-CSF administration prolonged the survival of developed DMD model mice (Fig. 9j, yellow line). Whole-body plethysmography then indicated that G-CSF administration significantly increased respiratory function, measured by tidal volume (TV), in the  $mdx/utrn^{-1}$  mice injected daily from 3 to 8 weeks of age (Supplementary Fig. 6e). The G-CSF-treated  $mdx/utrn^{-/-}$  mice also showed improved physical activity at 150 days of age (Supplementary Movie). Although cardiomyopathy is one of the fatal phenotypes of DMD, heart morphology is not affected by G-CSF treatment (Supplementary Fig. 6f,g). These data strongly suggested that G-CSF improves skeletal muscle regeneration and function, and supports the survival of DMD model mice.

#### Discussion

Research over many years has sought to understand the mechanism of skeletal muscle stem cell behaviour and elucidate the precise pathogenesis underlying DMD and, in turn, to develop effective therapies. Although many promising therapeutic strategies have been developed in animal experiments<sup>32</sup>, most human clinical trials failed to show significant efficacy in patients with  $DMD^{12,14,33-35}$ . In addition, with the exception of glucocorticoids, beneficial results with immunosuppressants were inconclusive<sup>36-39</sup>. Indeed, the precise mechanism by which glucocorticoids increase muscle strength in DMD is not known and its clinical effects are generally not sufficient. Improved or additional therapies for DMD are therefore urgently needed. G-CSF is a well-known cytokine that recruits hematopoietic cells<sup>40</sup>. The safety and side effects in humans were widely known from previously established clinical applications, which enable us to use G-CSF early in clinics. Our studies now highlight apparently encouraging the effects of G-CSF on DMD. In this work, we showed that G-CSFR is asymmetrically distributed in some activated SCs, and G-CSF successfully increases the population of SCs at the multi-differentiating stages. We successfully showed that G-CSF administration markedly improves the phenotype of DMD model mice.

The quiescent state in SC is required for the long-term maintenance of skeletal muscle<sup>41</sup>. We showed that G-CSF

increased the PAX7 + /MYOD – self-renewal SCs. There are quiescent SCs among PAX7 + /MYOD – SCs, but it remains unclear whether G-CSF would affect the quiescent SC population. Because G-CSFR is not expressed in PAX7 + /MYOD – SCs, G-CSF signalling would not be directly involved in the transition from activated SCs to PAX7 + /MYOD – SCs. Furthermore, it remains unclear how G-CSF signalling increases the population of PAX7 + /MYOD – SCs. And intrinsic differences among heterogenic satellite stem cells are still unclear and a practical marker to distinguish the subpopulations of satellite stem cells is still missing. Further precise elucidation of molecular mechanisms may directly facilitate control of the quiescent SC pool.

As a study limitation, we could not directly show that bone marrow cells would not be involved in the phenotype of DMD model. To determine whether bone marrow cells have a role in early lethality of haploinsufficiency for G-CSFR in the dystrophic background, we performed bone marrow transplantation using mdx/csf3r + / - mice of ~3-4 weeks of age. There is no established method of bone marrow transplantation for pups earlier than those ages. We selected active eight mdx/csf3r + / mice of  $\sim$  3-4 weeks of age and successfully performed bone marrow transplantation. One of eight mdx/csf3r + / - mice died at 3 weeks after wild-type bone marrow transplantation by 8 weeks of age, which is similar to the survival rate of active mdx/ csf3r + / - mice without bone marrow transplantation. Although wild-type bone marrow cells could not rescue the lethality of mdx/csf3r + / - mice, bone marrow transplantation would preferably be performed in mdx/csf3r + / - mice 0 days of age; alternatively, skeletal muscle-specific G-CSFR-deficient mice would ideally be generated.

We confirmed that the G-CSF/G-CSFR system is critically involved in muscle regeneration during the course of DMD in several mouse models. Given our findings, it is surprising that haploinsufficiency of G-CSFR leads to early lethality in *mdx* mice, indicating that dose reduction of the G-CSF/G-CSFR system cannot support the balance between degeneration and regeneration in these mouse models. We also showed that G-CSF administration markedly improves the phenotype of *mdx/*  $utrn^{-/-}$  mice. Together, our data suggested that G-CSF could improve the phenotype of human DMD and could consequently form the basis of novel and effective therapies in the near future.

#### Methods

**Mice.** C57BL/6-background *mdx* mice were provided by Dr T. Sasaoka (Niigata University Niigata, Japan). The *utrophin*-knockout mice were a kind gift from Dr Kay E Davies (University of Oxford, Oxford, UK)<sup>31</sup>. The *dystrophin–utrophin* double-knockout male homozygous (*dystrophin<sup>-/Y</sup>* (*mdx*), *utrophin<sup>-/-</sup>*) mice were obtained by crossing those heterozygous (*mdx*, *utrophin<sup>+/-</sup>*) mice. The csf3r - /- mice were kindly gifted by Dr Daniel C. Link (Washington University School of Medicine, St Louis)<sup>28</sup>. Mice deficient in both *dystrophin* and csf3r were derived by the following breeding program: male csf3r-knockout (csf3r - /-) mice were mated with female C57B/6 m*dx* (*dystrophin<sup>-/X</sup>*) mice, resulting in all F1-generation mice being heterozygous for csf3r ( $csf3r^{+/-}$ ) and either heterozygous for the *mdx* mutation (*dystrophin<sup>-/X</sup>*, females) or hemizygous for the *mdx* mutation (*dystrophin<sup>-/X</sup>*, females) or hemizygous for the *M* Animal Care and Use Committee of Keio University and conformed to the NIH Guidelines for the Care and Use of Laboratory Animals.

**Genotyping.** DNA was isolated from mouse tails using the Easy-DNA Kit (Invitrogen, CA, USA). PCR analysis to identify mutant dystrophin alleles used a forward primer complementary to mouse dystrophin (5'-CATAGTTATTAATG CATAGATATTCAG-3') and reverse primers complementary to the wild-type allele (5'-GTCACTCAGATAGTTGAAGCCATTTAG-3') or mouse mutant dystrophin allele (5'-GTCACTCAGATAGTTGAAGCCATTTAA-3'). PCR cycling conditions for both the reactions were as follows: 95 °C for 4 min; 34 cycles of 95 °C for 1 min, 55 °C for 1 min. Each reaction yielded a 275-bp product. All PCR reactions were performed using the GoTaq DNA Polymerase kit (Promega, Madison, WI, USA). PCR analysis to assess the

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*utrophin*-knockout status used a forward primer complementary to exon 7 of mouse utrophin (5'-GTGAAGGATGTCATGAAAG-3') and reverse primers complementary either to intron 7 (5'-TGAAGTCCGAAAGAGATACC-3') or to the PGK promoter located within the Neo-knockout cassette (5'-ACGAGACTAGT GAGACGTGC-3'). PCR reactions were carried out under the following conditions: 35 cycles of 94 °C for 30 s, 57 °C for 30 s and 72 °C for 25 s. PCR analysis to determine mutant *csf3r* alleles used a forward primer complementary to mouse csf3r (5'-AGTCACGAGCGAGGTGAGT-3') and reverse primers complementary to wild-type alleles (5'-GTAGGCCTAGTTCATACCTG-3') or mouse mutant *csf3r* alleles (5'-TCCAGACTGGCTTGGGAAAA-3'). The PCR cycling conditions for both reactions were as follows: 95 °C for 4 min, followed by 30 cycles of 95 °C for 30 sec, 60 °C for 30 sec and 72 °C for 45 sec. Each reaction yielded a 279-bp product for the wild-type allele and a 366-bp product for the mutant *csf3r* allele. All PCR reactions were conducted using the Taq DNA Polymerase kit (TaKaRa, Shiga, Iapan).

**G-CSF treatment**. G-CSF (Neutrogin, 100 µg; Chugai, Tokyo, Japan) was diluted in 1 ml of saline (concentration of 100 µg ml<sup>-1</sup>). Short-term treatment for mdx mice involved daily i.p. injections of 5 µg G-CSF from 3 to 5 weeks of age using a 27-gauge needle. Middle-term treatment for *mdx/utrn<sup>-/-</sup>* mice involved daily i.p. injections from 3 to 8 weeks of age at 5 µg using a 27-gauge needle. Long-term treatment for *mdx/utrn<sup>-/-</sup>* mice involved daily i.p. injections from 3 to 5 weeks of age at 5 µg using a 27-gauge needle. Long-term treatment for *mdx/utrn<sup>-/-</sup>* mice involved daily i.p. injections from 3 to 5 weeks of age at 5 µg using a 27-gauge needle. Late treatment for *mdx/utrn<sup>-/-</sup>* mice involved twice weekly i.p. injections from 10 weeks of age at 5 µg using a 27-gauge needle.

**Histological analysis.** Frozen section of TA muscle was stained by haematoxylin and eosin to measure the ratio of muscle regeneration. The fibre cross-sectional area was measured using software (BZ-H1C: Keyence, Osaka, Japan) from laminin-stained cross-sections of TA muscle. Micrographs were taken from each section at,  $\times$  10 and  $\times$  20 magnification with a digital camera (BIOREVO: Keyence). Evans blue (Sigma-Aldrich, MO, USA) was dissolved in PBS (0.15 M NaCl, 10 mM phosphate buffer, pH 7.0). Mice were i.p. injected with 0.5 ml 0.1% Evans blue at P1 using a 27-gauge needle and were killed 3 h later.

Immunofluorescence. All immunofluorescence was carried out on 6-µm-thick frozen sections, myofibres and SCs. The frozen sections were fixed with acetone (WAKO, Osaka, Japan) for 20 min at -30 °C. Myofibres and SCs were fixed with 4% paraformaldehyde for 20 min at room temperature. All samples were incubated with 0.1% Triton X-100 for 5 min at room temperature, washed and then incubated with the following primary antibodies: anti-G-CSF receptor (sc-9173, 1:50: Santa Cruz Biotechnology, CA, USA; sc-323898, 1:100, Santa Cruz Biotechnology), antilaminin 2 (L0663, 1:1000, Sigma-Aldrich), anti-laminin (L9393, 1:1000, Sigma-Aldrich), anti-BF-45 (1:30, Developmental Studies Hybridoma Bank, IA, USA), anti-MyoD (sc-32758, 1:200, mouse; sc-304, 1:200, Santa Cruz Biotechnology), anti-Pax7 (sc-81648, 1:100, 1:40, Santa Cruz Biotechnology), anti-myogenin (ab1835, 1:100, Abcam, Camb, UK; sc-576 1:100, Santa Cruz Biotechnology), anti-syndecan-4 (ab24511, 1:100, Abcam), anti-phospho-SAPK/JNK (#9251 1:100, rabbit, Cell Signaling Technology, MA, USA) and anti-phospho-p38 (sc-7973, 1:100, mouse, Santa Cruz Biotechnology). After overnight incubation, bound antibodies were visualized with a secondary antibody conjugated with Alexa 488, Alexa 546 or Alexa 633 (Life Technologies, CA, USA). Nuclei were stained with 4',6-diamidino-2-phenylindole dihydrochloride (Life technologies). For Pax7 staining, an M.O.M. kit (Vector Laboratories) was used to block the endogenous mouse IgG. The images were recorded using a confocal laser microscope system (Carl Zeiss, Jena, Germany) and BIOREVO (Keyence).

**Muscle force measurements.** EDL muscles were isolated and removed from 5-week-old mice. The muscles were carefully mounted in a chamber filled with PBS (95% O<sub>2</sub> and 5% CO<sub>2</sub>) and maintained at 30 °C. One tendon of the muscle was attached to a steel hook in the chamber, and the other was tied to the lever arm of a dual-mode servomotor system (Electronic Stimulator; NIHON KOHDEN, Tokyo, Japan) via 5-0 surgical silk. The muscles were stretched to the length at which a single twitch and tetanic force showed the highest amplitude (optimal length;  $L_o$ ). The corresponding tetanic force was then measured at 150 Hz over 5-s intervals for a total of 20 min, with a rest period of 120 s to change the buffer. The muscles were adjusted to the optimum length ( $L_o$ ) before all force measurements. For comparative purposes, all force measurements were expressed per the total muscle unit cross-sectional area, which was calculated by dividing the muscle mass by the product of the length and the mammalian skeletal muscle density (1.056 mg mm<sup>-3</sup>). The specific force (N cm<sup>-2</sup>) was calculated with the muscle density assumed as 1.056 g cm<sup>-3</sup>.

**Exercise tolerance tests.** Mice were subjected to an exhaustion treadmill test by placing them on the belt of a one-lane motorized treadmill (MK-680S Treadmill for Rats & Mice; Muromachi Kikai, Tokyo, Japan). The test was started at an incline of 0 at  $5 \text{ m min}^{-1}$  for 5 min; thereafter, the speed was increased by  $1 \text{ m min}^{-1}$  every

Whole-body plethysmography. Animals were placed in a free-moving chamber that was connected to a high-gain differential pressure transducer (Valydine MP45, Validyne, North Ridge, CA, USA). As the animal breathed, changes in the pressure were converted to signals representing the TV; these signals were amplified (BMA 830; CWE, Ardmore, PA, USA), recorded on a strip-chart recorder (Dash 10; Astro-Med, West Warwick, RI, USA) and stored in a computer with respiratory acquisition software for analysis. O<sub>2</sub> consumption and CO<sub>2</sub> production were measured by the open-circuit method using Beckman OM-14 and LB-2 analysers. The parameter TV was analysed in real time, then average values were calculated five times every 2 min for each serial 3-h period. The TV was normalized by dividing by the body weight.

**Myofibre isolation and culture.** Single myofibres were isolated from EDL muscles and digested in DMEM GltaMax (Gibco, CA, USA) with 0.2% type 1 collagenase at 37 °C for 90 min. Fibres were liberated by trituration in DMEM medium with Pasteur pipettes. Isolated myofibres were cultured in suspension in serum-coated dishes. The fibre medium contained 20% foetal bovine serum (Sigma-Aldrich), 1% chick embryo extract (US Biological, MA, USA) 1% penicillin–streptomycin (Gibco), and DMEM GltaMax (Gibco). Single myofibres were incubated at 37 °C with 5% CO<sub>2</sub> for the indicated times. For the treatments, G-CSF (Neutrogin; Chugai) was added to the fibre medium to a concentration of 0.375 ng ml<sup>-1</sup>, p38 inhibitor (SB203580, CST) was added to the fibre medium at a concentration of 10  $\mu$ M and JNK inhibitor (SP600125, Cell Signaling Technology) was added to the fibre medium at a concentration of 5  $\mu$ M.

**Isolation of SCs**. We obtained SCs using single myofibre culture, during which the SCs began to migrate on Matrigel (BD Biosciences, CA, USA)-coated dishes. Single SCs were then placed in Matrigel-coated glass-base dishes (Asahi Glass, Tokyo, Japan) and were maintained in fibre medium at 37 °C with 5% CO<sub>2</sub> for 3 days to allow SCs to migrate off the fibre and onto the Matrigel-coated dishes.

**Statistical analysis.** Values are reported as the means  $\pm$  s.d. The data were analysed using StatView J-4.5 software. Comparisons between the two groups were performed with Student's *t*-test. Comparisons among the groups were performed by one-way analysis of variance with Bonferroni's *post hoc* test. Scheffe's *F*-test was used to determine the level of significance. The probability level accepted for significance was \*P < 0.05, \*\*P < 0.01.

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#### **Author contributions**

S.Y. designed the research, N.H., S.Y., Y.S., M.H., N.I., H.H., D.K., T.S., S.T., M.K., A.K., S.K., M.T., Y.S., S.O., T.E., J.E. and T.S. performed the research, N.H., S.Y., Y.S., S.T. and K.F. analysed the data and S.Y. wrote the paper.

#### **Additional information**

Supplementary Information accompanies this paper at http://www.nature.com/ naturecommunications

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# Erratum: G-CSF supports long-term muscle regeneration in mouse models of muscular dystrophy

Nozomi Hayashiji, Shinsuke Yuasa, Yuko Miyagoe-Suzuki, Mie Hara, Naoki Ito, Hisayuki Hashimoto, Dai Kusumoto, Tomohisa Seki, Shugo Tohyama, Masaki Kodaira, Akira Kunitomi, Shin Kashimura, Makoto Takei, Yuki Saito, Shinichiro Okata, Toru Egashira, Jin Endo, Toshikuni Sasaoka, Shin'ichi Takeda & Keiichi Fukuda

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The immunofluorescence images in Fig. 1b of the PDF version of this Article were inadvertently duplicated from Fig. 1a during the production process. The correct version of Fig. 1 appears below; the HTML version of the paper was correct from the time of publication.

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#### ERRATUM





Figure 1

#### RESEARCH ARTICLE

#### **Targeting Notch Signaling and Autophagy Increases Cytotoxicity in Glioblastoma Neurospheres**

Manabu Natsumeda<sup>1,2</sup>; Kosuke Maitani<sup>1</sup>; Yang Liu<sup>1</sup>; Hiroaki Miyahara<sup>1</sup>; Harpreet Kaur<sup>1</sup>; Qian Chu<sup>1,3</sup>; Hongyan Zhang<sup>1</sup>; Ulf D. Kahlert<sup>1,4</sup>; Charles G. Eberhart<sup>1</sup>

<sup>1</sup> Department of Pathology, Division of Neuropathology, Johns Hopkins Hospital, Baltimore, MD.

<sup>2</sup> Department of Neurosurgery, Brain Research Institute, Niigata University, Niigata, Japan.

<sup>3</sup> Department of Oncology, Tongji Hospital, Wuhan, China.

<sup>4</sup> Department of Neurosurgery, University Medical Center Düsseldorf, Düsseldorf, Germany.

#### Keywords

autophagy, chloroquine, combination treatment, gamma-secretase inhibitor, glioblastoma.

#### Corresponding author:

Manabu Natsumeda, MD, PhD, Department of Pathology, Division of Neuropathology, Johns Hopkins University School of Medicine, Ross Building 558, 720 Rutland Ave., Baltimore, MD 21205 (E-mail: mnatsumeda@bri.niigata-u.ac.jp)

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#### Abstract

Glioblastomas are highly aggressive tumors that contain treatment resistant stem-like cells. Therapies targeting developmental pathways such as Notch eliminate many neoplastic glioma cells, including those with stem cell features, but their efficacy can be limited by various mechanisms. One potential avenue for chemotherapeutic resistance is the induction of autophagy, but little is known how it might modulate the response to Notch inhibitors. We used the  $\gamma$ -secretase inhibitor MRK003 to block Notch pathway activity in glioblastoma neurospheres and assessed its effects on autophagy. A dramatic, several fold increase of LC3B-II/LC3B-I autophagy marker was noted on western blots, along with the emergence of punctate LC3B immunostaining in cultured cells. By combining the late stage autophagy inhibitor chloroquine (CQ) with MRK003, a significant induction in apoptosis and reduction in growth was noted as compared to Notch inhibition alone. A similar beneficial effect on inhibition of cloogenicity in soft agar was seen using the combination treatment. These results demonstrated that pharmacological Notch blockade can induce protective autophagy in glioma neurospheres, resulting in chemoresistance, which can be abrogated by combination treatment with autophagy inhibitors.

#### INTRODUCTION

Glioblastomas are the most common primary adult brain malignancy, comprising more than 50% of all gliomas (23). Despite some advances in therapeutic options, outcomes remain dismal with a median overall survival of 14.6 months and a 2-year survival rate of 26.5% (33). Treatment resistant stem-like glioma cells have been proposed as one reason for treatment failure (4, 8, 10, 25, 36, 38). We (3, 9) and others (30, 34) have demonstrated the efficacy of  $\gamma$ -secretase inhibitors (GSI), which block Notch pathway activity, using *in vitro* and *in vivo* glioma models. However, success has been limited in clinical trials using GSIs as a single agent in gliomas (17), and in other solid tumors alone (17, 35) or in combination with other drugs (5, 26, 29). We, therefore, examined if induction of autophagy might be limiting the therapeutic efficacy of Notch inhibitors.

Autophagy is a dynamic process by which cellular organelles and proteins are sequestered, delivered to lysosomes and digested (37). Induction of autophagy has been identified as a potential mechanism for resistance to chemotherapy (16) including temozolomide (14, 18, 19, 22, 42) as well as radiation treatment (13, 41), which represent the standard therapies for glioblastoma. Manipulation of autophagy has increasingly been explored as a treatment strategy in many cancers (21). However, there is minimal data on the effects of the Notch pathway on autophagy in cancer (12). We examined if Notch pathway blockade using the GSI MRK003 would induce autophagy in glioma neurosphere lines, and found that it does. We next explored a therapeutic regimen using the late stage autophagy inhibitor, chloroquine (CQ), in conjunction with MRK003, and showed that the combination induced apoptosis and more effectively decreased cell growth, proliferation and clonogenicity.

#### **MATERIALS AND METHODS**

#### Cell culture conditions and drug preparation

HSR-GBM1 and JHH520 glioblastoma neurosphere lines were used, and their identity was authenticated at the Johns Hopkins Core Laboratory through short tandem repeat (STR) analysis. HSR-GBM1 was a kind gift from Dr. Angelo Vescovi, JHH520 from Dr. Gregory Riggins. The HSR-GBM1 is completely methylated at the MGMT locus but resistant to temozolomide *in vitro*, lacks mutation at the IDH1 and alterations in p53. For treatment studies, neurospheres were disassociated into single cell

suspensions using Accutase (Sigma Aldrich, St Louis, MO, USA), counted, plated and allowed to grow overnight in Neural Stem Cell medium supplemented with 20 ng/mL of human epidermal growth factor and 10 ng/mL of human fibroblast growth factor (Peprotech, Rocky Hill, NJ, USA). All cells were grown in a humidified atmosphere containing 5% CO2 at 37°C. The next morning, GSI MRK003, generously provided by Merck Research Laboratories (Boston, MA, USA), dissolved in dimethyl sulfoxide (DMSO) (Sigma Aldrich, St Louis, MO, USA) at final concentrations of 0.5 to 5 µM, chloroquine diphosphate (CQ) (Sigma Aldrich, St Louis, MO, USA), dissolved in phosphate buffered saline (PBS) at a final concentration of 10 µM, or DMSO (1:1000 dilution) was added to the media. Bafilomycin A1 (BafA1) (Sigma-Aldrich, St Louis, MO, USA) was dissolved in DMSO at final concentrations of 0.5-5 nM. 3-methyladenine (3-MA) (Selleckchem, Houston, TX, USA) was diluted in DMEM at a final concentration of 10 mM.

#### Quantitative real-time PCR analyses

Cells were collected after treatment with 0–5  $\mu$ L of MRK003 for 48 h. RNA extracted was performed using the RNeasy Mini Kit (Quiagen) with on-column DNA digestion to prevent amplification of genomic DNA. Reverse trancription into complementary DNA (cDNA) was performed. cDNA levels were analyzed by quantitative real-time PCR analysis with SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) on an iQ5 Multicolor real-time PCR detection system. Standard curves were used to determine expression levels and all values were normalized to β-actin. Statistical comparisons were between at least three biological replicates, each with triplicate technical replicates. Primer sequences were as follows: HES1 forward F: 5'-GTG-AAG-CAC-CTC-CGG-AAC-3', reverse R: 5'-CGT-TCA-TGC-ACT-CGC-TGA-3'; HES5 forward F: 5'-GTG-CCT-CCA-CTA-TGA-TCC-TTA-AA-3', reverse R: 5'-AGT-ACA-AAG-TCG-TGC-CCA-CA-3'; HEY1 forward F: 5'-TCT-GAG-CTG-AGA-AGG-CTG-GT-3', reverse R: 5'-CGA-AAT-CCC-AAA-CTC-CGA-TA-3'; β-actin forward F: 5'-CCC-AGC-ACA-ATG-AAG-ATC-AA-3', reverse R: 5'-CGA-TCC-ACA-CGG-AGT-ACT-TG- 3'.

#### Western blot analysis

Proteins were extracted from HSR-GBM1 and JHH520 after treatment with MRK003 and/or CQ for 48-72 h, unless otherwise indicated. The cells were lysed in RIPA buffer containing protease inhibitor cocktail (Sigma-Aldrich, St Louis, MO, USA) and phosphatase inhibitors on ice for 10 minutes then centrifuged at 4°C and 14,000 rpm for 5 minutes to remove cellular debris. Protein quantification was done using the Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA). Subsequently, protein separation was performed on 12% (for LC3B protein analysis) or 4%-12% (for analysis of other proteins) SDS-polyacrylamide gel, and transferred onto nitrocellulose membranes (Invitrogen, Carlsbad, CA, USA). Membranes were then probed with primary antibodies at 4°C overnight. The following primary antibodies were used: LC3B, P62/SQSTM1, phospho-AKT (Ser 473), AKT (C6E7), cleaved PARP, cleaved NOTCH1 (Val1744)(D3B8) (1:1000 dilution, Cell Signaling, Danvers, MA, USA), HES1 (1:800 dilution, Aviva Systems Biology, San Diego, CA, USA), β-actin (1:5000 dilution, Sigma-Aldrich, St Louis, MO, USA). Secondary antibodies conjugated to horseradish peroxidase (KPL, Gaitersberg, MD, USA) were incubated for 1 h at room temperature and detected with a Western Lightning Plus ECL chemiluminescent kit (PerkinElmer, Waltham, MA). All blots were repeated at least three times and representative blots are shown. Densitometry was performed using Image J Ver. 1.440 software (http://rsb.info.nih.gov/ij/) (31).

#### Immunofluorescence and immunohistochemical analyses

HSR-GBM1 cells were treated with DMSO or 5  $\mu$ M MRK003 for 48 h, or starved for 4 h in Earle's Basic Salt Solution (EBSS). The cells were subsequently pelleted, fixed in 10% formalin and embedded in paraffin. Antigen retrieval was performed by heating in a pressure cooker for 30 minutes in Target Retrieval Solution (Dako, Glostrup, Denmark). Sections were stained using anti-LC3B rabbit polyclonal antibodies (1:10,000 dilution, ab51520, Abcam, Cambridge, UK and 1:500 dilution, Cell Signaling, Danvers, MA, USA) at 4°C overnight. The sections were stained by avidin-biotin-peroxidase complex method (Vector, Burlingame, CA, USA) with secondary antibody incubation for 30 minutes at a dilution of 1:5000, diaminobenzidine as the chromogen and counterstained with hematoxylin.

For immunofluorescence staining, HSR-GBM1 and JHH520 cells were grown in 10% FBS DMEM in 12 well plates on coverslips. From the next day, the cells were treated with DMSO or 5  $\mu M$  MRK003 for 48 h. The cells were subsequently fixed in 4% paraformaldehyde, permeabilized in ice cold  $(-20^{\circ}C)$  methanol for 15 minutes, blocked for 30 minutes with 5% normal goat serum (NGS) with 0.3% Triton X solution. Sections were stained using anti-LC3B rabbit monoclonal antibody (1:2000 dilution, ab51520, Abcam, Cambridge, UK), which was added in 5% NGS with 0.3% Triton X solution at 4°C overnight. After washing with PBS, cells were incubated for 90 minutes at a dilution of 1:400 in Cy-3 (Jackson Immunoresearch Laboratories Inc., West Grove, PA, USA) conjugated secondary antibody. After washing with PBS, nuclei were counterstained with DAPI 4', 6-diamindino-2-phenylindole and coverslips were mounted on slides with antifade (Vectastain Elite, Vector Laboratories, Burlingame, CA, USA). Confocal imaging was done using the Fluoview 1000 (Olympus America, Melville, NY, USA).

#### **Determination of cell growth**

3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4sulfophenyl)-2H-tetrazolium (MTS) assays were performed to determine growth in viable cell mass. Cells were dissociated and seeded into 96-well plates at a density of 5000 per well in 200 µL of medium and treated with DMSO, 5 µM MRK003 and/or 10 µM CQ and incubated in 5% CO<sub>2</sub> at 37°C. For the plate readings, 20 µL of MTS solution was added to each well after 0–96 h postplating and incubated for 1 h, protected from light. The optical density at 490 nm was subsequently measured by spectrophotometer. The experiments were repeated at least three times for each cell line.

#### **Cell proliferation assay**

Cells were treated with DMSO, 5  $\mu$ M MRK003 and/or 10  $\mu$ M CQ for 72 h. Cells were subsequently fixed and stained for Ki67 per



**Figure 1.** *MRK003 inhibits Notch pathway.* **A.** Treatment with MRK003 inhibited downstream Notch pathway transcription factors HES1, HES5, HEY1 in JHH520 and HSR-GBM1 lines at the mRNA level. A statistically significant up to 50% reduction of HES1 was seen after treatment with 5  $\mu$ M MRK003. Likewise, an approximately 75% decrease of HES5 and approximately 80% decrease of HEY1

manufacturer's instructions using the Muse Ki67 Proliferation Assay (Millipore, Billerica, MA, USA). Cells incubated with IgG1 control in parallel were used to set the gates. The percentage of Ki67 positive cells was determined using the Muse Cell Analyzer. Analyses were repeated at least three separate times for each cell line.

#### **Cell cycle analyses**

Cells were treated with DMSO, 5  $\mu$ M MRK003 and/or 10  $\mu$ M CQ for 72 h. Cells were then fixed with 70% ethanol for at least 3 h and stained with Muse Cell Cycle Reagent. Cell cycle analyses were performed using the Muse Cell Analyzer and percentages of sub-G1, G0/G1, S and G2/M populations were determined using FlowJo software. Analyses were repeated at least three separate times for each cell line.

compared to DMSO control was seen after 2  $\mu$ M MRK003 treatment in both lines. Stars denote P values vs. DMSO. **B.** Treatment with 0.5  $\mu$ M MRK003 almost completely inhibited cleaved (active) Notch1 expression in JHH520 and HSR-GBM1 lines. Inhibition of HES1 was observed after treatment with 5  $\mu$ M MRK003 in both cell lines.

#### Apoptosis assay

Apoptosis was measured using the Muse Annexin V and Dead Cell Assay per the manufacturer's protocol. Neurospheres were treated with DMSO, 5  $\mu$ M MRK003 and/or 10  $\mu$ M CQ for 72 h. Cells were resuspended in DMEM/F12 without phenol red with 1% Bovine Serum Albumin (BSA) before analysis. Percentage of apoptotic and dead cells were analyzed using the Muse Cell Analyzer. Analyses were repeated at least three separate times for each cell line.

#### **Clonogenic assays**

Neurosphere media was mixed 3:1 with 4% agarose (Invitrogen, Carlsbad, CA, USA) to make the bottom layer, which was used to coat each well of a six-well plate. Neurospheres treated with DMSO, 2  $\mu$ M MRK003 or a combination of 2  $\mu$ M MRK003 and 10  $\mu$ M CQ for 72 h were dissociated and added to 7:1 media and





agarose mixture at a concentration of 5000 cells per 2 mL to make the top layer. After the agarose gelled, 2 mL of fresh media and DMSO or drug were added to the top of each well. Media and drug were changed every 1–4 days during the growth phase of the clonogenic assay. The cells were allowed to form colonies for 19–23 days. Colonies were visualized by staining with nitroblue Natsumeda et al

**Figure 2.** Autophagy is induced after MRK003 treatment in vitro. **A.** Western blot showed induction of autophagy as seen by the increase of the LC3B-II band after GSI treatment in JHH520 and HSR-GBM1 lines in a dose-dependent manner. **B.** Quantification of LC3B-II/LC3B-I from three separate blots. **C.** HSR-GBM1 cells were treated with DMSO, 5  $\mu$ M MRK003, or EBSS, then pelleted and stained for LC3B. Note the punctate staining of the cytoplasm of glioma cells after treatment with MRK003 and EBSS, but not DMSO (original magnification all ×400). **D.** HSR-GBM1 and JHH520 cells were treated with DMSO

tetrazolium (NBT) in a tissue culture incubator overnight at 37°C after which they were imaged. Colonies greater than 50  $\mu$ m in diameter were scanned and counted using MCID Elite software (Cambridge, England, UK). For each well, at least three images were scanned and counted. Triplicates of each condition were plated and the experiment was repeated three times.

#### **Statistical analyses**

Differences between three or more groups were assessed using one way ANOVA test with post hoc Tukey's multiple comparison test. Error bars represent standard error of means. All statistical tests were performed using the GraphPad Prism 6 software (GraphPad Software, La Jolla, CA, USA). *P* values less than 0.05 were considered statistically significant.

#### RESULTS

#### **MRK003 inhibits Notch pathway targets**

Notch pathway activity was assessed using quantitative RT PCR to measure levels of the canonical Notch pathway transcriptional targets HES1, HES5 and HEY1 after 48-h treatment of glioblastoma neurospheres with MRK003. A statistically significant, dose dependent, up to 50% reduction of HES1 was seen after treatment with 5  $\mu M$ MRK003. Likewise, an approximately 75% decrease of HES5 and approximately 80% decrease of HEY1 was seen after 2 µM MRK003 treatment in both cell lines (Figure 1A). Interestingly, the treatment with MRK003 at a high dose (5 µM) resulted in a parodoxical increase in the mRNA levels of HES5 and HEY1. We have previously observed this paradoxical increase when blocking Notch activity in brain tumors with relatively high concentrations of GSI or arsenic trioxide, and speculated a feedback or resistance mechanism of cells surviving maximal therapy [(6) and unpublished data]. We next assessed the effects of MRK003 on Notch pathway activity at the protein level. Treatment with 0.5 µM MRK003 almost completely inhibited cleaved (active) Notch1 expression in JHH520 and HSR-GBM1; inhibition of HES1 was also observed after treatment with 5 µM MRK003 in both neurosphere lines (Figure 1B).

### Notch inhibition induces autophagy in glioblastoma

Induction of autophagy, evidenced by a dose-dependent increase in LC3B-II/LC3B-I ratio, was seen after 48-h treatment with 0.5–5  $\mu$ M of MRK003 in both glioblastoma neurosphere lines (Figure 2A,B). In mature autophagosomes, LC3B-I undergoes a post-translational modification to LC3B-II increasing its hydrophobic

or 5  $\mu$ M MRK003 and stained for LC3B. Note the markedly increased punctate staining in the cytoplasm of glioma cells after treatment with MRK003 compared to DMSO by immunofluorescence analysis of LC3B (original magnification all ×640). **E.** Accumulation (loss of clearance) of LC3B-II after treatment with chloroquine (CQ). A decrease in p62 after MRK003 treatment alone and increase of p62 was observed after combined treatment with MRK003 and CQ, suggesting inhibition of autophagy by CQ.

nature resulting in further migration on electrophoresis. A significant (P < 0.05) increase in LC3B-II/LC3B-I ratio averaging over threefold was seen after 5  $\mu$ M treatment with MRK003 in JHH520; likewise, over fourfold (P < 0.05) and sixfold (P = 0.0008) increases were seen after 2 and 5  $\mu$ M treatment in HSR-GBM1 (Figure 2B).

Having observed an induction of autophagy after treatment with MRK003 by western blotting, we next sought to determine if induction of autophagy could be confirmed by immunohistochemical analysis in HSR-GBM1 cells and by immunoflourescence analysis in both cell lines. Increased punctate staining of LC3B in the cytoplasm of HSR-GBM1 cells, suggesting mature autophagosomes, was observed after 48 h treatment with 5  $\mu$ M MRK003 and 4 h starvation in EBSS media, but not in DMSO control (Figure 2C). Immunoflourescence analysis showed markedly increased punctate staining for LC3B in the cytoplasm of tumor cells compared to DMSO control in both cell lines (Figure 2D).

Given the induction of potentially protective autophagy after treatment with MRK003, a combinatorial regimen using the late stage autophagy inhibitor CQ in conjunction with MRK003 was explored. CQ blocks LC3B clearance via lysosomal inhibition, causing its accumulation in tumor cells (1). Consistent with this mechanism, we saw a very strong induction of the LC3B-II band after CQ treatment in both lines, indicating in this context a blockage of late autophagy (Figure 2E). We chose a dosage of 5 µM MRK003 for combination experiments, because maximal suppression of HES1 protein and induction of autophagy was seen at this level. An increase in LC3B-II/LC3B-I ratio was also seen after treatment with the two drugs in combination (Figure 2E). To help determine if this reflected an ongoing increase in autophagy or a late blockade of the process, we assessed expression of p62, which recognizes autophagic cargo and mediates formation of selective autophagy (27). p62 is known to be upregulated when autophagy is inhibited, resulting in intracellular waste accumulation. A 40% decrease in p62 was seen after induction of autophagy by MRK003 and was subsequently increased 2.4 fold after treatment with both 5 µM MRK003 and 10 µM CQ in HSR-GBM1, suggesting that the dominant process when both agents are used is inhibition of autophagy (Figure 2E). Interestingly, p62 was decreased by only 20% in JHH520 after treatment with 5 µM MRK003, and the increase in p62 after combination MRK003 and CQ treatment was a modest 1.8-fold compared to DMSO control (Figure 2E). Likewise, a similar lack of p62 decrease after MRK003 treatment and only modest (1.6-fold compared to 3.9-fold for HSR-GBM1) increase of p62 was seen after combination MRK003 and BafA1 treatment in JHH520 (Supporting Information Figure 1B), suggesting a cell line dependent variability in effects.

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**Figure 3.** Combination treatment with MRK003 and chloroquine more effectively inhibits glioma neurosphere growth and proliferation. **A.** 5,000 JHH520 and HSR-GBM1 cells per well were plated in a 96-well plate and cell viability was assessed by MTS assay after treatment with DMSO, 5  $\mu$ M MRK003, 10  $\mu$ M CQ, and a combination of 5  $\mu$ M MRK003 and 10  $\mu$ M CQ at days 0–5 of treatment. Combination treatment markedly reduced growth compared to DMSO (JHH520;

#### Combination treatment with MRK003 and chloroquine inhibits growth and proliferation

The effects of combination treatment with MRK003 and CQ on growth of the two glioblastoma neurosphere lines were examined using the MTS assay. In both lines, 10  $\mu$ M CQ did not have a significant effect on the viable cell mass, while Notch inhibition caused a moderate slowing (P < 0.05) in the glioma growth (Figure 3A). In both cell lines, culture growth was significantly reduced compared to DMSO controls (JHH520; P = 0.0004, HSR-GBM1; P = 0.0002) and MRK003 alone (JHH520; P < 0.01, HSR-GBM1; P < 0.05) when MRK003 treatment was paired with inhibition of autophagy (Figure 3A).

We sought to determine whether combination treatment of MRK003 with another late stage autophagy inhibitor, BafA1, would exhibit increased cytotoxicity. Combination treatment with 2  $\mu$ M of MRK003 and 0.5  $\mu$ M of BafA1 showed a drastic reduction of cell growth compared to DMSO (JHH520; *P* < 0.0001, HSR-GBM1; *P* < 0.001) (Supporting Information Figure 1A) after

P = 0.0004, HSR-GBM1; P = 0.0002) and MRK003 alone (JHH520; P < 0.01, HSR-GBM1; P < 0.05) in both neurosphere lines. Stars denote P values vs. DMSO unless otherwise specified. **B.** Ki67 MUSE assays were performed to assess effects on proliferation. A modest but significant decrease in the percentage of Ki67-positive cells was observed after combination treatment compared to DMSO control in the HSR-GBM1 line.

5 days of treatment. However, while the addition of BafA1 to 2  $\mu$ M MRK003 resulted in fewer viable cells, this increase over the GSI alone was not statistically significant, perhaps because the antigrowth effects of MRK003 were particularly prominent in these studies. Similar effects were seen with combination of 2  $\mu$ M MRK003 and 5  $\mu$ m of BafA1 (data not shown). However, no reduction in p62 was noted in JHH520 cells after MRK003 treatment. Increases in LC3B-II/LCB-I ratio and p62 were seen after combination treatment with MRK003 and BafA1, suggesting inhibitory effects of BafA1 at the late stage of autophagy similar to CQ (Supporting Information Figure 1B).

A combination of 2  $\mu$ M MRK003 and 10 mM of the early stage autophagy inhibitor, 3-MA, showed significant reduction of cell growth compared to DMSO control (JHH520; P = 0.002, HSR-GBM1; P = 0.002) and MRK003 alone (JHH520; P = 0.03, HSR-GBM1; P = 0.0009) after 5 days of treatment (data not shown). Western blots showed a 50% decrease in p62 expression compared to DMSO after MRK003 treatment and a 3.2-fold increase in p62 after combination MRK003 and 3-MA treatment in HSR-GBM1



**Figure 4.** Induction of apoptosis is observed after combination treatment with MRK003 and chloroquine. **A.** Western blot showed approximately fourfold increase of cleaved PARP after 5  $\mu$ M MRK003 treatment compared to DMSO control in both cell lines and 12.7-fold to 21.1-fold increase after combination treatment compared to DMSO

(Supporting Information Figure 2). A 40% reduction in LC3B-II/I ratio was seen after 10 mM 3-MA treatment compared to DMSO control and 30% decrease in LC3BII/I ratio was seen after combination MRK003 and 3-MA compared to MRK003 alone, suggesting the inhibition of early stage autophagy.

control. **B.** Cell cycle analysis and **C.** Annexin V MUSE assays were performed to assess effects on apoptosis. An approximately 10-fold increase in percentage of sub-G1 population and modest but significant increase in percentage of Annexin V-positive cells were seen after combination treatment compared to DMSO control.

To determine if combination treatment altered the proliferation of glioblastoma neurospheres, the percentage of Ki67-positive cells was assessed using the MUSE Ki67 assay. A modest but significant reduction in the percentage of Ki67-positive cells was observed after combination treatment with 5  $\mu$ M MRK003 and 10  $\mu$ M CQ

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**Figure 5.** Combination treatment with MRK003 and chloroquine reduces clonogenicity in glioblastoma neurosphere lines. **A.** Representative images of nitroblue tetrazolium-stained colonies formed by JHH520 and HSR-GBM1 cells after treatment with DMSO,

2  $\mu$ M MRK003, 10  $\mu$ M CQ and combination MRK003 and CQ are shown. **B.** Quantification of number of colonies depicted in A, showing markedly reduced colony formation after combination therapy.

compared to DMSO control in the HSR-GBM1 line (P < 0.05) (Figure 3B). However, this reduction in proliferation following combination therapy was not significantly greater than noted after Notch inhibition alone, suggesting that it was not the only mechanism by which overall growth was affected.

## Combination treatment with MRK003 and chloroquine induces apoptosis

Given the limited effect of combination treatment on proliferation, we examined changes in apoptosis. An approximately fourfold induction in the apoptotic mediator cleaved PARP was noted after 5  $\mu$ M MRK003 treatment in both lines, with a lesser increase seen after phamacological inhibition of autophagy (Figure 4A). In contrast, a dramatic 12-fold to 21-fold induction was seen after combination treatment. Only a minor, nonsignificant increase was observed in the percentage of sub-G1 population by cell cycle analysis after treatment with 5  $\mu$ M of MRK003 compared to DMSO. In contrast, an 8.9-fold to 10.6-fold increase in percentage of sub-G1 population was observed after combination treatment (Figure 4B, Supporting Information Figure 2A,B).

With respect to other cell cycle alterations, no significant change in percentage of G0/G1, S, or G2/M population was noted after 72h combination treatment of JHH520; a modest reduction of percentage of G0/G1 population was noted after 72-h combination treatment of HSR-GBM1 (Supporting Information Figure 2C). Although cell cycle arrest was not noted after 72-h combination treatment, G2/M arrest was seen after 48-h combination treatment in line (data not shown), suggesting that cells in G2/M arrest eventually undergo apoptosis. Finally, a significant but less pronounced increase in the percentage of total apoptotic cells was observed after combination treatment compared to DMSO control in both lines using the Annexin V assay (JHH520; P < 0.05, HSR-GBM1; P < 0.01) (Figure 4C). These data support the concept that induction of autophagy prevents apoptosis following Notch blockade in glioma cells, and that CQ can suppress this and increase the cytotoxicity of GSI.

#### Clonogenic capacity is reduced after combination treatment with MRK003 and chloroquine

Having confirmed the increase of apoptosis caused by inhibition of autophagy by CQ, we next looked at clonogenic capacity after monotherapy and combination treatment using the soft agar assay. These were performed using 2  $\mu$ M rather than 5  $\mu$ M MRK003, as the higher GSI dose resulted in too few colonies for an additive effect of the autophagy inhibitor to be examined. A representative



**Figure 6.** Phospho-AKT is inhibited by MRK003 and MRK003 induces autophagy in vivo. **A, B.** Phospho-AKT (Ser 473) was significantly inhibited by MRK003 after treatment with 0.5–5  $\mu$ M MRK003 in HSR-GBM1 for 48 h ( $P \le 0.0001$ ). However, inhibition of phospho-AKT was

not seen in JHH520 after 5  $\mu$ M MRK003 treatment for 48 h. **C.** Phospho-AKT was most inhibited by 5  $\mu$ M MRK003 treatment for 24 h, but subsequently upregulated after 48–72 h treatment in JHH520 line.

experiment shows an approximately 40%–50% decrease in the number of colonies after treatment with 2  $\mu$ M MRK003 alone compared to DMSO in both cell lines (Figure 5A,B). However, with combination therapy a 75%–85% reduction in clonogenicity was observed, and this was significantly more pronounced than the effect with 2  $\mu$ M MRK003 alone (both *P* < 0.0001). The clonogenic assays were repeated three times for each cell line, and all of these experiments showed a significantly lower number of colonies for combination treatment than the GSI alone.

#### Inhibition of AKT following Notch inhibition

AKT activity is known to suppress autophagy (39), and is also modulated by Notch in cancers other than glioma (7, 24). We have previously shown that GSIs other than MRK003 can inhibit phosphorylation and activation of AKT (9) in glioblastoma. Using MRK003, we found that phospho-AKT levels decreased dramatically 48 h after Notch inhibition at concentrations as low as 0.5 uM in HSR-GBM1 neurospheres (Figure 6A,B). JHH520 cells showed modest inhibition of AKT activity with greatest inhibition after 24 h treatment with 5  $\mu$ M MRK003 (Figure 6C). However, further investigation is needed to elucidate whether autophagy after MRK003 treatment is mediated by mTORC2 inhibition.

#### DISCUSSION

Despite some recent advances in treatment, the prognosis for glioblastoma patients remains dismal. Induction of autophagy has been implicated as a potential resistance mechanism to general treatments in gliomas, and its manipulation has actively been investigated as a therapeutic strategy (15). However, little is known about the role of Notch in regulating autophagy in gliomas, or the potential for autophagy to mediate resistance to pathway inhibitors such as GSIs. Increasing evidence suggests that the Notch pathway affects autophagosomal trafficking (2), and that inhibition of the Notch pathway induces autophagy in some types of cancer (12) and during differentiation of stem cells (32).

Our data provide initial evidence that pharmacological Notch pathway blockade can induce autophagy in gliomas. When neurosphere lines were treated with GSI, a dramatic several fold induction of the LC3B-II protein compared to LC3B-I was noted, as well as the emergence of punctate immunohistochemical LC3B staining in fixed cells, consistent with an increase in autophagic flux. However, a robust decrease in p62 following Notch blockade was only seen in one line, thus, clear induction of autophagy may be restricted to a subset of cases. Autophagy may also be present in a more complex in vivo treatment milieu, as analysis of orthotopic glioblastoma xenografts treated with the GSI from a prior study (3) has revealed focal punctate LC3B staining, but too little material was present for definitive conclusions to be drawn. Our findings are consistent with a very recent report demonstrating induction of autophagy after NOTCH1 knockdown by shRNA in a single glioma cell line (40).

The induction of autophagy is of potential clinical relevance, as combining the late stage autophagy inhibitor CQ with MRK003 resulted in a significant induction in apoptosis and reduction in growth and clonogenicity as compared to Notch inhibition alone. Similar growth changes were noted when combining Notch inhibition with the early stage autophagy inhibitor 3-MA. A few autophagy inhibitors have been clinically tested in gliomas. CQ is an antimalarial drug with lysosomotropic properties that inhibit lysosomal function. It is known to have central nervous system penetration and has been shown to effectively inhibit autophagy in vitro (28). A previous trial using radiation and carmustine with or without CQ in glioblastoma patients showed a trend toward increased median overall survival that was not significant (18). Hydroxychloroquine (HCQ), a derivative of CQ, shows equal potency to inhibit autophagy in vitro and has demonstrated less retinal toxicity. A trial of HCQ in conjunction with radiation therapy and temozolomide showed biological response caused by HCQ, but failed to improve overall survival in glioblastoma (28). New and more potent inhibitors of autophagy such as Lys05 (20) and VATG-027 (11) have been tested preclinically and await clinical validation.

We have demonstrated that pharmacological Notch blockade can induce protective autophagy in glioma neurospheres, which can be abrogated by combination treatment with CQ. Together, the two drugs efficiently induce apoptosis and decrease cell growth, proliferation and clonogenicity. A range of options for pharmacological inhibition of both Notch and autophagy exist, and combinatorial blockade of these two pathways represents a promising new treatment strategy for glioblastoma.

#### ACKNOWLEDGMENTS

We would like to acknowledge Dr. Jeff Mumm for assistance with the confocal microscope. The authors declare no conflict of interest. This study was funded by grant R01NS055089 to CGE.

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#### SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

**Figure 1. A** Combination treatment with 2  $\mu$ M MRK003 and Bafilomycin A1 (BafA1) significantly decreased cell growth compared to DMSO in both cell lines. **B** Increase in LC3B-II/LC3B-I ratio expression and marked elevation of p62 is noted after treatment with BafA1, suggesting late stage inhibition of autophagy. Conversely, LC3B-II/LC3B-I ratio decreases and p62 increases after treatment with 3-MA, suggesting early stage autophagy inhibition.

**Figure 2. A, B** Representative cell cycle analysis data depicting marked increases in percentages of sub-G1 population in JHH520 (A) and HSR-GBM1 (B) lines after combination treatment with MRK003 and CQ. C Cell cycle arrest was not observed after 72-hour treatment with MRK003 and CQ.

## 3. 社会との連携

知の拠点セミナー 夏期セミナー 共同研究拠点国際シンポジウム 脳研究所・生理学研究所・霊長類研究所合同シンポジウム 見てみようヒトの脳と心

知の拠点セミナー

国立大学共同利用・共同研究拠点協議会 第46回 知の拠点セミナー 「脳とは,認知症とは、そしてこころとは」

日 時:平成27年7月17日(金)17時30分~ 場 所:京都大学東京オフィス(東京都港区港南2-15-1品川インターシティA棟27階) 講演者:高橋 均(新潟大学脳研究所 新潟大学研究・社会連携担当理事) 参加者:71名

「知の拠点セミナー」

国立大学の共同利用・共同研究拠点が一体となって、毎月1回、第3金曜日の午後5時半から7時 半、東京・品川で開催される連続セミナーです。学問の最先端のようすを、広く一般の方々にお届け するとともに、その声を直接お聴きするのが目的です。第46回セミナーは、脳研究所が担当でした。 本講演では、脳の発達と加齢、アルツハイマー病とはどのような認知症か、発症を防ぐ対策はあ るのか、等をお話しいただきました。





# 新潟大専脳研究所 第45回新潟神経学 夏期セミナー

脳と心の基礎科学から臨床まで 最前線の研究者、臨床家に触れて体感しよう!

Brain Seminar

# 2015.7.23**★** 25±

場所:新潟大学脳研究所 統合脳機能研究センター(6F<u>)セミナーホール</u>

新潟大学脳研究所 新潟脳神経研究会

723	見学・体験	①奉颂伊栓	科学履修コース:A.フラビン園日虹元イン (新学術領域「メワ神経回) B.遺伝子改変動物作 C.神経細胞の培養と:	×ーンンク 踏」との共催 製の実際 遺伝子導	/実習(7/21~23)(8名) 約 §(7/22~23)(4名) 導入(7/23のみ)(6名)
thu	実習コース 共同利用・共同研究	②脳研レジデン 拠点プログラム	ント(臨床)体験コース:(7/23のみ)(10~20 脳外科、神経内科、病 3T-MRIなど脳研の臨 共同利用・共同研究拠点プログラム(旅費支給あ	)名) 理(Brai 京床を一日 らり)に応募	n Cutting. CPC)、 日で体験できるコース stétaろ方は下記HPをご覧ください
		10:00~10:30	「脳腫瘍におけるオートファジー誘導」	青木	<b>洋</b> (新潟大·脳研)
	オートファジート	10:30~11:20	「封入体の形成と分解」	若林	孝一(弘前大·医)
	オートノアノーと	11:20~12:10	「ミトコンドリアの動的な形態制御」	石原	直忠(久留米大·分子生命码
7.24 fri 細胞内小 ミクスの見	<b>神</b> 給疾息:	13:30~14:20	「ミトコンドリアオートファジー」	神吉	智 <b>丈</b> (新潟大·医)
	細胞内小器官ダイナ	14:20~15:10	「オートファジーとパーキンソン病病態」	斉木	<b>臣二</b> (順天堂大·医)
	ミクスの異常は疾患を	15:30~16:30	「オートファジーの分子機構」	水島	昇(東大·医)
	引き起こすか?	16:30~	ポスター発表(脳研究所の研究紹介)		
		18:10~	懇親会(講師・研究者,臨床家と語ろう!)		
	神経疾患の	9:00~ 9:50	「PDの新規治療ターゲット-異常タンパク伝播」	長谷	<b>川隆文</b> (東北大·医)
7 25	disease	9:50~10:40	「ALS-分子から病態パスウェイヘ-」	伊東	大介 (慶應大·医)
sat	modifying	10:50~11:40	「脳梗塞に対する神経・血管保護療法」	金澤	雅人(新潟大·脳研)
therapy	therapyを 日生して	11:40~12:30	「脳梗塞に対する細胞移植療法」	堀江	信貴 (長崎大·医)
申込み-	○事前申込みが必要です。氏名、	所属、連絡先、1日目	の希望コース(希望者のみ)を以下までお知らせくださ	L1.	受講科
申込み・	〒951-8585 新潟市中央区旭町	通1-757 新潟大学	学脳研究所 神経学夏期セミナー事務局・佐藤	• • •	

夏期セミナー













第6回新潟大学脳研究所共同研究拠点国際シンポジウム(プログラム) 6th BRI Symposium in Niigata (Program)	26th AM Session 2: Basic studies (chaired by Makiko Yamada & Shinii Nishimoto)
「意識を必要とする <b>脳機能</b> の神経メカニズム」 "Neural mechanisms of brain functions that require awareness"	9:00-9:45 Ryota Kanai (Univ Sussex/ATR) Roles of top-down prediction in visual awareness 9:45-10:30 Yutaka Komura (AIST)
25th PM - 27th AM, July, 2015	Linking metacognitive signals to vision in monkeys and humans
Center for integrated human brain science (6F) Brain Research Institute (BRI), Niigata University	<ul> <li>10:30-10:40 (Coffee break)</li> <li>10:40-11:25 Toshiyuki Hirabayashi (Univ Tokyo/NIRS)</li> <li>Towards understanding the circuit mechanisms underpinning conscious retrieval</li> </ul>
h PM 00-13:05 Masatovo Nichizawa Onennino remarks hv the director of BRI	of object memory in macaques 11:25-12:10 Atsushi Iriki (RIKEN)
	Neurobiological mechanisms for emergence of the objective-self during primate brain evolution
sion 1. Certutat/Morecutat mechanisms tenance by Kyota Nanat & Tutaka Nonutat) 05-13:50 Katsuei Shibuki (BRI, Niigata Univ)	
Bottom-up approach to visual awareness in mice 50-14:35 Takeshi Yagi (Osaka Univ)	12:10-13:10 (Lunch)
Genetic bases for generating the complex neural networks in the brain	26th PM Session 3: Amhlied studies (chaired hy Takeshi Yaoi & Katsuei Shihuki)
55-14:45 (Contee break) 45-15:30 Takahiro Furuta (Kyoto Univ)	13:10-13:55 Makiko Yamada (NIRS)
Introduction of morphological techniques for integrative brain science 30-16:15 Haruo Hosova (ATR)	Molecular mechanism of conscious self-perception 13:55:-14:40 Jun Miyata (Kyoto Univ)
Understanding representations in visual cortex with hierarchical statistical models	Psychosis as a disorder of consciousness integration 14:40-14:50 (Coffee break)
15-16:25 (Coffee break)	Special lecture 2 & 3 (chaired by Takahiro Furuta & Masafumi Oizumi)
cial lecture 1 (chaired by Toshiyuki Hirabayashi & Shinii Nishimoto)	14:50-15:50 Naoisugu Isucniya (Monasn Univ, Austrana) The no-report paradigm: an avenue for distilling the neural correlates
25-17:25 Biyu Jade He (NIH, USA)	of phenomenal consciousness
Slow cortical potentials in conscious perception and volition	Better believe the free energy principle
30- Reception	17-00 16-20 Doctor Carcion

17:30- Reception

16:15-16:25 (Coffee break)

16:25-17:25 Biyu Jade He (NIH, USA)

Free discussion with speakers

. . . . . . . . . . . . . . . . .

14:45-15:30 Takahiro Furuta (Kyoto Univ)

14:35-14:45 (Coffee break)

15:30-16:15 Haruo Hosoya (ATR)

25th PM

Bottom-up approach to visual awareness in mice 13:05-13:50 Katsuei Shibuki (BRI, Niigata Univ)

13:50-14:35 Takeshi Yagi (Osaka Univ)

# 27th AM

Session 4: Analytical studies (chaired by Ryota Kanai & Katsuei Shibuki)

9:00-9:45 Shinji Nishimoto (CiNet)

Modeling and decoding of human perceptual experiences

9:45-10:30 Masafumi Oizumi (RIKEN)

The integrated information theory of consciousness - theory and practice

10:30-10:40 (Coffee break)

# Special lecture 4 (chaired by Haruo Hosoya & Jun Miyata)

10:40-11:40 Andrew Haun (Univ Wisconsin, USA)Contents of consciousness and the structure of integrated information

11:40-11:45 Katsuei Shibuki Closing remarks by the organizer

#### 国際シンポジウム













17:00~18:00 ポスターセッション(中会議室)	18:00~20:00 懇親会(中会議室) ——————		3月2日(火) 8-30- 砌付		8:45~10:15 セッション3 座長:小松英彦(生理研)	5. 菱田竜一(脳研・システム脳生理学)	マウス頭頂連合野から一次視覚野への抑制投射とその機能 の ユニート (エーニー ユンシンの)	○. 大学 由(土荘町・大脳伴酢回路漏) 皮管回路におけるメシレーション活動	「大田である」で、「「「「「」」、「」、「」、「」、「」、「」、「」、「」、「」、「」、「」、「	ドーパミン受容体遺伝子操作マウスを用いた運動制御の仕組みの解明		10:15~10:30 コーヒーブレーク	10:30~12:00 セッション4 座長:柿田明美(脳研)		8. 吉村 — 武(生埋研・分子神経生埋) ###10 (#######11、11、日、一子: 特異化 ###10 (4 ##	随明の褶頭畔竹から見ん(さに航殿に糟頭の反剖・ (	<b>3. 価多具氏(脳好・ 脳体格外科子)</b>	脳神経外科手術における術中モニタリング	10.冨田拓郎(生理研・心循環シグナル)	運動を模倣する創薬		12:00   閉会の挨拶  那波宏之  新潟大学脳研究所長					
第5回 新潟脳研―生理研合同シンポジウム	平成 28 年 3 月 1 日(火)~2 日(水) 岡崎コンファレンスセンター 大会議室・中会議室	プログラム	3目1日(水)	5.21 日、2.27 13:00~ 受付 ポスター掲示	14:00 開会の挨拶 井本敬二 生理学研究所長		14:02~15:05 セッション1 座長:崎村健司(脳研)	1 江藤 圭(牛理研・牛体恒党機能発達機構)	・・・・ーーーーーーーーーーーーーーーーーーーーーーーーーーーーーーーーー	2. 内田仁司(脳研・細胞神経生物学)	神経障害性疼痛における RNA 編集異常	1 E・OE・1 E・OC 万香 裾影・ レーアー デー・イク		15:20~16:20 セッション2 座長:南部 篤(生理研)	1111111111111111111111111111111111111	O. 両田皿彡(韻枚呑・鴛口畳ン人アム) 十、ニュニュィー・ + E+ # E. WWA・ (F. + ▲ / コ ¥・ / ·	ワイルスヘンダーを用いた置反親脳への外米週位ナ導へ広と	その応用	4. 高桑徳宏(生理研・認知行動発達機構)	Visual input pathways for reward prediction signals	in dopamine neurons of blindsight monkeys		16:20~17:00 特別講演 座長:池中一裕(生理研)	回澤正豊 (脳研・神経内科学)	小脳性運動失調症の評価と治療		

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ポスター発表演題(会場:岡崎カンファレンスセンター 1階中会議室)	<b>P15. 鈴木清隆(脳研・統合脳機能研究センター)</b>
	7.0 Tesla MRI reveals electrostatic environment of the glia limitans
P1. 木田哲夫(生理研・感覚運動調節)	P16.小山総市朗(生理研・心理生理学)
注意制御に関わる脳機能ネットワーク特性の時空周波数動態	第二次体性感覚野に対する経頭蓋直流電気刺激が疼痛知覚に与える影響
P2. 菱田竜一(脳研・システム脳生理学)	P17. 原 範和(脳研・遺伝子機能解析学)
マウス頭頂連合野から一次視覚野への抑制投射とその機能	アルツハイマー病患者由来末梢血由来 RNA-seq ・網羅的発現解析
B3. 杉村岳俊(生理研・視覚情報処理)	P18. 菅原 翔(生理研・心理生理学)
片眼遮蔽により視覚野の興奮性シナプス伝達に誘発される可塑的変化	超高磁場 fMRI に最適化した multiband GE-EPI 法の検証
P4. 笹岡俊邦(脳研・動物資源開発研究)	P19. 井上謙一(霊長研・統合脳システム分野)
ドーパミン受容体遺伝子操作マウスを用いた運動制御の仕組みの解明	霊長類における光遺伝学を利用した神経回路の選択的操作
P5 陳 以珊 (生理研・神経機能素子)	Neuronal and behavioral modulations by pathway-selective
Activation mechanisms of an orphan metabotropic receptor	optogenetic stimulation of the primate oculomotor system
Prrt3	P20. 佐野裕美(生理研・生体システム)
P6. 大西 毅(脳研・システム脳生理学)	ジストニアモデルマウスの筋電図解析
マウス後肢血流遮断によって生じるNOは両側性脊髄応答増強を誘発する	P21. Sun Wuping(生理研・細胞生理)
P7. 秋元 望(生理研・神経シグナル)	Lack of TRPV2 impaired BAT thermogenesis in mice
自律神経系を介した内蔵感覚情報の中枢性制御機構	P22. 中本千尋(脳研・細胞神経生物学)
B8. 北浦弘樹(脳研・病理学)	る型グルタミン酸受容体 GluD1-KO マウスの情動異常とその生化学的解析
ヒト脳スライス標本を用いたてんかん病巣のイメージング解析	P23. 岡本士毅(生理研・生殖・内分泌系発達機構)
P9. 横井 功(生理研・感覚認知情報)	AMP-activated Protein Kinase in CRH Neurons in the PVH Controls
実物素材刺激によって引き起こされる素材カテゴリーに依存したサルの	Food Selection Behavior
行動反応	P24. 佐藤達也(生理研・生殖・内分泌系発達機構)
P10. 大石高生(霊長研・統合脳システム分野)	社会的ストレスによる食餌選択行動の変化に及ぼす
「早老症」自然発症ニホンザルについて	室傍核 CRH ニューロンの AMPK による調節作用
P11. 深田優子(生理研・生体膜)	P25. 中尾聡宏(脳研・動物資源開発研究)
PSD-95 パルミトイル化サイクルを担う酵素群の発見と機能解析	環状オリゴ糖による生体膜脂質環境の変化が精子の受精能を向上させる
P12. 成原 格(脳研・分子神経生物学)	P26. 穐吉亮平(生理研 • 生体恒常機能発達機構)
サイトカインによる統合失調症モデルラットの発声異常	大脳皮質ミクログリアによるシナプス活動制御と神経局所回路への関与
P13.野崎洋明(脳研・神経内科学)	P27. 永岡唯宏(生理研・脳形態解析)
Distinct molecular mechanisms of HTRA1 mutants	Role of planar cell polarity protein Vangl2 in synapse formation
in manifesting heterozygotes with CARASIL	P28. 横関明男(脳研・システム脳病態学)
P14. 國澤和生(生理研・分子神経生理)	TDP-43 関連 ALS の脊髄運動神経における Cajal 小体容積の解析
Analysis of neuronal responses against disruption of paranodal	P29. 二宮太平(霊長研・統合脳システム分野)
junction	多系統萎縮症関連遺伝子変異ザルの網羅的病態解析

#### 合同シンポジウム













脳の病気や障害そして心を知る重要性に ついて、世界的な共通の認識のもとアメリカを 中心とする脳神経科学者たちがDana脳科学推進 同盟(DABI)を結成しました。DABIは、ユネスコと国 際脳研究機構の後援をうけ、「世界脳週間」を設定して、 世界的に脳科学の重要性を広く社会に訴える啓蒙活動を企 画することになりました。日本でも、脳の最先端研究を実施し ている14以上の研究機関が、その趣旨に沿って、日本の科学 研究の将来を担うべき学生を対象に、わかりやすく最先端の脳研 究を紹介し、すこしでも脳と心の科学に興味を持ってもらおうと、研 究室/実験の公開と講演を予定しています。

当新潟大学脳研究所においてもこの趣旨に沿って、3月29日 (火)に「見てみようヒトの脳と心」という題の研究所公開と講演を企 画しました。この趣旨をご理解いただいて、学生の皆さんに積極的 に下記の行事に参加していただければ幸いです。

代表:新潟大学脳研究所長 那波宏之

I. **脳研究所長挨拶**(検討会室) 14:00 ~ 14:10

会場

研

究所

対 象

時

加

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II. 脳研究所公開/脳研究の実際(会場:各分野の研究室)
 14:10~15:40

1) 脳を観察する(病理学分野)	6名	
2) 活動する脳を見る (システム脳生理学分野)	10名	
3) 生きた神経細胞を育ててみる (分子神経生物学分野)	10名	
4) 記憶や学習の分子メカニズム(細胞神経生物学分野)	10名	計66名
5) 脳の働きを明らかにするモデル動物(動物資源開発研究分野)	10名	
6)遺伝子を調べて未来を予測する(遺伝子機能解析学分野)	-10名	
7) ヒトの脳と心を探る(脳機能解析学分野)	10名	

**II. ヒトの脳の不思議**(統合脳機能研究センター棟6Fセミナーホール)
 15:50~17:00

1) ヒトの脳発達と知能

2) 脳を治す

那波宏之 藤井幸彦 計**100**名



申込方法

の法人

脳の

世

紀推進会議

新潟大学脳研究所

後援

新潟県教育委員会

①氏名 ②住所・電話番号 ③学校名・学年
 ④公開コース1)~7)のうち第1&第2希望
 を明記してハガキまたはeメールで下記まで
 (先着順)

申込先-

〒951-8585 新潟市中央区旭町通り1-757 新潟大学脳研究所 担当:図書室・佐藤 E-mail:blib@bri.niigata-u.ac.jp TEL:025-227-0606 FAX:025-227-0814 URL:http://www.bri.niigata-u.ac.jp 見てみようヒトの脳と心













# 4. 科学研究費補助金決定一覧

#### 平成27年度 科学研究費助成事業(科学研究費補助金)決定一覧

種目	研究代表者	職名	課題名
新学術	那波 宏之	教授	精神疾患においてサイトカインがもたらす神経エンドフェノタ イプの変換と病態
新学術	池内健	教授	一次性ミクログリア病:CSF-1R変異関連HDLSにおけるミクログ リアの機能異常
新学術	小野寺理	教授	核酸代謝の乱れからみた蛋白質の老化基盤とその排除機構
基盤A	崎村 建司	教授	ヒト脳機能解明への道程としての遺伝子改変ラット作製法の開 発
基盤A	柿田明美	教授	内側側頭葉てんかんの病態病理 : パラドクスに挑む
基盤A	西澤 正豊	教授	TDP43の自己調整機能に注目したALSの病態機序の解明
基盤A	高橋 均	教授	筋萎縮性側索硬化症とTDP-43:その始まりと広がり方 の分子神 経病理学的解明
基盤B	那波 宏之	教授	中脳ドパミン神経の入出力発達を制御する内的・外的要因
基盤B	小野寺理	教授	アルツハイマー病の発症と進行を説明する脳内ターゲット分子 の探索
基盤B	笹岡 俊邦	教授	D1/D2ドーパミン受容体コンディショナル発現マウスによる 運動制御機構の解明
基盤B	池内健	教授	アルツハイマー病脳で生じるインスリン抵抗性に関する実証的 研究
基盤C	他田真理	助教	多系統萎縮症における認知機能障害の責任病巣:臨床病理学的 検討
基盤C	武井 延之	准教授	神経細胞のサイズ制御の機構とその破綻による病態の解析
基盤C	他田正義	助教	重合体毒性仮説に基づくポリグルタミン病の病態解明と新規治 療薬開発
基盤C	五十嵐 博中	教授	MMP特異的19F-MRトレーサーを用いた血栓溶解時の出 血性梗塞予測法確立
基盤C	福多 真史	准教授	近赤外分光法を用いた新しい術中モニタリングの開発-神経 ネットワークの可視化-
基盤C	藤井 幸彦	教授	脳機能再構築に基づく脳腫瘍新規治療法の開発
基盤C	阿部 学	准教授	記憶と社会的行動に果たす海馬CA2領域の機能解明
基盤C	菱田 竜一	准教授	注意の選択フィルターを形成するマウス頭頂連合野の機能解析
基盤C	豊島靖子	准教授	グリア細胞のタウ病理からタウオパチーの病態にせまる
基盤C	下畑 享良	准教授	脳梗塞に対する新規治療標的分子としてのプログラニュリンの 検討
基盤C	北浦 弘樹	助教	海馬硬化症のてんかん原性:神経活動異常と3次元的形態異常 の関連
基盤C	清水 宏	助教	多系統萎縮症: 乏突起膠細胞の鉄代謝異常と酸化ストレス亢進 の病的意義の解明
基盤C	辻田 実加	准教授	VWM型白質脳症の病態決定因子の探索
基盤C	難波 寿明	助教	ErbBシグナルを介する皮質高頻度発火型GABA神経の機能成熟調 節作用
挑戦的萌芽	伊藤浩介	助教	音楽の進化心理学:事象関連電位研究
挑戦的萌芽	小野寺 理	教授	C9FTD/ALSと弧発性ALSを繋ぐ病態機序の解明
挑戦的萌芽	崎村 建司	教授	脳機能解析に特化した全哺乳類胚への応用可能な一世代遺伝子 改変動物作成法の開発
挑戦的萌芽	高橋 均	教授	リン酸化TDP-43病理に準拠した「ALSからみたALS のための病型分類」の提唱
挑戦的萌芽	桑野 良三	フェロー	アルツハイマー病に特徴的な病巣が脳内に伝搬する分子機序の 解明

平成27年度 科学研究費助成事業(科学研究費補助金)決定一覧

種目	研究代表	者職名	課題名
挑戦的萌芽	那波 宏之	2 教授	セロトニン神経に対するR体サリドマイドの薬理作用機序とその新規受容体検索
挑戦的萌芽	池内 健	教授	血漿中アミノ酸プロファイルに着目したアルツハイマー病の血 液マーカー検索
若手B	塚野 浩明	引 助教	大脳聴覚野・記憶-連想回路の二光子イメージング解析
若手B	岩倉 百合	子 助教	セロトニン神経路の発達・成熟に対する栄養因子の作用機序の 解明
若手B	加藤泰分	▶ 特任助教	TGFβシグナルの亢進は脳小血管病を引き起こすか?
若手B	内田 仁司	引 助教	神経障害性疼痛におけるエピジェネティックサイレンシングの 統合的理解
若手B	吉武 講刊	立 助教	マウス頭頂連合野の予測誤差検出機能の光学イメージング解析
若手B	石原 智彦	愛 助教	介在ニューロンにおけるTDP-43機能に注目したALSシステム選択 性の解明

※ 職名は申請時のものです。

# 5. 共同利用·共同研究
# 平成27年度 共同利用・共同研究採択者一覧

プロジェクト型

エビックマ 部 日百 夕	申請者			所内対	对応教員	
训元味超石	所属	職名	氏名	分野名	氏名	
組換えウイルスを用いた筋萎縮性側索硬化症病 変の発症進展機序の解明	東京都医学総合研 究所	副参事 研究員	渡部 和彦	デジタル医学 分野	柿田 明美	
神経変性疾患:特異的異常蛋白はシナプスを越 えるのか	信州大学医学部	教授	小柳 清光	デジタル医学 分野	柿田 明美	
ドーパミン受容体変異マウスを用いた不安様行 動発症機序の解明	北里大学医学部	講師	山森 早織	動物資源開発 研究分野	笹岡 俊邦	
大規模アルツハイマー病ゲノムリソースを用い た系統的網羅的エピゲノム解析	東京大学医学部附 属病院	さきがけ 研究員	岩田 淳	遺伝子機能解 析学分野	池内健	
アルツハイマー病に関連するゲノム配列変異の 解析システムの構築	大阪大学大学院医 学系研究科	特任教授	中谷明弘	遺伝子機能解 析学分野	池内健	
バイオメタル・トランスポーターの動態と神経 変性疾患に関する研究	岐阜薬科大学	教授	保住功	デジタル医学 分野	柿田明美	
Gut microbiotaの制御が脳虚血病巣進展に及ぼ す影響	日本医科大学大学 院医学研究科	助教	西山康裕	生体磁気共鳴 学分野	五十嵐 博中	
異常凝集体の形成と伝播による神経細胞死機構 の解明	京都大学大学院医 学研究科	特定准教授	星 美奈子	デジタル医学 分野	柿田 明美	
セロトニン5a受容体の生理的役割の解明	名古屋大学環境医 学研究所	教授	山中 章弘	細胞神経生物 学分野	崎村 建司	
遺伝子ターゲティングによるChR2/ArchTレポー ターマウスの作成	東北大学大学院情 報科学研究科	教授	井樋 慶一	細胞神経生物 学分野	崎村 建司	
大脳におけるグルタミン酸受容体GluD1の入力選 択的回路形成と高次神経機能発現に関する共同 研究	北海道大学大学院 医学研究科	教授	渡辺 雅彦	細胞神経生物 学分野	崎村 建司	
限局性皮質異形成の分子遺伝学的発生機序の解 明	山形大学医学部附 属病院	講師	加藤 光広	デジタル医学 分野	柿田 明美	
オリーブ橋小脳委縮症における細胞障害機構の 解明	鳥取大学医学部	助教	瀧川 みき	デジタル医学 分野	柿田 明美	
統合失調症脳内タンパク質多項目同時測定解析 及び関連遺伝子発現解析	福島県立医科大学	講師	國井 泰人	デジタル医学 分野	柿田 明美	
CRISPR/Cas9システムを使った迅速なノックアウ トマウス作成	関西医科大学	准教授	赤間 智也	細胞神経生物 学分野	崎村 建司	
PNPLA6遺伝子の脳における機能一有機リン被爆 との関連から	東海大学医学部	教授	木村 穣	動物資源開発 研究分野	笹岡俊邦	
グリオーマの分子標的治療・放射線治療耐性機 構の解明と治療薬の開発	北海道大学大学院 医学研究科	講師	津田 真寿美	デジタル医学 分野	柿田明美	
タンパク質分解システムを標的とするシヌクレ イノパチーの分子病態解明と治療法の確立	弘前大学大学院医 学研究科	准教授	森 文秋	デジタル医学 分野	柿田明美	
生体リズムの遺伝子改変マウスによる解析	京都大学大学院薬 学研究科	教授	岡村 均	細胞神経生物 学分野	崎村 建司	
大脳基底核内情報伝達におけるドーパミン神経 伝達の機能の解析	自然科学研究機構 生理学研究所	教授	南部 篤	動物資源開発 研究分野	笹岡俊邦	
HtrA1欠損マウスにおける脳小血管の機能解析	国立循環器病研究 センター	医長	猪原 匡史	分子神経疾患資 源解析学分野	小野寺 理	
哺乳類中枢神経系における神経回路形成の遺伝 学的解析	国立遺伝学研究所	教授	岩里 琢治	動物資源開発 研究分野	笹岡 俊邦	
ヒトてんかん原性脳組織における酸化損傷タン パク質の網羅的探索	愛知県心身障害者 コロニー中央病院	部長	島田 厚良	デジタル医学 分野	柿田 明美	
optineurinタンパク質の研究	広島大学原爆放射 線医科学研究所	教授	川上 秀史	デジタル医学 分野	柿田 明美	
脳小血管病変モデルにおけるスタチンの脳組織 保護効果	日本医科大学大学 院医学研究科	講師	仁藤 智香子	生体磁気共鳴 学分野	五十嵐 博中	

神経変性疾患におけるアクアポリン(AQP)および AQP関連タンパクの解析	福島県立医科大学	講師	星明彦	デジタル医学 分野	柿田	明美
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悪性脳腫瘍の非コードRNAの機能解析を基盤とし た分子標的創薬の展開	京都府立医科大学 医学部	教授	山中 龍也	脳神経外科学 分野	藤井	幸彦
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免疫不全を伴わない患者に発生するEpstein- Barr virus関連の中枢神経原発悪性リンパ腫の 臨床病理学的検討	久留米大学医学部	教授	杉田 保雄	デジタル医学 分野	柿田	明美

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	申請者		所内対応教員		
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ゲノム編集技術と生殖工学技術を用いた効率的 な遺伝子改変マウス作製	熊本大学生命資源研 究・支援センター	教授	中潟 直己	動物資源開発 研究分野	笹岡俊邦
ALS/FTLDにおけるTDP-43関連軸索内mRNA輸送障 害の解析	国立精神・神経医療 研究センター神経研 究所	室長	長野 清一	分子神経疾患資 源解析学分野	小野寺 理
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アメリカ平原ハタネズミ(Prairie vole)から のES細胞樹立条件の検討,及びCrispr/Cas9法に よる遺伝子KOハタネズミ作出の試み	東北大学大学院農 学研究科	教授	西森 克彦	細胞神経生物 学分野	崎村 建司
パーキンソン病治療における標的タンパク質と しての Inhibitory PAS Domain Protein の検証	東北大学大学院生 命科学研究科	教授	十川和博	デジタル医学 分野	柿田 明美
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Astroblastomaの網羅的遺伝子解析	群馬大学大学院医 学系研究科	助教	信澤 純人	デジタル医学 分野	柿田 明美
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ヒト神経疾患脳におけるDAP12発現の病理学的検 討	埼玉医科大学	教授	佐々木 惇	デジタル医学 分野	柿田 明美
時間的空間的特異的Scrapperノックアウトマウ スの作製と解析	浜松医科大学	准教授	矢尾 育子	細胞神経生物 学分野	﨑村 建司

※所属・職名は、申請時のものです。

### 組換えウイルスを用いた筋萎縮性側索硬化症病変の発症進展機序の解明

### 研究代表者 渡部 和彦 1)

研究分担者 柿田 明美 2)

# 公益財団法人東京都医学総合研究所・神経変性病理 新潟大学脳研究所・デジタル病理学分野

#### 研究要旨

ヒト筋萎縮性側索硬化症(ALS)における運動ニューロン変性のメカニズムは依然として不明である が、ALS 関連遺伝子として TDP-43, FUS など様々なものが知られ、さらに蛋白分解系の異常によって 運動ニューロンにおける細胞内凝集体形成や細胞死が促進されることがわかってきた.本研究では、 各種の組換えウイルスベクターを用いて TDP-43, FUS などの ALS 関連遺伝子および蛋白分解系に対す る shRNA を培養系および成体マウス・ラット運動ニューロンに発現させ、ALS に特徴的な凝集体の 形成過程や細胞死、周囲の細胞への播種・進展様式を解析するとともに、ヒト ALS 剖検例と比較解析 することにより、ALS 病変の発症進展機序の解明を目指している.

### A. 研究目的

ヒト筋萎縮性側索硬化症(ALS)における運動ニ ューロン変性のメカニズムは依然として不明で あるが,近年, ALS 関連遺伝子として TDP-43, FUS, VCP, UBQLN2, C9orf72 など様々なものが知られ ている. さらにプロテアソームやオートファジー 経路の凝集体形成・細胞死への関与が指摘されて おり、これら蛋白分解系の異常が ALS の発症進行 にも影響を与えていると想定される、本研究では、 非増殖性組換えウイルスを用いて TDP-43, FUS な どの ALS 関連遺伝子,およびプロテアソーム・エ ンドソーム・オートファジーに対する抑制性ショ ートヘアピン(sh)RNA を培養系および成体ラッ ト・マウス運動ニューロンに単独または共発現さ せ,凝集体の形成過程や細胞死をヒトALS 剖検例 と比較解析することにより, ALS の運動ニューロ ン変性機序の解明を目指している.

我々はこれまで, TDP-43, FUS cDNA や蛋白分 解系を阻害する shRNA を発現する組換えアデノ ウイルスをラット,マウス末梢神経に混合接種し, ウイルスの軸索内逆行輸送により運動ニューロ ンに細胞質内凝集体を形成しうることを報告し た(Watabe et al., 2014).しかし組換えアデノウイ ルスによる外来遺伝子発現は一過性であるため, 昨年度は、より長期間安定した遺伝子導入発現が 可能な組換えアデノ随伴ウイルス 9 型(AAV9)を 用いて成体マウスに TDP-43 と蛋白分解系阻害 shRNA を逆行性に共発現させ、運動ニューロン細 胞体に凝集体の形成を認めた.そこで、今年度は 凝集体の周囲の細胞への播種・進展様式を解析す るための予備実験として、TDP-43 組換え AAV9 を新生仔マウス側脳室に接種し、脳脊髄における 外来性 TDP-43 の長期発現を検討した.

一方,正常 TDP-43 とその C 末断片を発現する 組換えアデノウイルスをプロテアソーム阻害条 件下で培養ニューロン,グリアに感染させると, 細胞質に粗大な凝集体が形成される(Watabe et al., 2014).しかし,凝集体形成と細胞死の関連性は 明確ではない.そこで,昨年度に引き続き,培養 ニューロンおよびグリア細胞における細胞質凝 集体の形成過程と細胞死をタイムラプス・イメー ジングにより経時的に観察した.

### B. 研究方法

 TDP-43 組換え AAV9 の新生仔マウス側脳室 接種実験;ヒト正常 TDP-43 を EGFP とともに 発現する組換え AAV9 を1日齢 ICR マウスの 側脳室に注入接種した. 接種 2,4 週~6 ヶ月後 に灌流固定し脳脊髄の凍結切片を作成, 観察した.

2. 組換えアデノウイルスによる培養ニューロ ン・グリア細胞質内 TDP-43 凝集体形成の経時 的観察;ニューロン,アストロサイト,または オリゴデンドロサイトに分化すると各々 tubulin beta-3 (TUBB3), GFAP, cyclic nucleotide phosphodiesterase (CNP) プロモータ制御下に EGFPを発現するプラスミドをラット神経前駆 細胞株 1464R に導入し各安定発現株を得た. これら細胞株をレチノイン酸負荷によって EGFP 陽性ニューロン、グリアに分化させたの ち,ヒト正常またはC末断片 (208-414) TDP-43 を DsRed とともに発現する組換えアデノウイ ルスを共感染させた. 感染 24 時間後, プロテ アソーム阻害剤 MG-132 を添加し, タイムラプ ス蛍光撮影を 72 時間行い、細胞質凝集体の形 成と細胞死を経時的に観察した.

### C. 研究結果

- TDP-43/EGFP 組換え AAV9 の側脳室注入接種 により,接種 2,4 週~6ヶ月後にわたって大脳, 脳幹,脊髄に瀰漫性に EGFP 陽性ニューロンを 認め,ヒト特異的 TDP-43 免疫組織化学では核 が陽性に染色された.
- 2. 培養タイムラプスの解析では、組換えアデノ ウイルスを EGFP 陽性分化ニューロン、グリアに 感染発現させ MG-132 を負荷すると、DsRed 陽性 TDP-43 凝集体が細胞質に徐々に充満し、やがて 細胞膜の破綻とともに細胞死に至り、残存した不 溶性の凝集体が長時間浮遊する像が観察された. この凝集体はsarkosyl不溶性の顆粒状構造物から なり、リン酸化 TDP-43 を含んでいた.また細胞 外に放出された凝集体が隣接する細胞に取り込 まれる像も観察された.

### D. 考察

TDP-43 組換え AAV9 の新生仔マウス側脳室接 種により,成体マウス脳脊髄ニューロンにおける ヒト TDP-43 の長期安定発現が可能となった. 今 後,このマウスにヒト TDP-43 および蛋白分解系 阻害 shRNA 組換えアデノウイルスを追感染させ て局所の運動ニューロンにヒト TDP-43 凝集体を 形成させ,凝集体が周囲の細胞にヒト TDP-43 を 介して伝播していくか否かを経時的に観察する 予定である.

一方,培養ニューロンにおける TDP-43 凝集体の

形成過程を経時的に観察しえた.現在,凝集体の細 胞間伝播の可視化に関しても検討を行っている.

これらの実験系を用いて、細胞変性や凝集体形 成のメカニズムを解析するとともに変性を抑止 する方法を見出し、ALSの治療法開発に繋げてい きたい.

#### E. 結論

TDP-43 組換え AAV9 の新生仔マウス側脳室接 種により,成体脳脊髄ニューロンにおけるヒト TDP-43 の長期発現が可能となった.また,培養ニ ューロン,グリアにおける TDP-43 凝集体の形成過 程と細胞死を経時的に観察した.

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G.知的財産権の出願・登録状況(予定を含む) 該当なし

### 神経変性疾患:特異的異常蛋白はシナプスを越えるのか

### 研究代表者 小栁 清光<sup>1)</sup>

研究分担者 鈴木 絵美<sup>2)</sup>、柿田 明美<sup>3)</sup>

信州大学医学部 <sup>1)</sup>神経難病学講座 <sup>2)</sup>分子神経生理学教室 <sup>3)</sup>新潟大学脳研究所脳疾患標本資源解析学分野

#### 研究要旨

古典型孤発性(s) 筋萎縮性側索硬化症(ALS)の疾患特異的なリン酸化(p) TDP-43 が神経細胞から神経細胞に連続的に伝播拡散するのかを明らかにする目的で、sALS 神経細胞の軸索内と胞体内とにおける pTDP-43 封入体の存在部位と形態を免疫組織学的に検索した。軸索内の pTDP-43 は、顔面神経核、舌下神経核の神経細胞、および前角細胞の軸索で見られ、顆粒網状と塊状の2 形態を示した。軸索内 pTDP-43 封入体は臨床経過が比較的短かい症例でのみ見られた。赤核では神経細胞を取り巻く軸索末端に pTDP-43 がみられ、pTDP-43 に取り巻かれた神経細胞では核内の TDP-43 が消失していた。しかし後シナプスであるこれら神経細胞胞体内に pTDP-43 の沈着は見られなかった。

### A. 研究目的

筋萎縮性側索硬化症(ALS)、パーキンソン病、 アルツハイマー病などの神経変性疾患における 特異的蛋白が脳内で神経細胞から神経細胞へ連 続的に伝播拡散するのかを解析する目的で、本 研究では、古典型孤発性(s)筋萎縮性側索硬化 症(sALS)で、リン酸化(p)TDP-43が神経細 胞から神経細胞に連続的に伝播拡散するのかを、 sALS 神経細胞の軸索内と胞体内とにおける pTDP-43 封入体の存在部位と形態について免疫 組織学的に検索した。

### B. 研究方法(倫理面への配慮を含む)

日本人古典型孤発性 ALS19 剖検例(男 11 例、 女 8 例、経過は 6 カ月から 72 カ月)および日本 人対照 3 例(男 3 例)を用いた。ホルマリン固定、 パラフィン包埋された脳と脊髄各箇所の切片を、 HE、KB 染色し、抗 pTDP-43 抗体、抗リン酸化非 依存性 TDP-43 抗体、抗リン酸化 neurofilament 抗 体、抗シナプトフィジン抗体等を用いて免疫染色 し(ABC 法および蛍光抗体法)、前角細胞を明視 野顕微鏡と共焦点顕微鏡(ツアイス LSM5)で観 察した。

#### C. 研究結果

軸索内 pTDP-43 陽性構造物は、sALS 症例の顔 面神経核、舌下神経核の神経細胞、および前角細 胞の軸索で比較的多数見られた。

その形態は、軸索外縁に存在する、大きさ 1µm 程度の顆粒-網状のものと、軸索内部に存在する、 大きさ 10µmx5µm 程度の塊状のものの 2 種類から なっていた。

軸索内 pTDP-43 陽性構造物は、顆粒-網状のものが概ね 30 カ月以内、塊状のものが概ね 10 ヶ月以内と、比較的経過が短い例でのみ認められた。 赤核では、神経細胞を取り巻く軸索末端に pTDP-43 がみられ、pTDP-43 に取り巻かれた神経 細胞では核内の TDP-43 が消失していた。しかし 後シナプスであるこれら神経細胞胞体内に pTDP-43 の沈着は見られなかった。

#### D. 考察

多くの sALS で胞体内の pTDP-43 封入体と神経 細胞脱落を呈する顔面神経核や舌下神経核、前角 細胞の軸索で pTDP-43 陽性構造物が認められた ことは、胞体内と軸索内で、何らかの関連性を有 して pTDP-43 陽性構造物が形成されていること を示している。しかし、どちらかが先に形成され、 他方に流動している、という解析は出来なかった。

軸索外縁の pTDP-43 は軸索表面の機能、とりわけ膜電位の変化を障害する可能性が考えられ、跳躍伝導に影響する可能性がある。また塊状の pTDP-43 は軸索流を阻害する可能性が考えられ、これはシナプス伝達を障害する可能性がある。これらの機能障害は、顔面や舌、四肢、呼吸筋など支配筋の麻痺を惹起する可能性が考えられ、軸索内に pTDP-43 陽性構造を有する症例の経過が短いことや、筋の線維束攣縮と関連しているのかもしれない。

経シナプス性 pTDP-43 の拡散の可能性につい ては、赤核の神経細胞が pTDP-43 陽性軸索に取り 巻かれていたが、それらの神経細胞胞体内後シナ プスに pTDP-43 陽性構造物は見られず、経シナプ ス的な pTDP-43 の拡散の所見は認められなかっ た。しかし、pTDP-43 陽性軸索に取り巻かれてい た神経細胞では核内のリン酸化非依存性の TDP-43 は消失していた。これは、前シナプスの pTDP-43 によって後シナプス神経細胞の核内のリ ン酸化非依存性 TDP-43 が影響されることをしめ している。核内リン酸化非依存性 TDP-43 は RNA のスプライシングはじめ種々の機能に関連して いると言われており、前シナプスの pTDP-43 によ って後シナプス神経細胞の蛋白合成が障害され ている可能性がある。

sALS 下位運動ニューロン軸索に見られた pTDP-43 凝集体は軸索の機能障害を惹起している 可能性が考えられる。赤核周囲軸索末端の pTDP-43 は、核内 TDP-43 を減量させ、細胞内蛋 白合成系の機能不全を惹起している事が考えら れる。

### E. 結論

古典型孤発性筋萎縮性側索硬化症(sALS)下位 運動ニューロン軸索に見られる pTDP-43 凝集体 は軸索の機能障害を惹起している可能性が考え られる。赤核周囲軸索末端の pTDP-43 は核内 TDP-43 を減量させ、細胞内蛋白合成系の機能不 全を惹起している事が考えられる。

#### F.研究発表(上記課題名に関するもの)

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ありません。

特許取得
 ありません。
 実用新案登録
 ありません。
 その他

G. 知的財産権の出願・登録状況(予定を含む)

ありません。

### ドーパミン受容体変異マウスを用いた不安様行動発症機序の解明

研究代表者 山森 早織 1)
 研究分担者 笹岡 (皮邦 2)
 研究分担者 板倉 誠 1)
 研究分担者 飯田 諭宜 1)

1) 北里大学医学部 2) 新潟大学脳研究所

#### 研究要旨

マウスの不安様行動の測定には、オープンフィールドテスト、明暗選択テスト、高架式十字迷路な ど、様々な試験が用いられる。C57/B6 マウスにキンピロール(ドーパミン D2/D3 受容体アゴニスト: D2 高選択性)を投与してこれらの行動試験を行うと、オープンフィールドテストでは壁走性、明暗選 択テストでは明所忌避の不安様行動が出現することが明らかとなった。D2 受容体ノックアウトマウス と D3 受容体ノックアウトマウスを用いて、各受容体と不安様行動の関係性を調べると、キンピロール によって生じる不安様行動のうち、明暗選択テストでの明所忌避は D2 受容体を介して発現し、オープ ンフィールドテストの壁走性は D3 受容体を介して発現する可能性が示唆された。さらに、野生型マウ スに PD-128907 (ドーパミン D2/D3 受容体アゴニスト:D3 高選択性)を投与しても、オープンフィー ルドテストでの壁走性が出現した。

### A. 研究目的

現代社会では、うつなどを代表とする不安障害 が問題となっているが、薬剤に対し難治性となる ものもあり早急な対応が求められている。不安障 害の克服には不安感の発症機序を分子的に明ら かにすることが必要だが、未だ明らかにはなって いない。本研究はドーパミン受容体の作用薬を用 いた薬理学的解析と、ドーパミン受容体の作用薬を用 いた薬理学的解析を行い、ある種の不安様行動の 発症がドーパミン受容体のどのサブタイプに起 因する可能性があるかを検証することを目的と する。

### B. 研究方法(倫理面への配慮を含む)

8 週齢の C67/BL6 マウスに、キンピロール(ド ーパミン D2/D3 受容体アゴニスト:D2 高選択性) や PD-128907 (ドーパミン D2/D3 受容体アゴニス ト:D3 高選択性)をそれぞれ腹腔内投与し、オー プンフィールドテスト、明暗選択テスト、高架式 十字迷路を行った。

さらに、笹岡らから供与を受けた D2 受容体ノ ックアウトマウスおよび D3 受容体ノックアウト マウスを用いて同様の実験を行った。

### C. 研究結果

C57/B6マウスにキンピロールを投与すると、オ ープンフィールドテストでは壁走性、明暗選択テ ストでは明所忌避の不安様行動が出現した。D2受 容体ノックアウトマウスにキンピロールを投与 すると明暗選択テストでの明所忌避は見られな くなるのに対し、オープンフィールドテストの壁 走性は観察された。これらのことから、明暗選択 テストでの明所忌避は D2 受容体を介して生じる 不安様行動であるがオープンフィールドテスト の壁走性は D3 受容体を介する可能性があると考えられた。

また C57/B6 マウスに PD-128907 を投与すると、 オープンフィールドテストでの壁走性と明暗選 択テストでの明所忌避の不安様行動が出現する が、オープンフィールドテストの方がより低濃度 から不安様行動が出現した。D3 受容体ノックアウ トマウスでは、PD-128907 の投与をしてもオープ ンフィールドテストでの壁走性行動の有意な出 現は見られなかった。これらの結果から、少なく ともオープンフィールドテストでの壁走性には D3 受容体が関与する可能性があることが推察で きた。

### D. 考察

以上の結果は、ドーパミン D2 および D3 受容体 が、マウスの不安様行動と密接な関連があること を明確に示している。非常に興味深いことに、不 安様行動実験の種類によって D2 受容体および D3 受容体シグナルの関与は大変異なっていた。この 結果はマウスの不安感を引き起こす脳内メカニ ズムが多種多様であることを示唆している。この 不安感を誘発する脳内の分子メカニズムの多様 性を明らかにするには、今後さらなる研究が必要 である。

#### E. 結論

D2 受容体ノックアウトマウスとD3 受容体ノッ クアウトマウスを用いることで、キンピロール (ドーパミン D2/D3 受容体アゴニスト:D2 高選 択性)によって生じる不安様行動のうち、明暗選 択テストでの明所忌避はD2 受容体を介して発現 し、オープンフィールドテストの壁走性はD3 受 容体を介して発現する可能性が示唆された。

さらに、D3 受容体を介してオープンフィールド テストでの壁走性が出現することは、低濃度の PD-128907 (ドーパミン D2/D3 受容体アゴニス ト:D3 高選択性) の投与によっても確認された。

F. 研究発表(上記課題名に関するもの)

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### ・投稿準備中

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G. 知的財産権の出願・登録状況(予定を含む) なし

大規模アルツハイマー病ゲノムリソースを用いた系統的網羅的エピゲノム解析

### 研究代表者 岩田 淳 1) 研究分担者 池内 健 2)

1) 東京大学医学部附属病院 神経内科 2) 新潟大学脳研究所遺伝子機能解析学分野

#### 研究要旨

アルツハイマー病発症の原因を探索するために, 死後脳の神経細胞核特異的 DNA メチル化解析を網羅 的に施行したところ, 複数の遺伝子の発現調整領域において DNA メチル化の異常を同定した. これによって 得られた遺伝子群の発現変化を死後脳で検討したところ, アルツハイマー病特異的に発現変化を生じている ものを同定した. この遺伝子の発現変化に至る DNA メチル化異常が中枢神経特異的かどうかの検討が次に 必要と考えたが, 使用した死後脳の脳以外の組織由来の DNA はなく, 他のサンプルセットでの検討が必要と 考え田. このため, 新潟大学脳研究所で蓄積されている大規模ゲノムリソースを用いて網羅的に解析する事 を提案した. それにより得られた結果が脳特異的なものなのか, また, 原集団以外のサンプルでも異常と検出 可能なのかを検討することとし, 末梢血での DNA メチル化解析を施行する事とした.

### A. 研究目的

東京大学において行われた神経細胞特異的 DNA メチル化解析では連続領域に DNA メチル化の際を 認めた遺伝子を3つ同定した.これらのうち2遺 伝子は遺伝子発現の差について mRNA, タンパク質 の双方で確認する事が出来た.DNA メチル化の変 化は 神経細胞以外のグリア細胞由来の DNA, 大脳 皮質ではない小脳由来の神経細胞の DNA でも同様 の傾向が見られた.すなわち,DNA メチル化変化 が中枢神経以外の細胞においても見られるかを 検討する必要があると考えられた.

### B. 研究方法(倫理面への配慮を含む)

新潟大学脳研究所付属生命科学リソース研究 センターバイオリソース研究部門の遺伝子機能 解析学分野において匿名化された臨床情報とと もに取得された末梢血ゲノムでの DNA メチル化を pyrosequencing 法で解析した.対象 DNA は認知機 能正常高齢者 40 名,臨床的に診断されたアルツ ハイマー病 36 名であり,22 の CpG においてメチ ル化の程度を測定し、診断、性別、年齢などで比 較検討した.

#### C. 研究結果

下図に示すように、測定した全ての CpG におい て正常■,アルツハイマー病□の間で有意な差は 見いだせなかった.



#### D. 考察

今回使用した末梢血サンプルはアルツハイマ 一病の診断は臨床診断である点,認知機能正常高 齢者の中には Preclinical アルツハイマー病と してアルツハイマー病病理の存在している者が 統計学的には 20-30\$程度存在していることを想 定すると,今回の解析で末梢血に DNA メチル化異 常がないと 100%結論づけるのは早計である可能 性は残る.

### E. 結論

今回の解析では、少なくとも臨床的に診断されるアルツハイマー病の末梢血では神経細胞で認められるような DNA メチル化異常は観察されなかった. 今後は脳サンプル取得対象者の脳以外の DNA での解析が考慮される.

**F. 研究発表**(上記課題名に関するもの) 1. 論文発表

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### G. 知的財産権の出願・登録状況(予定を含む)

 特許取得 なし
 実用新案登録 なし
 その他
 なし

### アルツハイマー病に関連するゲノム配列変異の解析システムの構築

### 研究代表者 中谷明弘 1)

### 研究分担者 池内健<sup>2)</sup>,春日健作<sup>2)</sup>,菊地正隆<sup>1)</sup>

1)大阪大学大学院医学系研究科 ゲノム情報学 2)新潟大学脳研究所 遺伝子機能解析学

#### 研究要旨

アルツハイマー病の2つの疾患群(家族性早期発症型/孤発性晩期発症型)および健常検体群に分類 される脳研究所が保有する約2,000名を想定して、ゲノム配列の変異情報(一塩基変異やコピー数変 異など)を俯瞰的に解析および可視化するソフトウェアを開発する。脳研究所にて既に蓄積している Affymetrix 社製アレイ系によるデータから配列変異情報の抽出を実現する処理パイプラインを構築 し、そこで抽出した変異情報のデータベースの構築を可能とするシステムの開発を行う。構築したデ ータベースを用いて疾患との関連解析を行った結果の可視化を行い、抽出した知見の当該疾患の研究 現場への還元を目指す。本研究では、コピー数変異(CNV)領域や一塩基変異(SNV)の連鎖不平衡(LD) 領域を例とした染色体上での範囲情報を伴う変異に注目する。それによって、個々の塩基位置のみで はなく、染色体領域と疾患との関連に注目することにより、検体群や疾患家系内でのゲノムワイドな 変異情報の特性を明らかにする。さらに、遺伝子の発現量や、脳研究所にて公開している変異情報の 文献データベースである JFADdb (Japanese Familial Alzheimer's Disease Database)等とのデータベ ースとの連携、および、次世代シーケンサによる全エクソーム解析/全ゲノム解析(WEA/WGA)情報と の連携も検討する。

#### A. 研究目的

アルツハイマー病の2つの疾患群(家族性早期 発症型/孤発性晩期発症型)および健常検体群に 分類される約2,000名のゲノム配列のAffymetrix 社製アレイ系による変異情報(一塩基変異やコピ 一数変異)をはじめとする網羅的なデータを俯瞰 的に解析および可視化するデータベースおよび ソフトウェアを開発する。コピー数変異領域や一 塩基変異の連鎖不平衡領域を例とした染色体上 での範囲情報を伴う変異の標的疾患との関連を 明らかにすることを目指す。また、本研究で整備 するリソースと脳研究所にて公開している変異 情報の文献データベースである JFADdb をはじめ とする既存のデータベースとを連携させること によって日本人・アルツハイマー病に関連するデ ータベースの統合化を行う。

#### B. 研究方法(倫理面への配慮を含む)

アルツハイマー病の2つの疾患群(家族性早期 発症型/孤発性晩期発症型)および健常検体群に 含まれる約2,000名のゲノム配列の変異情報(1 塩基変異およびコピー数変異)を俯瞰的に解析お よび可視化するソフトウェアを開発する。本研究 では、疾患群単位での変異情報の集計や疾患との 関連が期待される変異領域の抽出、および、そこ で抽出した変異情報のデータベースの構築する ための手法の開発を行う。構築したデータベース を用いて疾患との関連解析を行った結果の可視 化を行い、抽出した知見の当該疾患の研究現場へ の還元を目指す。 本研究では、コピー数変異(CNV)領域や一塩 基変異(SNV)の連鎖不平衡(LD)領域を例とし た染色体上での範囲情報を伴う変異に注目する。 各検体でのこれら変異領域の境界(始点と終点) の算出や断続する複数の変異領域の扱い方の検 討を行い、ゲノム配列上に「点」として散在する マーカー情報から「領域」としてのゲノム配列の 構造情報を再構築する。それらを検体群内で集計 することにより「領域と頻度」によって表現され る疾患に関連する変異情報の解析を進める。また、 アルツハイマー病で重要となることが想定され る稀少変異(例えば集団内変異頻度が1%以下)に 関しても検討を行う。

獲得した変異情報は関係データベースに格納 して検索用のインタフェースを開発する。遺伝子 の発現量や、脳研究所にて公開している変異情報 の文献データベースである JFADdb (Japanese Familial Alzheimer's Disease Database)等との データベース統合化を行う。また、次世代シーケ ンサによる全エクソーム解析/全ゲノム解析 (WEA/WGA) 情報との連携も検討する。

#### C. 研究結果

アルツハイマー病疾患群および健常検体群の ゲノム情報(コピー数変異や一塩基変異)を変異 の位置や頻度に基づいて可視化できるデータベ ースの構築手法を開発した(下図)。また本手法 に基づくデータベースでは、染色体領域の位置や、 遺伝子名、マーカー名等をクエリとしたデータベ ース参照のための API(検索のための URL)を備 えており、その APIを経由して他のデータベース



との連携を実現できる仕組みになっている。

また、次世代シーケンサを用いた Tethered Conformation Capture法(TCC法)による染色体 近接構造の同定を神経芽細胞腫および星状細胞 腫由来細胞株を用いて行ったデータの解析を併 せて実施して、より高次なゲノム構造の解明を進 めると共に、染色体セグメント間の複雑な相互関 係を把握するための手法の開発を進めた。

#### D. 考察

変異を「点」ではなく「領域」としてゲノムワ イドに評価することは変異情報の高密度化には 欠かせないものであると言える。また、同一染色 体上の連続した領域間の関係(≒連鎖不平衡)の みではなく、染色体を跨がるような位置関係にあ る領域間の相互関係の考慮も必要である。

#### E. 結論

検体における変異情報と疾患に関連した複雑 な形質の間の関係性を明らかにするためには、多 因子的な評価も必要になってくる。しかしながら、 単因子的な評価の総和では捉えきれない要因の 存在も想定されて、走査的処理や組合論的処理な どの「表計算ソフト」では扱い難い要素を含んで いるため、対象データに特化しての適切な処理手 法の開発とその実装系が必要となる。また、その 処理結果は必ずしも単純ではないため、適切な可 視化を行いながらデータの解釈や選別などを効 率良く実現する解析支援システムの構築も必要 となると考えられる。本研究で開発した解析シス テムは、これらの概念実証系として期待できるも のである。

### **F.研究発表**(上記課題名に関するもの) 1. 論文発表 該当なし。

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G.知的財産権の出願・登録状況(予定を含む)
1.特許取得
該当なし。
2.実用新案登録
該当なし。
3.その他
該当なし。

### バイオメタル・トランスポーターの動態と神経変性疾患に関する研究

### 研究代表者 保住 功1)

研究分担者 位田 雅俊 1)、栗田 尚佳 1)、柿田 明美 2)、豊島 靖子 2)

1) 岐阜薬科大学 薬物治療学研究室 2) 新潟大学 脳研究所

#### 研究要旨

これまでに筋萎縮性側索硬化症 (ALS)患者の脳髄液で亜鉛濃度の上昇、ALS患者脊髄中において亜 鉛輸送体である *SLC30A3*発現量の有意な低下を報告している。以上より、神経における亜鉛代謝異 常が ALS 発症に関わっている可能性がある。さらに近年、ALS の病因として小胞体ストレスの関与 が報告されていることから、本研究では、小胞体ストレスに対する *SLC30A3*の変動と防御効果につ いて検討した。ヒト神経芽腫 (SH-SY5Y)、ヒト胎児腎細胞 (HEK293) においてツニカマイシン処置に より、*SLC30A3* mRNA が有意に上昇した。また、siRNA を用いて *SLC30A3* をノックダウンした HEK293 に対してツニカマイシンを 24 時間処置したところ、細胞生存率が有意に低下した。これらの ことから、*SLC30A3*の小胞体ストレスに対する防御効果が示唆された。

### A. 研究目的

筋萎縮性側索硬化症 (ALS) は原因不明の神経 変性疾患であり、明確な発症機構は明らかにされ ていない。これまでに ALS 患者の脳髄液中で亜鉛 濃度の上昇を確認しており (Hozumi et al., J Neurol Sci, 2011)、また、我々は ALS 患者の脊髄中 において亜鉛トランスポーターである SLC30A3 発現量の有意な低下を報告している (Kaneko et al., J Neurosci Res, 2015)。以上より、神経における 亜鉛代謝異常が ALS 発症に関わっている可能性 がある。そこで、本研究では、小胞体ストレスを 与えたときの SLC30A3 の変動と防御効果につい て検討した。

### B. 研究方法(倫理面への配慮を含む)

ヒト神経芽腫細胞 (SH-SY5Y)、ヒト胎児腎細胞 (HEK293) にツニカマイシンを 0、1、3、5、10 µg/mL (SH-SY5Y)、または 0、0.5、1 µg/mL (HEK293)の 終濃度で 24 時間処置し、細胞生存率について調 べた。また、ツニカマイシンを 4 時間処置したと きの *SLC30A3* mRNA を Real time RT-PCR によって 測定した。そして、SLC30A3の細胞内局在につい て蛍光免疫染色法を用いて検討した。さらに、 siRNA を用いて SLC30A3 をノックダウンした細 胞に対しても同様にツニカマイシンを処置し、細 胞生存率について調べた。

### C. 研究結果

SH-SY5Y 細胞にツニカマイシンを 24 時間処置 したところ、10 µg/ml の濃度で細胞生存率は約 60%であり、一方、HEK293 細胞では 1 µg/ml の濃 度で細胞生存率は約 70%であった。次に、細胞が 約 80%生き残るツニカマイシン濃度 (SH-SY5Y: 3 µg/ml)、(HEK293: 0.5 µg/ml)の処置により *SLC30A3* mRNA が有意に上昇した。さらに蛍光 免疫染色法の結果から、SLC30A3 は SH-SY5Y 細 胞、HEK293 細胞ともに小胞体において局在して いた。また、siRNA を用いて *SLC30A3*をノック ダウンした HEK293 細胞に対してツニカマイシン を 24 時間処置したところ、1 µg/ml の濃度で細胞 生存率は約 70%であり、SLC30A3 のノックダウン により、ツニカマイシンによる細胞死が増強され ていることが示唆された。

### D. 考察

小胞体ストレスによって *SLC30A3* mRNA 発現 量が上昇した。また小胞体において SLC30A3 が 存在し、小胞体ストレスによる細胞死が *SLC30A3* のノックダウンによって増強されることを確認 した。これらのことから、SLC30A3 の小胞体スト レスに対する防御効果が示唆された。

### E. 結論

SLC30A3 は小胞体ストレスに対して応答し、防 御効果を示す可能性が示唆された。今後は、 SLC30A3 の防御効果のメカニズムの解明および、 ALS モデル細胞を用いた検討も必要と考えられ る。

F.研究発表(上記課題名に関するもの)

1. 論文発表

なし

### 2. 学会発表

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G. 知的財産権の出願・登録状況(予定を含む) なし

### Gut microbiota の制御が脳虚血病巣進展に及ぼす影響

# 研究代表者 西山 康裕<sup>1)</sup>

**研究分担者** 五十嵐 博中<sup>2)</sup>

1) 日本医科大学武蔵小杉病院 2) 新潟大学脳研究所

#### 研究要旨

脳梗塞における組織炎症に免疫系が大きく関与していることが解明されつつある。この免疫系の制 御に重要な役割を果たしている機構の一つに腸管免疫を修飾するGutmicrobiota(腸内細菌叢)があ るが、現時点においてmicrobiotaの制御により脳梗塞病巣の進展が変化する可能性についての研究は なされていない。本研究では、抗生物質を2週間経口投与した後に脳虚血モデルを作成し、24時間 後の脳梗塞サイズおよび神経スコアを検証した。結果は、vehicle群と抗生物質投与群の間にいずれ も有意な差を認めなかったが、梗塞サイズの検討ではvehicle群が健常側比48.5%であるのに対して、 抗生物質投与群では57.8%と19.0%の梗塞サイズ拡大を認めている。本実験系では虚血後急性期に観察 した梗塞サイズにおいては、両群での差が有意とならなかったものの、脳内に侵入した免疫担当細胞 がさらに活性化する72時間以降での現象を検討することが必要であると考えており、現在進行中で ある。

### A. 研究目的

臨床において、脳虚血による死亡・後遺症はその頻度の高さ、社会的な損失の大きさから重要な問題となっているが、予後改善が認められた治療は早期の血管再開通のみであるという現状である。一方で動物実験レベルでは、免疫寛容により梗塞巣の縮小・予後の改善が認められるなど、脳梗塞における組織炎症に免疫系が大きく関与していることが解明されつつある。この免疫系の制御に重要な役割を果たしている機構の一つに腸管免疫を修飾するGut microbiota (腸内細菌叢)があるが、現時点において microbiota の制御により脳梗塞病巣の進展が変化する可能性についての研究はなされていない。

我々は腸管免疫修飾が脳梗塞巣進展に及ぼす 影響を検討するために、種々の microbiota 修飾 モデルマウスに脳梗塞巣を作成し、腸管免疫と急 性期脳虚血における炎症の関連性について解明 する。 B. 研究方法(倫理面への配慮を含む)

- 急性脳虚血を惹起させるために、8週から1 0週齢のC57BL/6Jマウスを用いて、総頸動脈 からシリコンコートした塞栓糸を挿入して中 大脳動脈内を閉塞し、60分後に塞栓糸を抜 去し、急性脳梗塞モデルとした(中大脳動脈閉 塞術(MCA0モデル))。
- 腸管のmicrobiotaを修飾するため、抗生物 質含有水による下痢・食思不振・体重低下等の 報告を認めない(Li M. et al. Immunity, Vol. 43, 527-, 2015) Ampicillin, Neomycin, Vancomycin, Metronidazole剤の混合投与を行 った。具体的には Ampicillin(1g/L), Neomycin(1g/L), Vancomyci n(0.5g/L), Metronidazole(0.25g/L)を飲料水 に溶解し、14日間投与を続けた。
- 60分脳虚血作成24時間後に断頭した後、 TTC 染色を行い、TTC 染色で染色されない梗塞 巣を両群間で比較した。このとき、皮質領域、

基底核領域については各々測定した。また、同時に脳浮腫率も測定した。さらに、脳虚血前および24時間後に神経学的スコアを計測し、両群間で比較した。なお、脳浮腫率は脳浮腫率(%)=〔虚血半球-対側半球〕×100/対 側半球で計算した。

#### C. 研究結果

- 平均の飲水量は両群ともに一日 6mL で有意差 を認めなかった。
- 2. 抗生物質投与14日間でマウスの体調に変化 は認めず、死亡例はなかった。
- 3. 虚血後24時間の梗塞サイズにおいて、健常 側比にて皮質、基底核および全脳いずれにおい てもvehicle群と抗生物質投与群に統計学的有 意差を認めなかった(皮質;32.01±4.73 vs 37.1±7.01% of control, respectively, n=10) (基底核;16.53±3.79 vs 20.6±6.14% of control)(全脳;48.54±5.70 vs 57.80±12.49%)
  - of control)。脳浮腫率においても、両群間で 有意差を認めなかった。

### D. 考察

食物等を介して多数の異物が侵入する腸管は 体内で最も大きなリンパ器官の一つであり、我々 が摂取する様々な飲食物を通して、病原微生物だ けでなく、アレルゲンや毒性を持つ物質と恒常的 に接触する。こうした点から、腸管は侵入異物の 監視役を演じているものと推察される。腸管粘膜 には全末梢リンパ球の6割、抗体産生性B細胞の 8割が集結すると言われ、実際には腸管上皮細胞 や腸管上皮細胞間 T リンパ球 (intestinal intraepithelial lymphocytes: IEL) が恒常的あ るいは感染などの刺激に反応してサイトカイン を発現している。

最近、これらの粘膜免疫は消化管内だけではな く、全身の免疫システム制御に影響すると注目さ れている。腸管粘膜機構の恒常性が乱れ、腸内細 菌叢の構成が変化し、一旦バランスが乱れた状態 になると、全身の免疫系を過剰に活性化して自己 免疫疾患などの炎症を悪化させることがわかっ ている。実際に我々はB細胞を欠損するマウスに 腸管上皮細胞の再生速度が著しく亢進すること を発見したが、この現象は広域スペクトルを持つ 抗生物質を経口投与することにより正常化され ることがわかった。すなわち、腸内細菌叢の変化 が恒常性に変化を与えることが明らかとなった。

こうした現象に加え、近年脳梗塞は組織炎症の 一つであり、免疫系が大きく関与している可能性 があるとの報告が相次いでおり、腸内細菌叢の変 化が脳虚血のシステムに関連するかに注目した。 我々が preliminary に行った抗生物質を2週間経 口投与した後に脳虚血モデルを作成し、24時間 後の脳梗塞サイズおよび神経スコアを検証した 結果は、vehicle 群と抗生物質投与群の間にいず れも有意な差を認めなかった。しかしながら、梗 塞サイズの平均値では vehicle 群が対側比 48.5% であるのに対して、抗生物質投与群では 57.8%と 19.0%の梗塞サイズ拡大を認めている。本実験系 は薬剤投与や遺伝子操作を加えた実験などによ る特定の分子を標的とした実験系ではないため、 虚血後急性期に観察した梗塞サイズにおいては、 両群での差が有意とならなかったものの、脳内に 侵入した免疫担当細胞がさらに活性化する72 時間以降での現象を検討することが必要である と考えている。経時的に両群間で差が認められる ようになれば、この差を見いだす免疫担当細胞を 検証し、分子生物学的アプローチにより、さらに 病態を解明していくことが可能であると考えて いる。

#### E. 結論

- 1. 抗生物質を2週間経口投与した後に60分間 中大脳動脈閉塞術を作成し、24時間後の脳梗 塞サイズおよび神経スコアを検証したが、 vehicle 群と抗生物質投与群の間にいずれも有 意な差を認めなかったが、梗塞サイズでは vehicle 群が健常側比48.5%であるのに対して、 抗生物質投与群では57.8%と19.0%の梗塞サイ ズ拡大を認めた。
- 本実験系は薬剤投与や遺伝子操作を加えた実験などによる特定の分子を標的とした実験系ではないため、虚血後急性期においては、統計学的に梗塞サイズの差が有意とならなかったが、免疫担当細胞がさらに活性化する72時間以降での現象を検討することが必要と考え、今後の検討課題とする。

F. 研究発表

1. 論文発表 なし

2. 学会発表

なし

G. 知的財産権の出願・登録状況(予定を含む)

なし

### 異常凝集体の形成と伝播による神経細胞死機構の解明

### 研究代表者 星 美奈子 <sup>1)</sup> 研究分担者 柿田 明美 <sup>2)</sup>

1) 京都大学大学院医学研究科 2) 新潟大学脳研究所

#### 研究要旨

我々は、新潟大学脳研究所より剖検脳の供与を受けることで、世界で初めてアルツハイマー病患 者脳より極めて強い神経毒性を持つ球状の Aβ集合体=Amylospheroids(以下、ASPD)を単離した (Noguchi et al, JBC2009)。本研究では、これまでの新潟大学脳研究所との共同研究成果をさらに発 展させるため、ASPD が成熟神経細胞に選択的に結合することで細胞死を起こすことに着目し、神 経細胞膜上の ASPD 標的タンパク質を同定して下流の細胞死シグナル伝達経路を解明し、それによ り新たな治療法開発への基盤を提供することを目的に進めてきた。それに加えて、過去の共同研究 で培ったノウハウ及び成果を生かし、残された課題である、そもそも「なぜ異常凝集体が形成され広 まるのか」というアルツハイマー病の発症機構に迫るため、病理組織化学、生化学、構造生物学を駆 使した研究を進めたいと考えている。これについては、特に Aβ線維について、将来的に患者ごとの 構造プロファイリングを取ることを目指して、初年度は方法論の確立を目指した研究を進めた。

#### A.研究目的

アルツハイマー病の原因は、アミロイドβ(Aβ) の異常凝集体とされているが、臨床症状と最も相 関する神経細胞死が、どの凝集体でどのように起 こるかは不明であった。この状況で、様々な治療 薬が、神経細胞死を伴わない齧歯類疾患モデルを 用いて行われたが、多くは失敗に終わっている。 従って、安全で有効な治療法開発のためには、ヒ トでの病態を解明し、それに基づく治療法と早期 診断システムを開発することである。

研究代表者である星は、柿田博士との共同研究 により、患者脳から神経細胞死活性を持つ Aβ集 合体「アミロスフェロイド (ASPD)」を世界初め て単離するに至った (Noguchi *et al*, JBC2009)。そ の後の共同研究により、ASPD は、あるシナプス 膜タンパク質 (ターゲット分子)に特異的に結合 し、神経細胞死を誘導することを見出し、患者脳 では実際に ASPD 量と相関してターゲット分子 を持つ神経細胞が脱落することを明らかにした。 これにより、初めて神経細胞死の原因と機序について明快な説明が可能となった。更に、ASPDに結合するペプチド(ASPD結合ペプチド)を同定し、これがASPDの神経細胞死活性を中和することも明らかにした(Ohnishi et al. PNAS2015)。

本共同研究では、過去の共同研究で培ったノウ ハウ及び成果を生かし、残された課題である、そ もそもなぜ異常凝集体が形成され、広まるのかと いう、アルツハイマー病の発症機構の解明を試み る。ASPD については、我々は既にラット神経に おいて、特定のタイプの神経細胞内で形成される ことを見出しており(投稿準備中)、患者脳での 解析を行いたいと考えている。さらに、線維につ いても、特定のAβ種だけで形成される、過去に 報告のない新たな構造体を試験管において見出 しており(Xio et al. Nat. Str. Mol. Bio.2015)、これ が患者脳に存在するのかどうかを検証したい。こ れらの研究を足がかりに、ヒト脳で異常凝集体が どの部位でどのような機構で形成され、それがど のように伝播していくのかについて、病理組織化 学、生化学、構造生物学を駆使した研究で迫りた い。

#### B.研究方法

上記目的を達成するために、引き続き以下の研 究を実施する。

- (1) ASPD は特定の神経細胞内で形成され細胞 外に放出されることがラット神経細胞を 用いた研究から明らかになった(Komura, Hoshi et al.投稿準備中)。そこで、既に確立 した抗 APSD 抗体を用いた組織染色及び電 子顕微鏡観察により、ヒト患者脳において、 細胞内で ASPD 形成が起こるのかどうかを 検証する。
- (2) Aβは可溶性構造体以外に不溶性構造体である線維を形成するが、実はその構造にはバリエーションがあることが明らかになりつつある(Lu et a. Cell2013)。共同研究者である Prof. Ishii のラボでは、線維になる凝集核をシードとすることで、ある特定の線維構造を増幅し、NMR によって構造解析する手法を確立しており、これを用いて、新たに見出された特定の Aβ種だけで形成される構造体(Xio et al. Nat. Str. Mol. Bio.2015)が患者脳内に存在するのかどうか解析を行い、重症度との相関などについても検証を行う。

ヒト由来試料から ASPD を調製して用いる場 合は、剖検脳を新潟大学脳研究所より供与を受け る。これについては、既に科学技術・学術審議会 生命倫理・安全部会「機関内倫理審査委員会の在 り方に関する報告書」(平成 15 年 3 月 20 日)に 従い、各機関内倫理・安全委員会の審査を受け、 承認を受けている。実験に際しては、ご遺族の承 諾を得てその範囲を守り、連結可能匿名化により 個人情報を保護した上で、所定の設備の整った実 験室にて安全に配慮して行った。

### C.研究結果

初年度の研究により、ASPD については、ASPD が結合し毒性を発揮するターゲット分子が成熟 神経細胞にのみ発現するシナプス膜タンパク質 Na, K-ATPase ポンプの活性ユニットである α3 で あることを見出し、ASPD の結合によって Na, K-ATPase ポンプ活性が消失し、膜電位が上昇し神 経細胞死が起きるという分子メカニズムを解明 した。これにより、なぜアルツハイマー病におい て成熟した成人神経が脱落するかについて実際 の病態と合う明解な説明を与えた。さらに、ASPD 結合ペプチドは ASPD がターゲット分子 Na. K-ATPase α3 に結合することを阻止することで ASPDの神経毒性を抑制することも明らかにした。 以上により、治療法開発への道を拓くことに成功 した (Ohnishi et al. PNAS2015)。線維についても、 患者由来の線維をシーズとして、オリジナルな構 造をミミックした線維を試験管内で再構築出来 る手法を確立し、それにより特定の Aβ種だけで 形成される、過去に報告のない新たな構造体を試 験管において見出した (Xio et al. Nat. Str. Mol. Bio.2015)。この手法を生かして、次年度以降に複 数の患者脳由来の線維について解析を試みたい と考えている。

#### D.考察

上記の結果から、ASPD は病態と相関して患者 脳に蓄積し、その量と相関してターゲット分子を 発現する神経細胞の脱落が起きていることが強 く示唆された。次の課題として、ASPD の蓄積は いつから起きているのかという問題が残されて いる。アルツハイマー病の初期像を再現している 齧歯類モデルでは、神経細胞脱落が認められない が、そこでは ASPD も蓄積していないことなどを 考えると、発症の前、軽度認知症の段階で蓄積が 始まっているのではないかと考えられるが、軽度 認知障害の場合、その後必ずしもアルツハイマー 病を発症するとは限らないため、これについては 今後の課題と考えている。

### E.結論

上記のとおり、極めて順調に研究を進めること が出来た。上記の結果から、ASPD は病態と相関 して患者脳に蓄積し、その量と相関してターゲッ ト分子を発現する神経細胞の脱落が起きている ことが強く示唆された。次の課題として、ASPD の蓄積はいつから起きているのかという問題が 残されている。アルツハイマー病の初期像を再現 している齧歯類モデルでは、神経細胞脱落が認め られないが、そこでは ASPD も蓄積していないこ となどを考えると、発症の前、軽度認知症の段階 で蓄積が始まっているのではないかと考えられ るが、軽度認知障害の場合、その後必ずしもアル ツハイマー病を発症するとは限らないため、これ については今後の課題と考えている。これを是非、 継続課題として検証したい。また、線維について も方法論が確立出来、興味深い結果が得られつつ あるため、是非、患者脳由来の試料を用いた解析 を行いたいと考えている。いずれも、非常に高い レベルを持つ新潟大学脳研の試料であるからこ そ実施出来る課題である。

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1.特許取得
なし
2.実用新案登録
なし
3.その他
なし

### セロトニン 5A 受容体の生理的役割の解明

### 研究代表者 山中 章弘 1)

研究分担者 崎村 建司 2)、大束 歩 1)

1) 名古屋大学環境医学研究所 2) 新潟大学脳研究所

#### 研究要旨

セロトニン神経は脳内に幅広く投射しており、リズム、気分、情動や痛みの知覚など広範な生理機能を担っている。一つの伝達物質がこのように多くの生理機能に関わっている理由の一つして、受容体が 11 種類もあり多様なことが挙げられる。それら受容体のうち、セロトニン 5A 受容体は中枢神経系に発現するが、その機能はほとんど分かっていない。本研究では、セロトニン 5A 受容体の生理機能について部位特異的遺伝子欠損マウスと、セロトニン 5A 受容体発現神経機能操作マウスを用いて明らかにすることを目的としている。これらの研究に必要な遺伝子改変マウスを本研究において作出し、セロトニン神経の生理機能について明らかにする。

#### A. 研究目的

セロトニン神経は脳内に幅広く投射しており、 リズム、気分、情動や痛みの知覚など広範な生理 機能を担っている。一つの伝達物質がこのように 多くの生理機能に関わっている理由の一つして、 受容体が11種類もあり多様なことが挙げられる。 セロトニン 5A 受容体は中枢神経系に発現するが、 その機能はほとんど分かっていない。本研究では、 セロトニン 5A 受容体の生理機能について部位特 異的遺伝子欠損マウスと、セロトニン 5A 受容体 発現神経機能操作マウスを用いて明らかにする ことを目的としている。これらの研究に必要な遺 伝子改変マウスを本研究において作出し、セロト ニン神経の生理機能について明らかにする。

### B. 研究方法(倫理面への配慮を含む)

セロトニン 5A 受容体発現神経細胞の機能を操 作するために、セロトニン 5A 受容体発現細胞に Cre リコンビナーゼを発現する遺伝子改変マウス を作成する。セロトニン 5A 受容体遺伝子に Cre リコンビナーゼ遺伝子をノックインすることで、 セロトニン 5A 受容体発現細胞だけに Cre リコン ビナーゼを発現させる。Cre リコンビナーゼ依存 的に光遺伝学ツールであるチャネルロドプシン 2(ChR2)やアーキロドプシン(Arch)を発現するア デノ随伴ウイルスベクターと組み合わせること によって、セロトニン 5A 受容体発現神経細胞機 能を操作する。その時に表出する個体レベルでの 行動発現を解析することに依って、セロトニン 5A 受容体発現神経細胞の機能を明らかにする。また、 このマウスは全身性のノックアウトマウスとし ても使用することが可能であり、セロトニン 5A 受容体機能が消失したときの表現型についても 解析する。

次に、セロトニン 5A 受容体を部位特異的にノ ックアウトするために、flox マウスを作成する。 特定神経だけに Cre リコンビナーゼ発現細胞と交 配させることによって、Cre リコンビナーゼ発現 細胞においてセロトニン 5A 受容体遺伝子を欠損 させることができる。特に、視交叉上核において セロトニン 5A 受容体遺伝子を脱落させた時に個 体レベルでの日内リズム発現がどのように変化 するのかについて解析する。

### C. 研究結果

ES 細胞を用いた相同組み替えによって、セロト ニン 5A 受容体遺伝子座に Cre リコンビナーゼを ノックインしたキメラマウス(5ht5a-iCre)マウ スを作成した。交配によりノックインマウスを得 た。また、部位特異的にセロトニン 5A 受容体を ノックアウトするために、相同組み替えを用いて セロトニン 5A 受容体の開始コドン含むエクソン を loxP 配列で挟んだキメラマウス(flox-5ht5a) を作成した。交配によりノックインマウスを得た。 これらの系統を確立したマウスを名古屋大学 動物実験支援センターに搬入するために、クリー ニングを行った。♂2匹から精子を採取し、体外 受精を行い、受精卵を仮親の子宮に戻して産仔を 得た。仮親の微生物モニタリングを行ったところ 陰性であったため、飼育室への搬入を行った。野 生型マウスとの交配を行い、産仔をジェノタイピ ングして SPF 施設においてラインの確立をおこな った。

### D. 考察

セロトニン受容体の一つである 5HT5A 受容体の 生理機能を明らかにするための遺伝子改変動物 を作成することができた。セロトニン受容体は非 常に数多く存在するが、受容体の構造が似ている などから、特異的抗体を用いた発現部位の確認や、 特異的拮抗薬等を用いた機能阻害が行えないた めに、その機能を明らかにすることが出来なかっ た。遺伝子工学の発展によって、特定遺伝子の遺 伝子座にノックインを行うことで、細胞種特異的 に外来遺伝子を発現させること、また組織特異的 に脱落させることが可能となってきた。特に多く は Cre/loxP を用いた組換えを応用することで達 成されており、目的遺伝子のプロモーターで Cre リコンビナーゼを発現する遺伝子改変動物の作 成が機能同定に大きな役割を果たすことが十分 証明されてきている。そこで、本研究では、5HT5A 発現細胞特異的に遺伝子を発現させて、その神経 機能を操作することで、生理機能を明らかにする ための 5HT5A-iCre マウスを作成した。また、細 胞種特異的に Cre を発現する動物と交配させるこ とで、その部位における 5HT5A 受容体遺伝子を脱 落させて機能欠損を引き起こすことが出来るマ ウス floxed-5HT5A マウスを作成した。今後これ

らのマウスを用いて 5HT5A 受容体の機能が明らか になることが予想される。

### E. 結論

生理的役割が十分分かっていないセロトニン 受容体の一つである、5HT5A 受容体を解析するた めに、5HT5A 発現細胞特異的に遺伝子発現が可能 な5HT5A-iCreマウスおよび、部位特異的に5HT5A 遺伝子を脱落させることが可能な5HT5A-floxマ ウスを作成しラインの確立に成功した。

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- 山中章弘、犬束 歩、山下 哲、第89回日本薬理 学会年会(平成28年3月9日~11日)
- 5. <u>山中章弘</u>、千里ライフサイエンスセミナー(平成 28 年 2 月 26 日)
- <u>山中章弘</u>、第67回 埼玉大学脳科学セミナー(平 成28年2月24日)
- <u>山中章弘</u>、大阪精神科診療所協会学術研究会 (平成 28 年 2 月 27 日)
- 8. <u>山中章弘</u>、大脳新皮質構築終了国際シンポジウム 「Neocortical Organization III」(平成 28 年 2 月 11 日~12 日)
- 9. <u>山中章弘</u>、第 29 回上田記念講演会(平成 28 年 1 月 9 日)
- 10. <u>山中章弘</u>、日米科学技術協力事業「脳研究」分野 研究成果報告会(平成 27 年 12 月 19 日)
- 11. <u>山中章弘</u>、International Symposium Optogenetics 2015(平成 27 年 12 月 4 日~5 日)
- <u>山中章弘</u>、医用光学·分光学系合同研究会(平成 27年12月3日)
- 山中章弘、平成27年度生理学研究所研究会大脳皮質の機能原理を探る(平成27年12月3日~4日)
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- <u>山中章弘</u>、第68回日本自立神経学会総会(平成 27年10月29日~30日)
- 山中章弘、比較記憶研究会(平成 27 年 10 月 8 日 ~9 日)
- 18. <u>山中章弘</u>、岡山睡眠薬理研究会(平成 27 年 10 月 2 日)

- 山中章弘、不眠症とオレキシン研究会(平成 27 年 9月 26 日)
- <u>山中章弘</u>、第42回日本神経内分泌学会(平成27 年9月18日~19日)
- <u>山中章弘</u>、2015年光化学討論会(平成 27 年 9 月 9日~11日)
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- 23. <u>山中章弘</u>、第 38 回日本神経科学大会(平成 27 年 7月 28 日~31 日)
- <u>山中章弘</u>、田中謙二、喫煙科学財団第 30 回平成 25 年度助成研究発表会(平成 27 年 7 月 23 日)
- 25. <u>山中章弘</u>、RIKEN BSI Summer Program Lecture Course (平成 27 年 7 月 20 日~24 日)
- 26. <u>山中章弘</u>、犬束 歩、山下 哲、日本睡眠学会第 40回定期学術集会(平成 27 年 7 月 2 日~3 日)
- 27. <u>山中章弘</u>、第11回帝京大学バイオカフェ 特別セ ミナー(平成27年7月2日)
- <u>山中章弘</u>、山陰周術期管理研究会(平成 27 年 6 月 27 日)
- <u>山中章弘</u>、第56回日本神経学会学術大会(平成 27年5月20日~23日)
- 30. <u>山中章弘</u>、尾北医師会学術講演会(平成 27 年 5 月 16 日)
- <u>山中章弘</u>、Seoul International Congress of Endocrinology and Metabolism(平成 27 年 4 月 30 日~5 月 3 日)
- 32. <u>山中章弘</u>、岡崎内科医会学術講演会(平成27年4 月 24 日)
- 33. <u>山中章弘</u>、犬束 歩、他 第88回日本内分泌学会 学術総会(平成27年4月23日~25日)
- 34. <u>山中章弘</u>、Tokyo Mental Disorder Conference(平 成 27 年 4 月 4 日)

G.知的財産権の出願・登録状況(予定を含む)
1.特許取得なし
2.実用新案登録なし
3.その他なし

### 遺伝子ターゲティングによる ChR2/ArchT レポーターマウスの作成

### 研究代表者 井樋 慶一<sup>1)</sup> 研究分担者 崎村 建司<sup>2)</sup>

1) 東北大学大学院情報科学研究科 2) 新潟大学脳研究所

#### 研究要旨

チャネルロドプシンは今や脳科学の実験手法として不可欠であり、チャネルロドプシン-floxed 遺伝 子を特定のニューロンに発現させた実験系の必要性が日増しに高まっている. 微量注入法を用いてウイ ルスベクターをドライバーマウス脳内に注入するという従来の方法にはいくつか問題点が指摘されて おり、 Cre-loxP 系の作動原理によりチャネルロドプシンを発現させるレポーターマウスの作成が望 まれる. そこで、本研究課題では従来の問題を克服し、Cre ドライバーマウスと交配することにより組 織(細胞)特異的に ChR2 が発現するレポーターマウスを作成した.また、ストレス研究に不可欠なコ ルチコトロピン放出因子(CRF)遺伝子座に ChR2 遺伝子がノックインされたマウスも新たに作成した. これらのマウスは今後、脳科学領域に広く用いられ未解決の問題を解くためのブレイクスルーとなるで あろう.

### A. 研究目的

チャネルロドプシンは今や脳科学の実験手法 として不可欠であり, チャネルロドプシン -floxed 遺伝子を特定のニューロンに発現させた 実験系の必要性が日増しに高まっている.しかし ながら,微量注入法を用いてウイルスベクターを ドライバーマウス脳内に注入するという従来の 方法にはいくつか問題点が指摘されている. たと えば,この技術にはウイルスベクター導入方法 (微量注入法)に起因したいくつかの欠点がある. すなわち、注入量や拡散範囲が個体ごとに異なる ため同一条件を再現することができない.また, 拡散範囲が不十分で目的とするニューロンにチ ャネルロドプシンが発現しない場合がある.加え て,実験終了後全個体で注入領域を組織学的に確 認する労力が要る. そこで, Cre-loxP 系の作動 原理によりチャネルロドプシンを発現させるレ ポーターマウスの作成が望まれる.本研究課題で は従来の問題を克服し, Cre ドライバーマウスと

交配することにより組織(細胞)特異的に ChR2 が発現するレポーターマウスを作成することを 目的とする.また,ストレス研究に不可欠なコル チコトロピン放出因子(CRF)遺伝子座に ChR2遺 伝子がノックインされたマウスも新たに作成す る.

#### B. 研究方法(倫理面への配慮を含む)

まず相同組換えのためのターゲティングベク ターとして、CAG プロモーター下に floxstop-ChR2 (H134R),または、floxstop-ArchT 遺伝子, さらに標識マーカーとして mCherry,または、変 異型 YFP (Venus)遺伝子を結合した DNA コンスト ラクトを作成する.これらのベクターを RENKA 株 ES 細胞内にエレクトロポレーションし,相同組換 えによって  $\beta$ -actin 遺伝子下流に位置する non-coding 領域に挿入する.Southern blot によ り同定された陽性クローンを胚盤胞に注入し、得 られたキメラマウスから germ line transmitted line を選別しヘテロ接合体を得る. これらを交配 しホモ接合体を得てライン化する.

本研究で作成されたチャネルロドプシンレポ ーターマウスと様々な Cre ドライバーマウスとの 交配によりニューロン選択的光操作実験系を構 築することができる.これは従来ウイルスベクタ ーを用いて行われていたほとんど全ての光操作 実験に応用可能であり,高い汎用性を具えた極め て有用な実験ツールである.

同様の手法を用い, CRF 遺伝子座に ChR2 遺伝子 をノックインする.

### C. 研究結果

本研究課題では従来の問題を克服し、Cre ドラ イバーマウスと交配することにより組織(細胞) 特異的に ChR2 が発現するレポーターマウスを作 成した.また、ストレス研究に不可欠なコルチコ トロピン放出因子(CRF)遺伝子座に ChR2 遺伝子 がノックインされたマウスも新たに作成した.

### D. 考察

これらのマウスは今後, 脳科学領域に広く用い られ未解決の問題を解くためのブレイクスルー となるであろう.現在, これらのマウスを用い, 細胞特異的に ChR2 が発現することを検証中であ る.

### E. 結論

Cre-loxP 系で作動する ChR2 が発現レポーター マウス,および,コルチコトロピン放出因子 (CRF) 遺伝子座に ChR2 遺伝子がノックインされたマウ スを作成した.

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### G. 知的財産権の出願・登録状況(予定を含む)

 特許取得 なし.
 実用新案登録 なし.
 その他 なし.

### 大脳におけるグルタミン酸受容体 GluD1 の入力選択的回路形成と

### 高次神経機能発現に関する共同研究

研究代表者 渡辺雅彦 1) 研究分担者 崎村建司 2)

1) 北海道大学大学院医学研究科 2) 新潟大学脳研究所

### 研究要旨

GluD1はグルタミン酸との結合能を失いシナプス伝達機能を失ってしまったGluDファミリーの1つである。これまで小脳プルキンエ細胞に豊富なGluD2についての研究から、この分子がシナプスの構造的および機能的結合性を制御する重要な分子であり、その遺伝子異常により小脳性の運動障害が起こることを遺伝子変異を有するヒト家系やマウスの研究から明らかになっている。しかし、その類縁分子であるGluD1については、シナプス発現から分子機能に至るまで、これまで全く不明な分子であった。GluD1 欠損マウスの作成と供給による本共同研究を通して、特異的な発現プローブを開発することができ、この分子が成体マウスの大脳を含む脳の広範な領域に発現し、特に大脳皮質、海馬、歯状回、視床 VPM 核、扁桃体、分界条床核、小脳皮質に強く発現し、小脳では特定の入力を受けるシナプスに発現し、その神経回路形成に関与していることが明らかとなった。

### A. 研究目的

GluD1 の大脳における細胞発現と回路発現を解 析し、その解析結果から想定される GluD1 のシナプ ス回路形成制御と高次脳機能発現に対する機能 について GluD1 欠損マウスを用いて解析するこ とを共同研究の目的とした。具体的には、GluD1 の細胞発現と神経回路発現を大脳を含むマウス 脳において明らかにし、GluD1 欠損マウスの回路 表現型を追求した。

### B. 研究方法(倫理面への配慮を含む)

GluD1 の細胞発現をジゴキシゲニン標識の in situ ハイブリダイゼーション法により検討し、 GluD1 の神経回路発現を特異抗体を用いた免疫 組織化学法により解析した。用いたプローブと抗 体の特異性を共同研究により作成・供給された GluD1 欠損マウスを用いて証明した。さらに、 GluD1 欠損マウスにおける回路表現型を電子顕 微鏡を用いて解析した。

#### C. 研究結果

1. GluD1 の細胞発現

GluD1 の全長をコードするリボプローブを用い ることにより、特異的で高感度の in situ ハイブ リダイゼーション法が可能となった。その結果、 GluD1 は大脳を含む広範な領域に発現し、特に大 脳皮質、海馬、歯状回、視床 VPM 核、扁桃体、 分界条床核、小脳皮質に強く発現していた。

#### 2. GluD1の神経回路発現

平成27年度の共同研究では、神経回路が最もよ くわかっている小脳皮質を回路発現の解析対象 として行った。その結果、GluD1は分子層の介在 ニューロンに選択的に発現し、平行線維シナプス の後部に局在していた。同様の入力選択的な回路 発現は視床 VPM 核や分界条床核においても確認で きた。

### 3. GluD1 欠損マウスの回路表現型

GluD1 欠損マウスの小脳皮質を電子顕微鏡で解 析したところ、この分子が局在する平行線維・介 在ニューロンシナプスが半減していた。同様の表 現型は視床 VPM 核においても得られつつある。 以上の観察結果より、GluD1 は特定の入力を受け るシナプスに発現し、その接着を強化しているこ とが判明した。

### D. 考察

これまで小脳プルキンエ細胞の平行線維シナ プスに豊富な GluD2 についての研究から、この 分子がこのシナプスの構造的および機能的結合 性を制御する重要な分子であり、その遺伝子異常 により小脳性の運動障害が起こることを遺伝子 変異を有するヒト家系やマウスの研究から明ら かになっている。一方、GluD1 の遺伝子変異も 様々な精神・神経疾患と関連することが報告され ているが、その細胞発現、回路発現、生理機能は 全く不明であった。

本共同研究の推進により、GluD1 も特定のニュ ーロンに発現し、特定の神経入力を受けるシナプ ス後部に選択的に発現し、その結合を制御する分 子であることが判明した。しかも、この GluD1 の発現特性と分子機能は視床 VPM や分界条床核 の解析から、小脳以外の脳領域でも保たれており、 脳に普遍的なものであることもクローズアップ されてきた。今後、本共同研究が最終目的とする 大脳皮質や海馬などの大脳領域における研究成 果へと発展・結実することにより、この分子の発 現と機能の全貌が明らかとなることが期待され る。

### E. 結論

GluD1 および GluD2 からなる GluD ファミリー は、特定の神経入力を受けるシナプス後部に発現 し、そのシナプス接着を強化する。

## F. 研究発表(上記課題名に関するもの)

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### G. 知的財産権の出願・登録状況(予定を含む)

- 1. 特許取得
- なし
- 2. 実用新案登録
- なし
- 3. その他
- なし

### 限局性皮質異形成の分子遺伝学的発生機序の解明

研究代表者	加藤	<sub>光広</sub> 1)
研究分担者	柿田	<sub>明美</sub> 2)
	武井	延之 <b>2)</b>
	松本	<sub>直通</sub> 3)
	才津	浩智 <b>3)</b>
	中島	<sub>光子</sub> 3)
	亀山	茂樹 <b>4)</b>
	遠山	潤 4)

昭和大学医学部小児科学講座
 新潟大学脳研究所
 横浜市立大学遺伝学講座
 国立病院
 機構西新潟中央病院

### 研究要旨

限局性皮質異形成(FCD)の原因を明らかにするために、FCDと病理診断されたてんかん患者の脳手術標本24検体および同患者の血液からgDNAを抽出し、次世代シーケンサーによる deep sequence を行ない、体細胞モザイク変異の有無を検討した。FCD IIb型13 例中6 例で MTOR に体細胞モザイクのミスセンス変異を検出した。MTOR 変異体を導入した細胞ではmTORの標的である 4EBP のリン酸化が促進されていた。変異症例の脳組織ではmTOR のもう一つの標的である S6 キナーゼのリン酸化が促進されていた。FCD IIb型における MTOR 変異は機能が亢進する活性型変異であった。mTOR 信号伝達経路の上流には、結節性硬化症や巨脳症関連疾患の原因分子 TSC1/TSC2, PIK3, AKT3 が存在する。三者は病理所見の類似性から共通病態が示唆されていたが、分子レベルで共通性が証明された。

### A. 研究目的

限局性皮質異形成(FCD)は、1971年に Taylor らが focal dysplasia of the cerebral cortex in epilepsy として報告され、難治性てんかんの外 科切除病変で認められる特異的な病理像を呈す る疾患単位である。てんかん発作の発症時期は病 理変化の程度に反比例し、皮質神経細胞の配列の 乱れのみの I 型では中高生以降が多く、異常細胞 を伴う II 型では乳幼児期から学童期に発症する ことが多い。発作型は年齢によって変化し、部分 発作だけでなく、発症時には強直発作、強直間代 発作も多く認められ、症状の進行に伴い、認知機 能の障害や片麻痺など局所運動症状の出現がみ られる。発作は薬剤抵抗性を示し、多くは病変切 除を必要とする。病理的には皮質層構造の異常に よる神経細胞の配列の乱れと、異型細胞や balloon cell などの異常細胞の出現を特徴とす る。皮質の局所的な発生異常が病態として推測さ れているが、その原因は全く不明である。本研究 では FCD の分子遺伝学的発生機序を明らかにす ることを目的とする。初年度の研究で脳病理組織 に存在し、血液には認められない体細胞モザイク 変異の有無を検討し、1名に細胞外カルシウム濃 度の減少による神経細胞興奮に関与する遺伝子 X を同定したが、他の8名の患者に共通する変異 は認められなかった。昨年度も脳組織で発現する 遺伝子の体細胞モザイク変異が原因と推定し、病 理学的に形成異常の強い FCD II 型と診断された 脳組織標本と血液のペア検体を増やして、FCD の 原因遺伝子の特定をめざし、遺伝子 Y を同定した。 遺伝子 Y は MTOR であり、同定された mTOR 変異体 の機能を解析し、FCD の発生機序を解明する。

### B. 研究方法(倫理面への配慮を含む)

FCD と病理診断されたてんかん患者の脳手術 標本 24 例の凍結組織もしくはパラフィン標本か らゲノム DNA を抽出した。FCD IIb 型の 9 例に対 してライブラリー調整後、次世代シーケンサーに よるエクソーム解析を行ない、体細胞変異が検出 された遺伝子を FCD の原因候補として、他の 15 例について変異解析を行った。また、新たに FCDIIb 型 4 例から同意を得て、体細胞モザイク 変異の有無を比較検討した。また、ヒト培養細胞 株に MTOR 変異体を発現するプラスミドをトラン スフェクションし、同定された各変異が mTOR 経 路に及ぼす影響を調べた。

(倫理面への配慮)

山形大学医学部倫理委員会(平成24年8月6日第71号)および昭和大学医学部ヒトゲノム・遺伝子解析倫理審査委員会(平成27年7月1日第220号)の承認を受け、患者もしくは保護者から研究に対する同意を得た。

### C. 研究結果

FCD IIb型13 例中6 例で MTOR の体細胞モザイ ク変異を同定した。脳組織のモザイク率が1-9% に対し、血液における頻度は0.1%未満であり、 明らかに差を認めた。脳組織の他のタイプのFCD では変異が認められなかった。MTOR 変異体を発 現するプラスミドをトランスフェクションした 細胞株は MTOR 正常体を発現するプラスミドをト ランスフェクションした細胞株に比べて、4EBP の発現が亢進し、mTOR 経路の活性が著明に上昇 していた。

### D. 考察

FCD IIb 型の約半数で MTOR の体細胞モザイク 変異を同定し、FCD IIb 型の主要な原因と考えら れた。MTOR は PI3K-AKT3-mTOR の信号伝達経路を 構成するタンパク質をコードする遺伝子である。 FCD は従来から病理学的に結節性硬化症の脳病 理標本所見と類似しており、結節性硬化症との病 態の共通性が示唆されていた。結節性硬化症の原 因遺伝子である TSC1/TSC2 は PI3K-AKT3-mTOR 経 路の AKT3 と mTOR の間に位置しており、FCD と結 節性硬化症の分子機構が共通することを直接的 に示唆する。また、同じく病理学的に類似所見を 示す片側巨脳症も PI3K-AKT3-mTOR 経路の遺伝子 変異が原因である。さらに、画像的に両側性の巨 脳症と脳形成異常をきたす皮膚毛細管異常を伴 う巨脳症(MCAP)や多小脳回と多指症を伴う巨脳 症 (MPPH) も PIK3R2, PIK3CA, AKT3 など PI3K-AKT3-mTOR 経路の遺伝子変異が原因である ことを我々と他のグループが明らかにしている。 病理所見から推測されていた病態の共通性が、原 因遺伝子の同定によって分子レベルで共通する 病態が示されたことは「脳神経病理標本資源活 用」の本プロジェクトの趣旨からも意義深いと考 えられる。

MTOR の変異は FCD IIb 型のみに同定され、他 のタイプ(I型、IIa型)では MTOR の変異は同定さ れなかった。上述した結節性硬化症や片側巨脳症、 MCAP, MPPH のいずれもが PI3K-AKT3-mTOR 経路の 複数の遺伝子が原因に関与する。FCD は PI3K-AKT3-mTOR 経路の局所的な体細胞変異が原 因と考えられ、MTOR 以外の PI3K-AKT3-mTOR 経路 の遺伝子や他の関連する遺伝子変異が原因で生 じている可能性がある。今後さらに他の症例で解 析を行うとともに、限局性皮質異形成の病態と分 子基盤を細胞や臓器・個体レベルで精査する必要 がある。

#### E. 結論

FCD 24 例の脳と血液を用いて体細胞モザイク 変異解析を行い、FCD IIb 型 13 例中 6 例で PI3K-AKT3-mTOR 経路に関与する MTOR に体細胞突 然変異を検出した。FCD が結節性硬化症等と共通 の病態を有することが分子レベルで示された。

### F.研究発表(上記課題名に関するもの)

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G. 知的財産権の出願・登録状況(予定を含む)

1. 特許取得

なし

2. 実用新案登録

なし

3.その他

なし

### オリーブ橋小脳萎縮症における細胞障害機構の解明

研究代表者	瀧川	みき
研究分担者	柿田	明美 <sup>2)</sup>
	加藤	信介1)

鳥取大学医学部病理学講座脳病態医科学分野
 新潟大学脳研究所脳科学リソース研究部門脳疾患標本資源解析学分野

#### 研究要旨

オリーブ橋小脳萎縮症(MSA-C)21 例、年齢層を一致させた正常対照 10 例を用い、免疫組織学的解析 として、p53 抗体、caspase3 を用いた。

MSA-Cの残存プルキンエ細胞は、p53 抗体で陰性を呈した。一方、caspase3 は発症後 6~9 年頃に発現が認められる傾向があった。

これまでの我々の研究により、MSA-C の残存プルキンエ細胞の一部では、酸化ストレスによる酸化型 SOD1の形成が認められ、Ca++代謝を含めた神経細胞の機能が低下し、やがて、酸化ストレスを一要因として、変性・細胞死に至ることが推測された。今回の研究からは、酸化ストレスと関連して p53 は MSA-C のプルキンエ細胞死に関与しないことが判明した。一方、caspase3 を介して、酸化ストレス は病態の進行に関与する可能性が示唆された。

### A. 研究目的

MSA-C は日本に多い多系統萎縮症 (MSA)の一亜 型であり、プルキンエ細胞の脱落が主たる病理学 的所見の一つである。しかし、MSA の基本病態と される α-シヌクレインの蓄積はプルキンエ細胞 そのものには認められず、細胞死の原因は未だ不 明である。これまでの我々の研究で、MSA-C にお けるプルキンエ細胞死には、酸化ストレスが関与 している事が判明した。今回は、酸化ストレスが どのような機序で細胞死に関与しているかを、 apoptosisの観点から検討した。

### B. 研究方法

MSA-C21 例、年齢層を一致させた正常対照 10 例 を用い、通常染色に加え、免疫組織学的解析とし て、我々が開発した酸化型 SOD1 抗体、カルビン ジン抗体、SOD1 抗体、p 53 抗体、caspase3 抗体 を用いた。

#### C. 研究結果

MSA-C の残存プルキンエ細胞は、p53 抗体には 陰性を呈した。一方 caspsase3 抗体に対しては、 発症後 6~9 年の症例は、一部の残存プルキンエ 細胞で陽性を呈した。

残存プルキンエ細胞における酸化型 SOD1 の発 現は、発症後より徐々に増加し、発症後6年頃を ピークとして横ばいとなり、発症後9年以降は減 少する傾向が見られた。

#### D. 考察

これまでの我々の研究で、MSA-C の残存プルキ ンエ細胞の一部では、酸化ストレスによる酸化型 SOD1の形成が認められ、Ca++代謝を含めた神経細 胞の機能が低下し、やがて、酸化ストレスにより、
変性・細胞死に至る機序が推測されていた。今回 apoptosisの観点から、p53 および caspase3 抗体 を用いて検討したが、p53 は明らかな発現を認め ず、細胞死への関与は低い事が推測された。一方、 casepase3 は、発症後 6~9 年の症例における残存 プルキンエ細胞に発現が多く見られ、この時期の 細胞死に深く関与していると考えられた。

酸化型 SOD1 発現様式と caspase3 発現様式が比較的似通っている点から、酸化ストレスが apoptosis を介して、残存プルキンエ細胞死を加速している可能性が示唆される。

### E. 結論

MSA-C における原因不明のプルキンエ細胞死に は、酸化ストレスが関与しており、酸化ストレス による apoptosis を介した細胞死の機序が示唆さ れた。

F.研究発表(上記課題名に関するもの)

1. 論文発表

なし

2. 学会発表

第 56 回神経病理学会 2015 年 6 月 3 日~5 日、 福岡

G. 知的財産権の出願・登録状況(予定を含む)

1. 特許取得

なし

2. 実用新案登録

なし

3.その他

なし

# 統合失調症脳内タンパク質多項目同時測定解析及び関連遺伝子発現解析

# 研究代表者 國井 泰人 1)

研究分担者 柿田明美<sup>2</sup>) 矢部博興<sup>1</sup>)、丹羽真一<sup>3</sup>)、和田明<sup>1</sup>)、松本純弥<sup>1</sup>)、日野瑞城<sup>1</sup>)、 那波宏之<sup>2</sup>)、高橋均<sup>2</sup>)

1) 福島県立医科大学 2) 新潟大学脳研究所 3) 会津医療センター

#### 研究要旨

本研究では、統合失調症発症のメカニズムの解明を目的として患者死後脳内タンパク質の発現解析 を行う。ドパミン関連分子、グルタミン酸関連分子、GABA 関連分子を標的タンパク質として、統合失 調症群と健常対照群の死後脳凍結組織における発現量の差異を大槻法で用いてスクリーニングし、タ ンパク質 10 種について脳内の複数の部位についてルミネックス法によるタンパク定量を行う。更に、 免疫組織化学的手法を用いて、より詳細な標的タンパク質の組織内分布を観察し発現している細胞を 同定する。統合失調症群に関しては、当バンク特有の詳細な臨床情報(罹病期間、抗精神病服薬量、 生活歴・既往歴・手術歴・鎮痛薬を含む全服薬歴等)を駆使して関連を検討する。また、タンパク質 発現解析から得られた情報を元に遺伝子発現解析を行う。

### A. 研究目的

本研究の目的は、統合失調症病態におけるドパ ミン系・グルタミン酸・GABA 系の異常を、動物実 験や画像研究などの間接的アプローチではなく、 死後脳組織を用いて病態の現場を直接的に検討 することである。これまで代表者らが統合失調症 死後脳組織を用いて分析を進めてきたドパミン 系・グルタミン酸・GABA 系の分子を中心に、ごく 少量のサンプルでタンパク質の多項目同時測定 を可能にするルミネックス法という革新的新手 法から新たな脳内表現型の創出し、更に免疫組織 化学、遺伝発現解析などを通して精神疾患病態の 鍵となる分子や治療標的分子を同定する。

### B. 研究方法(倫理面への配慮を含む)

当講座の精神疾患死後脳バンクにおいて凍結 保存された死後脳及び新潟大学脳研究所保管の 年齢、性別、死後時間等をマッチさせた非精神神 経疾患対照例を測定対象として、ルミネックス法 による死後脳タンパク質群の多項目同時測定を 行う。測定対象のタンパク質は大槻法によって統 合失調症患者死後脳で健常対照群に対して発現 量に変化が認められたタンパク質 10 種程度とす る。さらに免疫組織化学的手法を用いて、より詳 細な標的タンパク質の組織内分布を観察し発現 している細胞を同定する。統合失調症群に関して は、当バンク特有の詳細な臨床情報を駆使して関 連を検討する。解析した分子の中で異常が捉えら れたものについては、レーザーマイクロダイセク ション等により、免疫組織化学解析と同じ解析部 位の組織を抽出し、局所的遺伝子発現解析も実施 する。

なおこの研究は各施設の倫理委員会の承認を 得ており、ヘルシンキ宣言に基づいた倫理的原則 に則って実施され、発表にあたっては死後脳提供 遺族から十分なインフォームド・コンセントを得 て、プライバシーに関する守秘義務を遵守し、匿 名性の保持に十分な配慮をした。

### C. 研究結果

年齢、性別、死後時間をマッチさせた統合失調 症死後脳 19 例、健常対照死後脳 21 例の凍結脳 サンプルの前頭前皮質(BA10)および側坐核につ  $\mathcal{VT}$  vascular endothelial growth factor receptor 2 (VEGFR2), epidermal growth factor receptor (EGFR), Akt1、リン酸化 Akt1 (Ser473)、 extracellular signal-regulated kinase 1/2 (ERK1/2)、リン酸化 ERK1/2 (Thr185/Tyr187), glycogen synthase kinase 3 β(GSK3β)、リン酸化 GSK3β(Ser9)、および cAMP response element binding protein (CREB)の蛋白質発 現 量 を Bead-Based Multiplex Immunoassay (Luminex システム)により多項目同時測定した。 さらに統合失調症群と対照群間で発現量の差が みられた VEGFR2 については、発現量と生前の臨 床症状スコアとの関連について、またその遺伝子 多型と各蛋白質の発現量、統合失調症発症の有無 との相関を解析した。同時多項目測定の結果、 VEGFR2 が統合失調症群の前頭前皮質において有 意に減少していた。統合失調症群において前頭前 皮質の VEGFR2 発現量と生前の陽性症状スコア の間に負の相関がみられた。また、統合失調症群 の前頭前皮質ではリン酸化 Aktl が増加していた。 遺伝子多型解析の結果、VEGFR2 遺伝子の多型 (rs7692791)はAkt1の発現量とERK1/2およびAkt1 のリン酸化レベルを予測することが示された。ま た VEGFR2 遺伝子の 2 多型(rs17709898 および rs1870377)が統合失調症と相関していた。

### D. 考察

### E. 結論

VEGFR2 は細胞外からの血管増殖刺激を Akt シ グナル伝達系に伝える因子であることから、統合 失調症群の前頭前皮質の VEGFR2 発現量低下は 統合失調症の病因が微小血液循環の異常にある とする仮説を説明するものと考えられる。

### F. 研究発表(上記課題名に関するもの) 1. 論文発表

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日野瑞城、國井泰人ら;統合失調症患者死後脳前 頭前皮質における Akt シグナル伝達系蛋白質群の 多項目同時測定.第11回日本統合失調症学会,群 馬,2016/3/26

(国際学会) なし。

#### G. 知的財産権の出願・登録状況(予定を含む)

- 1. 特許取得 なし。
- 2. 実用新案登録

なし。

- 3. その他
  - なし。

# CRISPR/Cas9 システムを使った迅速なノックアウトマウス作成

# 研究代表者 赤間智也 1) 研究分担者 崎村建司 2)

1) 関西医科大学薬理学 2) 新潟大学脳研究所細胞神経生物学分野

### 研究要旨

我々は角膜や脳に存在する直鎖状糖鎖であるケラタン硫酸グリコサミノグリカンの生合成経路とその生物学的機能解明に向けた研究を行なっている。ケラタン硫酸糖鎖合成の律速段階となる酵素は β3GnT2 およびβ3GnT7 と考えられており、この遺伝子をノックアウトした変異マウスは角膜の細胞外 マトリックスの異常や新生仔致死の表現型を示すことが明らかとなった。現在我々はタモキシフェン 誘導型のコンディショナルノックアウトマウスを作成して成体でのケラタン硫酸合成酵素の必要性を 調べており、成体でβ3GnT2 の発現を抑制した場合、急性の変化は見られなかったことから少なくとも β3GnT2 の発現の消失は成体マウスの生存に必須ではないということが明らかとなった。

### A. 研究目的

ポリNアセチルラクトサミンやケラタン硫酸は 細胞表面や細胞外マトリックスタンパク質に見 られる直鎖状の糖鎖構造であり、細胞間あるいは タンパク質間相互作用に影響を与えていると考 えられている。我々はこれら直鎖状糖鎖の生物学 的機能を解析する目的でその合成酵素である β3GnT2 およびβ3GnT7 の遺伝子ノックアウトマウ スを作成しその表現型を解析した。また化合物誘 導型のコンディショナルノックアウトマウスを 作成することで成体における酵素遺伝子および 直鎖状糖鎖の必要性についても解析を試みた。

### B. 研究方法(倫理面への配慮を含む)

我々は既に新潟大学脳研究所との共同研究に てβ3GnT2 をコードする遺伝子である *B3gnt2* の floxed マウスおよびβ3GnT7 をコードする遺伝子 である *B3gnt7*の floxed マウスを作成済みである。 これらのマウスに Cre 発現マウスを掛け合わせる ことで全身性のノックアウトマウスを作成して その表現型を解析した。また *B3gnt2*の floxed マ ウスを ROSA26-CreERT2 マウスと掛け合わせるこ とでタモキシフェン誘導型の変異マウスを作成 し、15週齢の段階で腹腔内にタモキシフェンを投 与することでβ3GnT2の発現を抑制してその後の マウスの変化を調べた。一方で別のケラタン硫酸 合成酵素である *Chst1や Chst5*遺伝子を迅速に破 壊する CRISPR/Cas9 システムの構築も行った。

### C. 研究結果

- B3gnt2ノックアウトマウス
   B3gnt2の全身性ノックアウトマウスは生後2
   日以内にそのほとんどが死んでしまう新生仔 致死であることからβ3GnT2の合成するポリN
  - アセチルラクトサミンおよびケラタン硫酸は 新生仔の発生に重要であることが明らかとな ったが、成体でも必須であるのかどうかは不明 であった。そこで

*B3gnt2*(flox/-)/ROSA26(+/CreERT2)マウス

(*B3gnt2* CK0 マウス)を作成し、成体(15週 齢)の段階で腹腔内にタモキシフェンを投与 (150 mg/kg/日で5日連続投与)し、その後の

影響を調べた。*B3gnt2* CK0 マウスは投与後1 週間以上経過しても特に変化を見せず生存し ていたことからβ3GnT2の発現抑制は成体マウ スの生存にとって急性の影響を与えるもので はないことが分かった。このマウス(投与後1 ヶ月経過)はさらに飼育を続け、β3GnT2発現 抑制の影響の観察を続けている。

2) B3gnt7ノックアウトマウス

B3gnt7 全身性ノックアウトマウスは正常に成 育し交配も行うが、詳細な解析の結果、ケラタ ン硫酸が豊富に存在する角膜実質においてそ の厚さが減少し、細胞外マトリックスの構造が より乱雑になるという表現型が確認された。こ れはヒトの斑状角膜ジストロフィーに類似す る表現型であり、この疾病を有する患者ではケ ラタン硫酸の生合成が不全となりケラタン硫 酸鎖の伸長が抑制されていることが明らかと なっている。B3gnt7 ノックアウトマウスでも 角膜実質のケラタン硫酸合成が阻害されてお り、マウスにおいてもケラタン硫酸の合成阻害 が角膜実質細胞外マトリックスの構築不全を 引き起こして角膜厚の減少を示していること が分かった。そこで成体の角膜においてもケラ タン硫酸糖鎖のターンオーバーがあるとすれ ば成体角膜でB3GnT7 の発現を抑制することで 角膜厚が減少するのではないかと予想し、 B3gnt7(flox/−)/ROSA26(pA-*lacZ*/CreERT2) マ ウスを作成して眼球に直接タモキシフェンを 投与することを計画している。予備的実験とし て ROSA26(pA-lacZ/CreERT2)マウスを作成し、 このマウスの眼球に活性型タモキシフェンで ある 4-hydroxytamoxifen (OHT)をワセリンに 懸濁して塗布(麻酔下で 5-10 μg/眼/日で5日 連続投与)したところ CreERT2 の活性化とそれ に伴う lacZの発現が角膜上皮細胞および実質 細胞で確認されたことからこの条件での OHT 投与で角膜実質細胞にβ3GnT7 の発現抑制を誘 導することが出来るものと考えられた。現在実 験に必要な数のマウスの繁殖を進めている。

3) CRISPR/Cas9 による迅速な遺伝子発現抑制系の 構築

Chst1および Chst5遺伝子はマウスにおいてケ ラタン硫酸の硫酸化を行う酵素をコードする 遺伝子である。これらの遺伝子の発現していな い細胞を入手するべく、ヒトの遺伝子のオーソ ログである CHST1と CHST6をヒト HEK293T 細胞

で破壊することを目的として CRISPR/Cas9 シ ステムの構築を行った。それぞれの遺伝子に対 応する gRNA 発現ベクターを 3 種類作成して Cas9 発現ベクターと共に細胞内で発現させ、 発現細胞をピューロマイシン選択培地で選択 すると同時にクローニングし、この培養細胞に おける遺伝子破壊の効率をシークエンスにて 調べた。その結果、CRISPR/Cas9 による遺伝子 の破壊効率は充分高く(CHST1 20 アレル中 15 アレル、CHST6 20 アレル中 10 アレル)、ケラ タン硫酸の産生を行わない細胞を入手するこ とに成功した。一方でクローニングしたはずの 細胞の遺伝子変異を調べたところ3アレル以 上の変異を検出し、クローニングしたはずの細 胞株は2 種類以上の細胞が混在することが明 らかとなった。

### D. 考察

β3GnT2 とβ3GnT7 は *in vitro* では共にポリNア セチルラクトサミンおよびケラタン硫酸の合成 に関わることが知られているが、その全身性ノッ クアウトマウスの表現型を調べるとβ3GnT2 は新 生仔の発生に重要であるがB3GnT7 は必須ではな いことが明らかとなった。これはβ3GnT2 は主に N 型糖鎖のポリNアセチルラクトサミン合成に関与 し、β3GnT7 は主に角膜におけるケラタン硫酸合成 に関わっているからで、B3GnT7 が合成を行なう糖 鎖は機能が限局していて発生に致命的な影響を 与えないからではないかと考えられる。B3GnT2は 新生仔発生に必須であることが分かったが、成体 で発現を抑制しても急性の影響が出ないことが 今回の研究で明らかとなった。成体での糖タンパ ク質のターンオーバーは長いものから短いもの まで幅があり、β3GnT2の酵素活性が寄与する糖タ ンパク質のターンオーバーは比較的長いため急 性の影響が表れないのかも知れない。あるいは通 常のマウスの生存には必須でないが、炎症など病 的な状態で必要とされることも考えられるので 更なる研究が必要であると考える。

CRISPR/Cas9 システムによる遺伝子破壊はシス テムの構築が簡単で変異導入細胞を調製するだ けであれば非常に迅速に行なえることが分かっ た。しかしながら均一なゲノムを有するクローン 化細胞の作成はクローニングが容易ではなく比 較的煩雑であるのでこの点を改良することが必 要であると考えられた。この手法を遺伝子破壊マ ウスの作成に使用することはそれほど難しくな く、少なくとも変異 ES 細胞の作成は簡単である と考えられる。但し得られた変異細胞はクローン ではなく、その細胞群の中からフレームシフトを 生じている細胞のみをマウス作成に使う必要が あるので注意が必要である。

### E. 結論

糖鎖合成酵素のノックアウトマウスの作成に より、これまでに知られていない糖鎖の生体内に おける機能が明らかとなり、この手法が機能解析 に有用であることが分かった。またコンディショ ナルノックアウトマウスを作成することで成体 になってからの機能を解析することができ、発生 過程での機能と区別することが可能である。 CRISPR/Cas9 システムは細胞内の遺伝子を破壊す ることは容易であり、この方法を用いた全身性遺 伝子ノックアウトの作成は難しくないものと考 えられるが、コンディショナルノックアウト型の ES 細胞を作成するには *loxP*の挿入などいくつか の課題があると考えられる。今後の研究でこれら の問題点を克服することが出来ることを期待す る。

F. 研究発表(上記課題名に関するもの)

1. 論文発表

該当なし

2. 学会発表

該当なし

G. 知的財産権の出願・登録状況(予定を含む) 該当なし

# PNPLA6遺伝子の脳における機能-有機リン被爆との関連から

### 研究代表者 木村穰1

研究分担者 笹岡俊邦<sup>2</sup>、畑中朋美<sup>1</sup>

1) 東海大学医学部基礎医学系分子生命科学 2) 新潟大学脳研究所

#### 研究要旨

我々は有機リンやホルムアルデヒド等の被爆が主原因とされるシックハウス症候群の患者単球において Neuropathy Target Esterase(以下 NTE)の活性が健常者に比べて高いことを報告した(2013)。有機リンの曝露 後に神経症状が生じることが知られているが、そのメカニズムについては良くわかっていない。本研究では、 有機リン関連疾患の発症機構解明を目指し、まず NTE をコードする遺伝子 PNPLA6を導入したマウスを作製 し、有機リンやホルムアルデヒド投与実験を開始した。シックハウス症候群の原因物質の一つとされるフタル酸 エステルの経皮吸収とエステラーゼの関わりについては良いモデルができた。有機リンと NTE の複合体検出 は進行中である。最近、いくつかの小脳疾患で PNPLA6 に点突然変異が見いだされており、マウスモデルを 作製すべく、CRISPR/Cas9 システムを整えた。

### A. 研究目的

本研究ではシックハウス症候群やその原因物質と して指針値が定められている13種の化学物質-中で も有機リンに対しての発症メカニズムを遺伝子操作マ ウスを駆使して探求することを目的とした。また、対象 とする酵素は有機リンと共有結合を形成することから、 その複合体が種々の症状を生み出す可能性を考え、 複合体検出への取り組みを行っている。すでに確立 した NTE 高発現マウスと、CRISPR(Clustered regulatory Interspaced short parindromic repeats)  $\gg$ ステムによる遺伝子変異導入技術をマウス pnpla6 遺 伝子に応用することにより最終的には pnpla6 変異個 体を得て、pnpla6 遺伝子の個体レベルでの機能を、 神経系を中心に明らかにしたい。また、農薬等に良く 使用されるジクロルボス(DDVP)をこれらのマウスに投 与し、中枢および末梢神経系における有機リンの影 響を、NTE に対する急性および遅延性反応という観 点から解析することが本研究の最終目的である。

B. 研究方法(倫理面への配慮を含む)

ヒト PNPLA6 遺伝子導入マウスを用いた実験計 画は東海大学遺伝子組換え実験安全委員会およ び東海大学動物実験委員会を通じて大学より承認 されている。マウスは我々独自の方法で、 ROSA26 部位に CAG プロモーター下にヒト PNPLA6遺伝子 cDNA および同時に EGFP遺伝子 を導入したマウスである。Foマウスと Cre マウスとの 交配により発現を誘導し、F1以降の系統化は C57BL/6 との交配によった。ヒト皮膚を用いた経皮 吸収実験計画は医学部医の倫理委員会の審議後、 承認を受けている。

マウスのホルムアルデヒド曝露実験および経皮 吸収実験は独自の装置を用いて行い、酵素活性 測定は常法に従った。

複合体検出では島津製作所の質量分析器 (Shimazu LC-MS)を使用した。眼球運動は高速度 デジタルカメラを用いた瞳孔追跡システム、空 気中のホルムアルデヒド濃度は新規に開発した レーザーを用いたガスセンシング法及び高速液 体クロマトグラフィを用いて測定した。

### C. 研究結果

ヒト PNPLA6 遺伝子導入マウスでは PNPLA6 遺 伝子がコードするNTEの発現がモザイク状態となる ようであり、全身発現性の CAG プロモーターを用い ているせいか、成長遅延の個体も見られる。しかし ながら各臓器で遺伝子導入個体では通常のマウス に比べ少なくとも数倍の活性上昇があった。

有機リンの一種で殺虫剤等にも使用されている ジクロルボス(DDVP)を投与したが、成体では今の ところ特に変化はないが、妊娠マウスに投与した場 合には、遺伝子導入マウスの死亡胎仔の割合が高 い(以上既報告)。

東海大学准教授加藤明先生の協力を得て 60-80 ppm の蒸散ホルムアルデヒドガス曝露後 (18 時間 / 日、7日間、n = 1)、HE 染色した 脳切片を用いて遺伝子導入マウス脳の形態異常 を解析したところ、小脳プルキンエ細胞の委縮 及び海馬歯状回-CA3 周辺の層構造異常を示唆 する結果が得られた。

経皮吸収に関しては表皮と真皮の境界領域で NTE の発現を免疫組織学的に認めた。ついでフ タル酸ベンジルブチルの結合切断の種特異性を 利用してその皮膚の透過実験からこのマウス皮 膚では NTE 活性が 100 倍上昇し、かつヒト型の 主代謝物の特性を備えていることを解明した。

NTE 活性は吉草酸エステルを基質として paraoxon 耐性で mipafox 感受性のエステラーゼ 活性として定義されるが、より簡便な検出法開 発に取組んだ。ヒト単核球中での総エステラー ゼ活性が paraoxon 耐性のエステラーゼ活性と 比例することを確認し、マウス皮膚と脳で NTE 活性と paraoxon 耐性のエステラーゼ活性は比 例した。しかし、フタル酸エステル代謝活性と は必ずしも比例しなかった。

ー過性のNTE 高発現 293 細胞を利用し、ゲルより抽出したバンドがNTE であることを質量分析系で確認していたが、このクルードな系ではNTE-DDVP 複合体の検出には至らなかった。そこで、現在は大腸菌で活性を有するペプチド部分のN 末および C 末にタグを配置して大量産生し

た"NTE"の精製を試みている。当初のタグでは2種 の分子が生じるため、ゲル上で単一バンドとするべ く再度トライしている。大量精製ののち in vitro で DDVPとの複合体を形成させ、質量分析を行う予定 である。

CRISPR(Clustered regulatory Interspaced short parindromic repeat)技術は、遺伝子の特定の配列 を利用して、任意の動植物種や細胞に変異を導入 することができるとされる方法だが、我々も今年度 は複数のマウス遺伝子系で条件を検討しているが、 点突然変異の導入に関しては難しい例もある。 pnpla6遺伝子の改変を進行中である。

脳研柿田教授のご協力により、ヒト農薬被爆者の 標本が1例ある事が判明した。NTE 抗体で Western blot に用いることが可能な抗体が準備出来てきてい るので、今後、他の例も含めて年齢ごとの脳内 NTE 発現量を検討し、基礎データを得ていきたい。なお、 貴学ヒト脳標本の利用については、本学では2016年 3月に承認が得られた段階である。

### D. 考察

我々が開発した独自の方法で、全身でNTEの高 発現を示すトランスジェニックマスの系統化に成功し たが、DDVP 投与やホルムアルデヒド曝露では神経 症状や行動上の顕著な外見上の変化はない。もとも とマウスはヒトでは致死量のホルムアルデヒド濃度で も耐性であることや、そのくせDDVPの腹腔内投与等 では微妙な投与量の違いですぐに死亡する個体が 出現することから、マウスはこの課題において、あまり 適切な実験動物とは言えないのではないかと考えて いる。今後は感受性の高い鳥類の利用等も視野に 入れて検討したい。

しかしながら、ヒト PNPLA6 遺伝子導入マウス ではフタル酸エステルの代謝について、見事にマ ウス型とヒト型を区別することができ、個体レベ ルでヒト型皮膚の経皮吸収実験が行える道が開 けた。また、手術時のヒト皮膚を用いた活性測定 では NTE 活性は低いものの、paraoxon 耐性の活性 で代用出来る見通しがある程度出来ることとな った。

DDVP-NTE 複合体の質量分析器での検出は現在 大量精製している大腸菌に産生させた NTE によっ て実現可能になると思われる。その先では複合体 に特異的な抗体作成に挑戦したい。

NTEをコードする PNPLA6遺伝子の変異が運動神 経系関連疾患で続々報告されているので、CRISPR 技術でマウスの相同遺伝子に変異導入する取り 組みを開始した。

### E. 結論

有機リン関連疾患の発症機構解明を目指すため に、有機リンと活性中心で共有結合を形成するヒト NTE を発現するマウスを系統化した。発現の程度は 臓器や週齢によって異なるがいずれも高発現で ある。DDVP 投与やホルムアルデヒド曝露では少数 例ではあるが影響は見られている。シックハウス症候 群の原因物質とされるフタル酸エステルの経皮吸収 については良いヒト型モデルができ、論文にまとめる ことができた。有機リンと NTE の複合体検出は動物 細胞使用例ではうまく行かず、現在は大腸菌でのタ グ付き NTE 産生に切り替えた。

現在、ヒトで報告される PNPLA6 遺伝子変異に 相当するマウスを CRISPR 法で作製するべく、 CRISPR/Cas9 システムを整えているが、DNA の塩基 配列によって効率がかなり変化する可能性を示唆す るデータを得ている。

### F.研究発表(上記課題名に関するもの)

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G. 知的財産権の出願・登録状況(予定を含む) なし。

# グリオーマの分子標的治療・放射線治療耐性機構の解明と治療薬の開発

### 研究代表者 津田 真寿美 1)

### 研究分担者 田中 伸哉 1,2)、谷野 美智枝 1)、王 磊 2)、長嶋 和郎 3)、柿田 明美 4)

北海道大学大学院医学研究科腫瘍病理学分野
 北海道大学大学院医学研究科探索病理学講座
 礼幌東徳洲会病院、北海道大学名誉教授
 新潟大学脳研究所病理学分野

### 研究要旨

膠芽腫は極めて予後不良の悪性腫瘍であり、この一因は腫瘍細胞が治療経過中に放射線や化学療法 に対して治療抵抗性を獲得するためである。当該研究の目的は、放射線や今後高い治療効果が期待さ れる分子標的治療薬に対する抵抗性獲得機構を解明し、より有効な新規治療法を臨床現場に届けるた めの基盤を構築することである。具体的には、治療抵抗性細胞株を樹立し、遺伝子プロファイリング、蛋白 プロファイリングを行い、治療抵抗性獲得シグナル経路および責任分子を同定、これらの結果の普遍性を病 理組織検体にて検証する。これまでの研究により、分子標的治療薬耐性株は上皮間葉移行から幹細胞様 へと形質変化を遂げることを明らかとし、耐性獲得の責任分子として IGFBP2 を同定した。平成 27 年 度の研究では、分子標的治療薬耐性に直接関与する分子として MSX1 を新たに同定し、ヒストンのメ チル化を含むエピジェネティクスとの関連性について解明した。

### A. 研究目的

膠芽腫は WHO grade IV の極めて予後不良の原 発性脳腫瘍である。近年、化学療法および放射線 治療の進歩に伴い一時的な腫瘍退縮が認められる ようになってきたが、診療経過中に治療抵抗性を獲 得し悪性転化するため、依然として長期予後は不良 である。昨年、アルキル化剤による治療後の再発に 際して、特定の遺伝子変異が高頻度に生じることが 報告されたが (Science 343, 189-193, 2014)、その他 の悪性転化の機構は未だ不明な点が多く、再発時の 治療法選択が困難となっている。

近年、膠芽腫において受容体型チロシンキナー ゼ(RTK)のEGFR、c-Met、PDGFRの遺伝子増 幅や変異が報告されており、これらを標的とした 分子標的治療の臨床治験が試みられている。阻害 剤投与により一時的な治療効果は認められるも のの、EGFR 阻害後の c-Met 発現亢進に伴う腫瘍 幹細胞性の獲得、PDGFR 阻害による副側経路の 持続的な活性化や RTK 間のクロストーク等が生 じて薬剤耐性や腫瘍幹細胞性を獲得することが 報告されており、分子標的治療を標準治療とする には未だ多くの課題が残されている。

本研究では、膠芽腫の分子標的治療において異 なる種類の RTK 阻害剤に対する共通の治療耐性 獲得メカニズムを解明し、耐性解除に有用な新規 治療標的分子を同定することを目的とした。

### B. 研究方法(倫理面への配慮を含む)

ヒト膠芽腫細胞株 KMG4 において、RTK 阻害剤 (EGFR、c-Met、PDGFR 阻害剤)に対する3種類 の耐性株を樹立した(平成25 年度の研究で樹立 済み)。定量的 RT-PCR 法にて耐性細胞における幹 細胞性を規定する遺伝子の発現を検討後、幹細胞 関連 PCR アレイ(QIAGEN)を実施し、その結果 を定量的 RT-PCR 法にて検証した。耐性細胞特異 的に発現亢進を認めた分子に対し、siRNA を用い て発現を抑制後、高濃度の阻害剤を添加し、Cell Death Detection ELISA キット(Roche)を用いてア ポトーシス誘導能を検討した。発現抑制により 3 種類の耐性細胞の薬剤耐性を共通して解除する ことができる分子を同定後、耐性獲得機序を解析 するために、定量的 RT-PCR 及びイムノブロット 法により、アポトーシス関連分子や薬剤耐性関連 分子、幹細胞性を規定する分子との関連性を検討 した。

### C. 研究結果

EGFR、c-Met、PDGFR 阻害剤耐性 KMG4 を用 いた定量的 RT-PCR により、全ての薬剤耐性細胞 において幹細胞性遺伝子 Sox2、Oct3/4、Nanog の 発現が亢進していることが明らかとなった。幹細 胞関連 PCR アレイ解析より、EGFR、PDGFR 阻害 剤耐性細胞において転写抑制因子 Msh homeobox 1 (MSX1)の発現亢進を認めた。

MSX1 はアポトーシス誘導遺伝子 p53 と相関し て発現し、本来は腫瘍抑制遺伝子として報告され ている (Park et al., 2005, Cancer Res)。ヒト子宮頸 癌細胞株 HeLa に MSX1 を過剰発現させるとアポ トーシス誘導遺伝子 p53 の発現を誘導し、腫瘍形 成を抑制する (Shien et al., 2013, Cancer Res)。 MSX1 特異的 siRNA を用いて MSX1 の発現を抑制 すると、全ての薬剤耐性細胞株において幹細胞性 遺伝子 Sox2 の発現が低下した。一方、アポトー シス誘導能は有意に亢進し、チロシンキナーゼ阻 害剤に対する感受性が回復した。また MSX1 の発 現抑制により、ヒストン H3K27 トリメチル化 (H3K27me3) が抑制された。H3K27 トリメチル 化酵素 Enhancer of zeste homolog (EZH2) を特異 的阻害剤 GSK343 により抑制すると、H3K27me3 の低下に伴いアポトーシス関連遺伝子 Bcl-2、p53、 薬剤耐性に関与する ALDH1A1 の発現が亢進した。 上記研究成果は、第74回日本癌学会学術総会 (2015.10.8-10.10、名古屋市)において報告され た。

#### D. 考察

H27 年度の研究により、MSX1 は幹細胞マーカ ー遺伝子の発現上昇に寄与し、また腫瘍のエピゲ ノム異常に関わる因子の一つである H3K27me3 酵 素の EZH2 を競合的に阻害することで、薬剤耐性 遺伝子やアポトーシス抑制遺伝子の発現上昇に 関与する可能性を見出した。

ヒストン H3 のメチル化酵素である EZH2 は膠 芽腫幹細胞の維持に重要であり(Suva et al., 2009, Cancer Res)、既に他の癌腫においても EZH2 に対 する阻害剤を用いて基礎実験が実施されている。 EZH2 を阻害し H3K27me3 を抑制すると、大腸癌 では EGFR 阻害剤の効果が向上し細胞死が誘導さ れ (Katona et al., 2014, Cancer Biol Ther)、BRG1 や EGFR の遺伝子変異が検出された肺癌において は多剤併用による抗腫瘍作用の向上が報告され ている (Fillmore et al., 2015, Nature)。一方で、 H3K27me3 活性の減少が及ぼす抗腫瘍作用機序の 解析は不明であった。当該研究により、分子標的 治療薬に対する耐性細胞ではMSX1の発現が亢進 しており、EZH2 を介して H3K27me3 を制御する ことにより薬剤耐性に関する遺伝子の発現を変 化させていることが明らかとなった。

将来的にMSX1を分子標的治療の新規ターゲットとして臨床応用を目指すためには、網羅的な分子解析によって詳細な機序解析を行うと共に、生体内でも *in vitro* と同様の薬剤耐性解除効果が認められるかを検討する必要がある。また、エピジェネティックな変化が薬剤耐性に及ぼす機序を解析することは、膠芽腫のみならず、他の癌腫の新規治療法開発においてもブレイクスルーとなり得る可能性を持ち合わせていると期待される。

### E. 結論

膠芽腫において、MSX1 はチロシンキナーゼ阻 害剤に対する治療抵抗性獲得に重要な分子であ ることが明らかとなった。MSX1 および MSX1 と協調して作用する EZH2 は、膠芽腫の分子標的 治療の展開に大きく貢献するものと期待される。

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# (シンポジウム、特別講演など)

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- 田中伸哉: Neuro-Oncology Challenges in a Diverse Asia「Pathological Features of Tumors of the Pineal Region」2015.9.17 St.Luke's Medical Center (Manila, Philippines)
- 田中伸哉:第74回日本癌学会学術総会 「Molecular machinery for agaisition of TKI resistance in glioblastoma」2015.10.8 名古屋国 際会議場(名古屋市)
- 4) 田中伸哉:第8回香川県脳腫瘍学術研究会「悪 性グリオーマの分子病理学的研究:治療耐性 メカニズムに挑む」2015.10.30 高松国際ホテ ル(高松市)

# G.知的財産権の出願・登録状況

1.特許取得

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2.実用新案登録

- なし
- 3.その他
- なし

# タンパク質分解システムを標的とするシヌクレイノパチーの分子病態解明 と治療法の確立

#### 研究代表者 森 文秋 1)

研究分担者 丹治 邦和<sup>1)</sup>、三木 康生<sup>1)</sup>、柿田 明美<sup>2)</sup>、高橋 均<sup>3)</sup>、若林 孝一<sup>1)</sup>

<sup>1)</sup>弘前大学大学院医学研究科 脳神経病理学講座 <sup>2)</sup>新潟大学脳研究所 脳疾患リソース解析部門 <sup>3)</sup>新潟大学脳研究所 病理学分野

#### 研究要旨

シヌクレイノパチー(パーキンソン病、レビー小体型認知症、多系統萎縮症)を含む神経変性疾患 では、疾患特異的な分子の蓄積が認められる。特に異常な翻訳後修飾を受けたαシヌクレインは蓄積 しやすく、病態と深く関連している。今回、多系統萎縮症の延髄および脊髄腹側の軟膜下にリン酸化α シヌクレインの蓄積を認め、さらなる解析の結果、これらはアストロサイトの突起に局在することを見出 した。一方、シヌクレイノパチーの治療法開発を目的に「脳内オートファジーの活性化」を進めた。こ れまでトレハロースにより脳内のどこでオートファジーが活性化しているのかは不明であった。そこ で今回オートファジー可視化マウスを利用して解析を進めた結果、海馬および大脳皮質の錐体細胞に おいて、特に強いオートファジー活性化シグナルを検出した。今後もヒト剖検例およびモデル動物の 双方の利点を生かした研究を進めることにより、シヌクレイノパチーの病態を改善する新たな治療法 に結び付く可能性がある。

#### A. 研究目的

レビー小体病(パーキンソン病、レビー小体型 認知症)と多系統萎縮症(MSA)は、神経細胞お よびグリア細胞内におけるαシヌクレインの異 常凝集(封入体形成)を特徴とする神経難病であ り、その進行を遅延・阻止する治療法は確立して いない。我々はこれまでにヒト凍結脳組織を用い た検討などから、シヌクレイノパチーの病態には タンパク質分解システムの障害が深く関与して いることを報告してきた。実際に、MSA および正 常対照のホルマリン固定パラフィン包埋組織を 用いて網羅的にマイクロ RNA (miRNA)の変動を検 討したところ、複数のタンパク質分解システム関 連分子群が変動している可能性を得た。以上より 本年度は以下二点を目的として研究を遂行した。

- 1. miRNA 解析によって同定された分子群と病態 との関連性。
- 2. シヌクレイノパチーの治療を視野に入れ、オ

ートファジーを活性化した際の病態への影響。

#### B. 研究方法

 ヒト剖検脳を用いたタンパク質分解システム関連分子群の病理学的解析 MSA および正常対照のホルマリン固定パラフィン包埋組織の miRNA 解析によって得られた病態関連タンパク質のうち細胞内のタンパク 質分解系、特にオートファジー関連分子に対する抗体を用い、神経変性疾患の剖検脳組織を免 疫組織化学的に解析した。

2.マウスを用いた『オートファジー活性化』の 生化学的および病理学的解析 オートファジーはカロリー制限、飢餓状態、さ らには種々の薬物により活性化される。既に二 糖のトレハロースによる脳内オートファジー 活性化を確認しており、より効果的な投与法や 投与物質を検討した。活性化のスクリーニング にはオートファゴソーム膜可視化マウス (GFP-LC3 マウス)を用い、病態への効果は シヌクレイン過剰発現 TGマウス (SNCA-TG) お よび対照マウスを用いて、病理学的、生化学的 および行動学的に解析した。

### C. 研究結果

1. タンパク質分解システム関連分子群の病理 学的解析

オートファゴソーム膜形成の調節分子として 働く AMBRAI は、異常シヌクレインと病理学的 に密接に関連していることを見出した。さらに、 AMBRAI はリン酸化 α シヌクレインと病理学的、 生化学的に一致した挙動を示した。また、我々 は今回、MSA の軟膜下アストロサイトにリン酸 化 α シヌクレインの蓄積を認めた。これらは p62、ユビキチンまたは Gallyas-Braak 染色に 陰性であり、線維性凝集を形成していないとい う点で、MSA における既知の封入体 (グリア細 胞および神経細胞)とは組織学的に異なってい ることを見出した。

2. マウスを用いた『オートファジー活性化』の 生化学的、病理学的解析

マウスに天然二糖のトレハロースを経口投与 すると、効率よく脳内のオートファジーを誘導 する(前年度に報告)。しかし、脳内のどこで オートファジーが誘導されているのかは不明 であった。そこでオートファジー可視化マウス を用いて検討を行った。パラフィン切片および 凍結切片でオートファジーのシグナル(GFPお よびLC3 染色)を比較したところ、凍結切片に おけるシグナルの方がより明瞭であった(図 1)。特に、海馬および大脳皮質の錐体細胞に おいて、オートファジーのシグナルが顕著であ った。トレハロース給水投与の効果を検討した ところ、前述した領域において、特に神経細胞 の樹状突起におけるシグナルの増強が確認で きた。



<u>図1 パラフィン(A, B)</u>及び凍結切片(C)に おけるオートファジーシグナル(GFP-LC3)の 比較 パラフィン切片における GFP 抗体(A) と LC3 抗体(B)による免疫染色の結果から、 LC3 抗体の方がより感度良く検出できた。一方、 凍結切片では GFP シグナル(C)を直接可視化 できるため最も感度良く検出できることを確 認した。Bars = 10 µm.

#### D. 考察

細胞内のタンパク質分解システム(オートファ ジー・リソソーム系、ユビキチン・プロテアソー ム系)はシヌクレイノパチーの病態と密接に関連 していることをこれまでに報告してきた。

そこで細胞内の分解システムの活性化を生か して病態を改善できる可能性を探ってきた。特に 天然二糖のトレハロースは脳内オートファジー を活性化し、アルツハイマー病やポリグルタミン 病のモデルマウスに対して効果がある証拠を複 数のグループが示している。これらの報告と一致 して、我々もトレハロースがレビー小体病モデル マウスの脳内オートファジーを活性化すること を報告した。しかし、トレハロースが脳内のどこ で活性化しているのかについての報告はこれま でになかった。今回、オートファジー可視化マウ スを利用することで海馬および大脳皮質の神経 細胞において、LC3 は特に高発現していることを 見出した。また、トレハロース給水投与により、 これらのシグナルが増強することに加え、樹状突 起部のシグナル増強が観察された。トレハロース によるオートファジー活性化をより増強する手 法を探索することで、シヌクレインの異常蓄積を 抑制するばかりでなく、記憶の改善も期待できる。

### E. 結論

昨年度に引きつづきシヌクレイノパチーを含

む神経変性疾患の病態解明および治療法の開発 を視野に入れ研究を遂行した。天然二糖のトレハ ロースは、これらのオートファジー形成分子を修 飾することにより、脳内のオートファジー、特に 海馬や大脳皮質の神経細胞に影響を与えている 知見を得ることができた。トレハロースはすでに 食品添加物として認可されており、安定して供給 されている。今後、トレハロースと相乗効果を示 す活性化剤を探索することができれば、治療法と して有用であり、他の神経変性疾患への応用も可 能である。

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G. 知的財産権の出願・登録状況(予定を含む)

 特許取得 なし
 実用新案登録 なし
 その他 なし

# 生体リズムの遺伝子改変マウスによる解析

# 研究代表者 岡村 均1)

### 研究分担者 崎村 建司 2)

京都大学大学院薬学研究科・医薬創成情報科学・システムバイオロジー
 新潟大学脳研究所・基礎神経科学部門・細胞神経生物学

### 研究要旨

24時間周期の生体リズムは生命にとって最も根源的な「時間」の仕組みであり、細胞の代謝や基本 機能と密接にリンクしている。哺乳類生体リズムの中枢は、脳の視交叉上核(Suprachiasmatic nucleus: SCN)である。SCN は強力なリズム信号を発し、他の脳部位や全身の細胞で発生するリズム を調律・統合している。我々は SCN に発現する遺伝子を組織化学で同定し、それがリズムに及ぼす影 響をノックアウトマウスの表現型を解析することで検定する網羅的プロジェクト(SCN-Gene Project) を推進しているが、本研究では、我々が近年見出した、SCN でリズミックに発現振動するアセチルコ リン受容体の生体リズムへの影響を、SCN 特異的ノックアウトマウスを作製することで特定する。

### A. 研究目的

網膜で受容された光シグナルは、網膜-視床下 部路を介し、体内で独自のリズムを刻む概日時計 の中枢である視交叉上核(Suprachiasmatic nucleus: SCN) に到達し、体内時計の位相を外界 の明暗周期に同調させている。この伝達の主力に なっているのは、メラノプシン含有網膜神経節細 胞―グルタミン酸受容体系であることは、よく知 られている。一方、1970年代より、アセチルコリ ン受容体作動薬であるカルバコール (carbachol)を用いた薬理学的研究が行われ、カ ルバコールの脳室内投与では行動リズムの位相 が変動するとの報告がなされた。しかし、その分 子メカニズムは未だ不明である。そこで、我々は 概日リズムを制御するアセチルコリン受容体を 特定し、カルバコールの生体リズムへの生理作用 におけるアセチルコリン受容体の役割を解明す る。

### B. 研究方法

具体的な SCN-Gene Project の実施過程は以下 の通りである。

1) 一次スクリーニング: 直径 0.5 mm の球形の 視交叉上核を顕微鏡下でパンチアウトし、 Affimetrix GeneChip Mouse Genome 430 2.0 array により、SCN での発現量を測定したところ、これ までに、12,379 個の遺伝子が SCN で発現している ことを同定した。このうち、ターゲット分子、制 御因子といった分子機能が未知の受容体を 2 次 スクリーニングへと進めた。

2) 二次スクリーニング:SCNは、0.5 mm 程度の 小核であり、上記のパンチアウト法では SCN 以外 の部分も試料に含有されるため、false positive が含まれる。そこで、in situ hybridization あ るいは免疫組織化学の手法を用い、SCN での発現 を定量形態学的に確認した。これにより、昼(CT4) と夜(CT16) で遺伝子発現の差も検出できる。こ の二次スクリーニングにより、ターゲット遺伝子 を約2000個へと絞った。

3) 三次・四次スクリーニングとして、ターゲッ ト遺伝子のノックアウトマウスを作成する。特に、 SCN 特異的 Cre マウスを用いて、SCN 特異的ノッ クアウトマウスを作成する。続いて、専用の明暗 コントロールボックスにて、個体ごとのマウスの 24 時間行動リズムを一ヶ月以上にわたって測定 し、リズム異常をきたすマウスを同定する。既に、 この手法にて、バソプレッシン V1 受容体、RGS16 のリズムへの機能を明らかにしている。

本研究で行う動物実験は全て、京都大学動物実 験委員会の承認を得ている(2015-21)。また、本 研究で行う組換え DNA実験についても、京都大学 組換え DNA実験委員会の承認を得ている(140157)。 また、研究機関等における動物実験等の実施に関 する基本指針(平成 18 年文部科学省告示第 71 号) を遵守して遂行する。

### C. 研究結果

本研究課題にて、SCN 特異的マイクロアレイを 用いて、SCN に発現する 24 時間の発現変動がある アセチルコリン受容体の候補分子、AChS01を得た。 続いて、定量的 RI in situ hybridization 法に より、SCN においてこの分子が明期にピークを迎 える明瞭な24時間リズムを示すことを見出した。 そこで、AChS01 が生体リズム形成に関与をするか どうかを検定するため、SCN 特異的な AChS01 コン ディショナルノックアウトマウスの作成を開始 した。AChS01の機能の重要と考えられる Exon を 1oxP 配列で挟んだ targeting vector を作成し、 ES 細胞へとエレクトロポレーションにより導入 した。計画通り、AChS01の遺伝子座にて相同組み 換えを起こした ES 細胞をサザンブロッティング により選別し、この変異 ES 細胞を胚盤胞へ注入 した。これにより得た、高寄与率のキメラマウス と野生型マウスを交配させ、ヘテロ変異体 F1 マ ウスを得た。F1 マウスのゲノムが、計画通り遺伝 子変異されたかを、サザンブロッティングにより 確認した。続いて、全身で flippase を発現する マウスと交配させることで AChS01-flox マウスを 得た。さらに、AChS01-flox マウスを、全身で Cre を発現するマウスとかけあわせ、全身 AChSO1-KO マウスを作成した。しかし、AChS01の全身ホモノ ックアウトマウスは成長障害がみられ、およそ生

後2週—4週で全匹死亡した。そこで、SCNでCre を発現するマウスとかけあわせ、SCN 特異的 AChS01-KO マウスを作成した。SCN 特異的 AChS01-KO マウスは、外見上の成長障害は確認さ れず、成体となり、繁殖能力も正常であった。

#### D. 考察

生体リズムの中枢である SCN において、大量に、 かつリズミックに発現変動するアセチルコリン 受容体 AChSO1 の SCN 特異的コンディショナルノ ックアウトマウスを作成できた。今後、この変異 マウスを用いて、AChSO1 のリズム機能を特定する。 まず、明暗環境下、恒暗条件下、時差環境下での 概日行動リズムや短時間光パルス照射に対する 反応などの時間行動学的検索を行う。また、カル バコールによる概日行動リズム位相変動の応答 性を測定する。また、SCN で AChSO1 のリズムを生 み出す転写調節領域をプロモーターアッセイに より同定する。

### E. 結論

我々は、SCN-Gene Project により同定した遺伝 子の解析を行い、SCN でリズミックに発現振動す るアセチルコリン受容体 AChS01 を、新たに同定 した。続いて、AChSO1の概日リズムにおける生理 機能を同定するために、AChS01の全身ノックアウ トマウスを作成したが、顕著な成長障害のために、 成体を得ることができなかった。そこで、我々は、 SCN 特異的 Cre マウスを利用して、SCN 特異的 AChS01 ノックアウトマウスを作成した。このコン ディショナルノックアウトマウスは、成長障害が 観察されず、成体となり繁殖も可能であった。よ って、この変異マウスを用いることにより、SCN における AChSO1 の生理的意義が解明されるもの と期待できる。さらに、カルバコールを用いて概 日行動リズムを検証することで、SCN のリズム発 振の分子機構の解明や概日リズム障害に対する 創薬が多いに期待される。

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G. 知的財産権の出願・登録状況(予定を含む)

1. 特許取得

なし

2. 実用新案登録

なし

3. その他

なし

# 大脳基底核内情報伝達におけるドーパミン神経伝達の機能の解析

### 研究代表者 南部 篤 1)

### 研究分担者 知見 聡美<sup>1)</sup>、笹岡 俊邦<sup>2)</sup>

# 自然科学研究機構 生理学研究所 生体システム研究部門 新潟大学 脳研究所 生命科学リソース研究センター 動物資源開発研究分野

#### 研究要旨

パーキンソン病では、中脳のドーパミン作動性ニューロンが変性、脱落し、重篤な運動障害、非運 動症状が生じるが、その病態生理については不明な部分が多い。ドーパミン受容体はD1様受容体ファ ミリーとD2様受容体ファミリーとに大きく分類されるが、ドーパミン受容体を無くしたノックアウト マウスの大脳基底核内情報伝達と運動症状を解析することは、パーキンソン病の病態解明につながる と考えられる。本研究においては、昨年度に引き続き、ドキシサイクリンの投与によりドーパミンD1 受容体の発現を制御することができるコンディショナルノックダウン(KD)マウスを用い、覚醒下神 経活動の記録と行動解析を行った。その結果、D1受容体を介したドーパミン神経伝達が、大脳基底核 の直接路を介した情報伝達と正常な運動の発現に必須であることが示された。これらのことから、D1 受容体を介した神経伝達の消失がパーキンソン病における無動の少なくとも一部を説明できると考え られる。

#### A. 研究目的

パーキンソン病では、中脳のドーパミン作動性 ニューロンが変性、脱落し、無動、筋強剛、振戦 などの重篤な運動障害を生じる疾患である。有病 率は10万人あたり100~150人程度と高 いが、その病態生理については、まだ不明な部分 が多い。本研究では、遺伝子改変マウスを用いる ことにより、D1受容体(D1R)を介したドーパミ ン神経伝達が、大脳基底核内情報伝達と運動制御 において果たす機能を解析することにより、パー キンソン病の病態解明とより効果的な治療法の 開発を目指す。

### B. 研究方法(倫理面への配慮を含む)

昨年度に引き続き、笹岡らが作製した D1R コンデ ィショナルノックダウン(KD)マウスを用いた。 本マウスは、D2R の発現は正常であるが、D1R の 発現をドキシサイクリン (Dox) 投与によって on/off することが可能である (Tet-Off)。

マウスに十分なハンドリングを行い馴化させた後、 ケタミン (100 mg/kg,腹腔内投与)・キシラジン (2-5 mg/kg)麻酔下において、頭部固定器具をマウスの頭 蓋骨に装着する手術を行った。また、大脳皮質運 動野に刺激電極を埋め込み留置した。手術から回 復後、神経活動の記録を開始した。

マウス頭部を固定器具により無痛的にステレ オ装置に固定し、大脳基底核ニューロンの活動を 覚醒下で記録した。ドーパミン作動性ニューロン の主な投射先は、大脳基底核の入力部である線条 体であり、線条体の直接路ニューロンはD1Rを発 現し淡蒼球内節 (GPi)に投射しているのに対し、 間接路ニューロンは D2R を発現し淡蒼球外節 (GPe) に投射している。金属記録電極を、GPi お よび GPe に刺入することにより、これらニューロ ンの自発発火の頻度とパターン、大脳皮質の電気 刺激に対する応答パターンを記録した。また、ケ ージ内における自発運動量、ロタロッドテストに よる運動機能の測定も行った。これらの計測を、 D1R が発現している状態と、ドキシサイクリン (Dox)を投与することによってD1Rの発現を抑え た状態で行った。

本研究計画は所属研究機関の動物実験委員会 の審査、承認を受けており、関連する法令、所属 研究機関の指針などを遵守して行った。動物実験 および飼養保管は、動物実験委員会の承認を受け た実験室で行った。また、本研究計画は P1A レベル の遺伝子組換え実験を含むため、所属研究機関の 組換え DNA 実験安全委員会の審査、承認を受け、 関連する法令、ならびに、これに基づいて作成された 所属研究機関の規定を遵守して行った。実験場所に 関しては、組換え DNA 実験安全委員会の審査、承 認を受けた実験室で行った。実験動物飼育中は注 意深く様子を観察し、健康状態を維持するように努め、 動物が苦痛を感じる状態が長期に亘り回復が困難な 場合には、安楽死の処置をとるようにした。

### C. 研究結果

まず、D1R が発現している状態において調べた。 自発運動量、ロタロッドの滞在時間とも野生型マ ウスと変わらなかった。GPi、GPeの神経活動を調 べたところ、大脳皮質刺激に対して、早い興奮-抑制-遅い興奮という3相性の応答パターンを 示した。また、GPi、GPeともに、約50Hzでラン ダムな自発発火を示した。これら神経活動は、野 生型マウスと同様であった。

次に、Dox を投与し D1R の発現が抑制された状 態で調べた。Dox 投与により、ケージ内の自発運 動量が減少した。また、ロタロッド上の滞在時間 は野生側マウスより短く、訓練によって増加する も、野生型マウスの成績には至らなかった。野生 型マウスに Dox を投与しても、また D1RKD マウス に Dox 非投与の場合でも、このような行動変化は 起こらなかった。GPi、GPe の神経活動を調べたと ころ、大脳皮質刺激に対して、GPi では多くのニ ューロンが興奮ー興奮という 2 相性の応答パタ ーンを示すようになり、抑制が消失していた。一 方、GPe では有意な変化は見られなかった。GPi、 GPe ともに、自発発火頻度とパターンにおいて有 意な変化は示さなかった。

Dox 投与を止めると、自発運動量も正常化した。 また、大脳皮質刺激によって GPi に誘発される反応も3相性になり、正常化した。

### D. 考察

GPi および GPe で記録される大脳皮質由来の3 相性の応答のうち、早い興奮は大脳皮質ー視床下 核-GPi/GPe 路を、抑制は大脳皮質-線条体-GPi/GPe 路を、遅い興奮は大脳皮質-線条体-GPe -視床下核-GPi/GPe 路を介して伝達されること が明らかにされている。D1R が発現していない状 態では、GPi における抑制がほとんど消失したこ とと、マウスの運動量が低下したことから、D1R を介したドーパミン神経伝達が、直接路(大脳皮 質-線条体-GPi 路)を介した情報伝達と正常な 運動の発現に必須であることが示された(図参 照)。これらのことから、D1Rを介したドーパミン 神経伝達の消失が、パーキンソン病における無動 や寡動の症状発現の少なくとも一部は説明して いると考えられる。一方、自発発射頻度、パター ンとも変化しなかったことから、これらの変化は 症状発現への寄与は少ないと思われる。



### E. 結論

D1R を介したドーパミン神経伝達が、直接路を 介した情報伝達と正常な運動の発現に必須であ る。また、D1R を介したドーパミン神経伝達が、 パーキンソン病における無動や寡動の症状発現 に寄与すると考えられる。

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# G. 知的財産権の出願・登録状況(予定を含む)

 特許取得 なし
 実用新案登録 なし
 その他
 なし

# HtrA1 欠損マウスにおける脳小血管の機能解析

# 研究代表者 猪原 匡史 1)

研究分担者 齊藤 聡<sup>2)</sup> 山本 由美<sup>2</sup>) 上村 麻衣子<sup>3</sup>) 小野寺 理<sup>4</sup>)

- 1) 国立循環器病研究センター 脳神経内科
- 2) 国立循環器病研究センター 再生医療部
- 3) 京都大学大学院医学研究科 臨床神経学
- 4) 新潟大学脳研究所 神経内科分野

### 研究要旨

CARASIL (Cerebral Autosomal Recessive Arteriopathy with Subcortical Infarcts and Leukoencephalopathy) の原因として *HtrAI* 遺伝子変異が同定されている. 今回我々は,新潟大学脳研 究所で作りだされた *HtrAI* 遺伝子欠損 (*HtrAI* KO) マウスについて, CARASIL モデルマウスとしての有 用性を検証した. その結果,組織学的解析や行動解析では有意な異常は認められなかったが, *HtrAI* KO マウスは野生型マウスに比して,脳血流量の減少と,脳循環予備能の異常が示された.

HtrA1 KOマウスは CARASIL や認知症の病態解析に有用なモデルであると考えられた.

### A. 研究目的

脳血管障害はわが国における三大死因の一つ であると同時に、認知症による要介護・寝たきり 状態の最大の原因である.近年、血管病変のアル ツハイマー病への関与も示唆され、血管性認知症 への関心も高まっている.しかし、血管性認知症 研究の障壁となるのが、その多くが孤発性である が故の危険因子あるいは病型の多様性である.そ こで、単一遺伝子疾患 CARASIL を突破口に血管性 認知症の病態を明らかにすることを目的として、 本研究を開始した.

### B. 研究方法(倫理面への配慮を含む)

### 1. 実験動物

新潟大学脳研究所で作りだされた HtrA1 遺伝 子欠損(HtrA1 K0)マウスと C57BL/6Jマウスを 使用した.すべての動物実験は国立循環器病研 究センター動物実験管理委員会で審査され,承 認された内容である.

#### 2. 脳血流量の測定

16ヶ月齢の *HtrA1* K0 マウスおよび野生型マウ スを解析した. 脳血流はレーザースペックル血 流計 (Omegazone-2; Omegawave 社, 日本) にて測 定した. 麻酔は 2%イソフルレンで導入し, 1.5% イソフルレンで維持した. bregma から 2mm 外側, 1mm 後方を中心とした直径 1mm の円を関心領域と して設定し, 左右の測定値の平均値を記録した.

### 3. 脳循環予備能の評価

16 ヶ月齢の *HtrA1* K0 マウスおよび野生型マウ スを解析した.マウスの麻酔は α-chloralose (50 mg/kg)および urethane (750 mg/kg)の腹腔内 投与で行った.気管内挿管の上,5%二酸化炭素を 10 分間吸入させ、レーザースペックル血流計にて 脳血流を継時的に測定した.

### <u>4.</u> 行動解析

24 ヶ月齢の HtrA1 KO マウスおよび野生型マウ

スについて、Y 迷路試験を行い、総進入回数と自 発的交替行動数を評価した.

### 5. 組織学的解析

*HtrA1* KO マウスおよび野生型マウスについて, H&E 染色, KB 染色および抗 BMP-4 抗体を用いた免 疫染色を施し, 解剖学的評価を行った.

### C. 研究結果

#### 1. 脳血流量の測定

*HtrA1*K0マウスは野生型マウスに比して有意に 脳血流量が減少していた(p < 0.05).

### 2. 脳循環予備能の評価

 $\alpha$ -chloralose およびurethane 麻酔下では、炭酸ガス投与前のベースラインの脳血流量について、*HtrA1*K0マウスと野生型マウスの間で有意な差異を認めなかった.一方、炭酸ガスの投与後、*HtrA1*K0マウスは、野生型マウスに比して、脳血流量の増加が鈍化しており、脳血管反応性の異常が示唆された(p < 0.05).

#### 3. 行動解析

*HtrA1* K0 マウスは野生型マウスに比べ,Y迷路 試験での総進入回数が少なかったが,作業記憶 の指標である自発的交替行動数には有意差がな かった.

#### 4. 組織学的解析

H&E 染色等の一般染色や抗 BMP-4 抗体等の免疫 染色では *HtrA1* KO マウスと野生型マウスの間で 有意な差異を認めなかった.

#### D. 考察

2009年,新潟大学で CARASIL の原因遺伝子 HtrA1 遺伝子が同定された.その後,欧州や中国 など世界各国で CARASIL の報告が相次ぎ,本疾患 は孤発性の血管性認知症全体を含む難治性血管 障害疾患の病態解明や治療法開発に与すること が期待され,近年ますます注目を集めている.

CARASIL における脳梗塞の発症機序は未だ不明 であるが,病理学的に血管壁細胞の変性が報告 されており,血管反応性の異常が本疾患の病態 機序に深く関連していている可能性が考えられている.本研究においても,*HtrA1*K0マウスでは脳血流量の減少に加え,血管反応性の障害が認められた.

本研究では *HtrA1* K0 マウスが CARASIL の病態 を再現していることが確認され, CARASIL の治療 法開発に有用なモデル動物となりうる可能性が 示された.また本モデルは CARASIL にとどまらず, 認知症の病態解明にも有用であり,さらなる研 究が必要と考えられた.

### E. 結論

*HtrA1*K0マウスはCARASILや認知症の病態解析 に有用なモデルであると考えられた.

### F. 研究発表(上記課題名に関するもの)

1. 論文発表 なし.

### 2. 学会発表

なし.

### G. 知的財産権の出願・登録状況

 特許取得 なし.
 実用新案登録 なし.
 その他 なし.

# 哺乳類中枢神経系における神経回路形成の遺伝学的解析

# 研究代表者 岩里 琢治1),2)

研究分担者 笹岡 俊邦 3) 研究分担者 香取 将太 1)

1) 国立遺伝学研究所形質遺伝研究部門
 2) 総合研究大学院大学生命科学研究科遺伝学専攻
 3) 新潟大学脳研究所

### 研究要旨

マウス遺伝学を用いた研究によって哺乳類の中枢神経系回路形成のメカニズムを解明することは、 ヒトの脳高次機能の基盤を理解するために重要である。本課題では、細胞骨格制御に重要な Rho ファ ミリーの一つである Rac に特異的な GTPase activating protein (GAP)である αキメリンに焦点を当 てて研究を行っている。昨年度までの研究において、様々なタイプの αキメリン変異マウスを作製し、 それらのマウスの包括的な行動解析を行うことにより、 αキメリンの α 2イソフォーム (α 2キメリ ン)がある種の海馬依存的学習において重要な働きをすることを明らかにした。今年度はその回路レ ベルの基盤について解析を行った。

### A. 研究目的

昨年度までの研究により、RacGAP  $\alpha$  キメリンの  $\alpha$  2型イソフォーム( $\alpha$  2キメリン)を発達期に 欠損したマウスではある種の海馬依存的記憶(文 脈型恐怖条件付けなど)に亢進が見られるが、成 体になってから欠損したマウスは正常レベルの 記憶を示すことを報告した。すなわち、発達期の  $\alpha$  2キメリンが成体での海馬依存的記憶の能力 を調節していることが明らかになった。本年度は、 その神経回路レベルの基盤を明らかにすること を目的に解析を行った。

### B. 研究方法(倫理面への配慮を含む)

各種のαキメリン変異マウス(α1キメリンお よびα2キメリンの全身性ノックアウトマウスと 各種の条件的ノックアウトマウス)を用いて、ス パインに特に注目して詳細な形態学的解析を行 った。 組換えDNA実験は、法律と所属機関の規程に従 い、機関長に承認された計画に沿って行った。動 物を使用する実験は、文科省の定める指針と所属 研究機関の規程に従い、機関長に承認された計画 に沿って行った。特定化学物質等の取り扱いにつ いては、所属研究機関の規程に従い使用した。研 究に用いる実験動物・試薬などの提供に関する MTAは提携済みである。

### C.研究結果と考察

これまでの我々の研究によって、α2キメリン が皮質脊髄路の回路形成において軸索誘導を制 御していることが明らかになっている(Iwasato et al., Cell 2007)。同様のことが海馬でも起き ている可能性がある。それを検証する目的で、終 脳(海馬、大脳皮質)特異的にα2キメリンを欠 損するマウス(DT-α2ChnKOマウス)の海馬にお

いて、軸索投射パターンを解析したが、それらに 顕著な異常が見られなかった。また、樹状突起の 形態にも顕著な異常はみられなかった。

一方、αキメリンがスパイン形成に関与してい るという報告はこれまでになかったが、アクチン 細胞骨格を制御するという性質から考えて、αキ メリンがスパイン形態に影響する可能性がある。 それを検証する目的で、スパイン形成を解析した ところ、3 週齢において、 α 2 キメリン変異マウ スの海馬 CA1 の錐体細胞のスパインは、コントロ ールマウスのものと比較して、大きいことがわか った。ただし、密度には違いがみられなかった。 成体で解析したところ、大きさも密度もどちらも コントロールマウスのものよりも有意に増加し ていることが明らかになった。一方、α1キメリ ンノックアウトマウスではこうした異常は見ら れなかった。これらの結果によって、α2キメリ ンがスパイン形態を制御していることが初めて 明らかになった。

興味深いことに、成体になってからα2キメリ ンを欠損した場合ではスパインは正常であった。 すなわち、発達期のα2キメリンが成体における スパイン形態を制御していることがわかった。さ らにα2キメリンの作用する時期を特定する目 的で、生後10日目のα2キメリン floxed マウス に Cre 組換え酵素を発現する AAV ウイルスベクタ ーをインジェクションすることによって、海馬特 異的に *α* 2 キメリンをノックアウトしたところ、 3 週齢においてスパインのサイズの増大が見られ た。なお、2週目以降はスパイン成熟が活発に起 きる時期であり、α2キメリンがスパイン形態形 成に重要な働きをすることがさらに確認された。

なお、海馬への AAV ベクター導入によって生後 10日目以降の海馬特異的にα2キメリンをノック アウトしたマウスでは、記憶の亢進も見られた。 一方、成体になってからα2キメリンをノックア ウトしても記憶は正常である。

すなわち、発達期のα2キメリンが海馬のスパ イン形態を制御することによって成体での記憶 能力を制御している可能性が強く示唆された。

遺伝子変異マウスを用いた神経回路レベルの 脳高次機能研究に造詣の深い研究分担者の笹岡 教授との議論の機会を得られたことは本研究の 推進に有効であった。

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T. Iwasato.

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E. 知的財産権の出願・登録状況(予定を含む) 特になし

# ヒトてんかん原性脳組織における酸化損傷タンパク質の網羅的探索

### 研究代表者 島田 厚良1)

### 研究分担者 古川 絢子 2)、千葉 陽一 3)、柿田 明美 4)

1) 愛知県心身障害者コロニー 中央病院 中央検査部

2) 鈴鹿医療科学大学 薬学部

- 3) 香川大学 医学部
- 4) 新潟大学脳研究所

### 研究要旨

内側側頭葉てんかんに代表されるてんかん原性脳組織では、繰り返すけいれん発作に伴う興奮毒性 によって神経細胞死が生じる。興奮毒性による神経細胞死のメカニズムには酸化ストレスが関与する と報告されているが、てんかん焦点においてどのようなタンパク質が酸化損傷を受けるかは不明であ る。本研究は、内側側頭葉てんかんで外科的切除手術適応となった症例のうち、海馬硬化が認められ る症例と、海馬硬化が認められない症例を比較し、神経細胞死が顕著で海馬硬化をきたした海馬組織 において酸化損傷を受けたタンパク質の同定を行った。組織から抽出したタンパク質サンプルを二次 元電気泳動にて展開し、タンパク質酸化損傷の指標のひとつであるカルボニル化を検出する抗体を用 いて、酸化損傷タンパク質をウェスタンブロットにて検出した。定量解析の結果、海馬硬化組織にお いて共通して酸化損傷を受けるタンパク質スポットが3個検出された。従って、てんかん原性脳組織 においては、酸化ストレスによって特定のたんぱく質が損傷され、機能低下をきたすことが病態形成 に関与するものと考えられる。現在、3個のスポットについて、飛行時間型質量分析装置を用いたタ ンパク質同定を試みている。

### A. 研究目的

興奮毒性による神経細胞死は、新生児では低酸 素性虚血性脳症や新生児仮死において認められ る。この急性期の障害を脱した後は、重篤な神経 学的後遺症を引き起こす事が知られている。興奮 毒性による細胞死の原因のひとつとして、酸化ス トレスの関与が示唆されている。我々は興奮毒で あるカイニン酸を投与した3週齢ラットの海馬 において、投与3時間後に特定のタンパク質に酸 化損傷が生じる事を報告した(Neurobiol Dis. 43(3):706-714, 2011)。内側側頭葉てんかんに代 表されるてんかんの焦点となる脳組織において は、繰り返すけいれん発作に伴う興奮毒性によっ て、組織は慢性的な興奮毒性に曝されていると考 えられ、タンパク質などの生体高分子が酸化損傷 を受けている可能性がある。しかし、どのタンパ ク質が特異的に酸化されるかなどは明らかでは なく、酸化ストレスが興奮毒性に起因する神経細 胞死に果たす役割は不明な点が多い。本研究では、 薬剤抵抗性の内側側頭葉てんかん患者から治療 目的で外科的に切除された脳組織を用い、酸化損 傷タンパク質の網羅的解析を行って、てんかん焦 点において特異的に酸化傷害を受けるタンパク 質を同定することが目的である。てんかん原性病 態形成において中心的となり得る分子をタンパ ク質損傷の観点から同定し、酸化ストレスが神経 細胞死に果たす役割を解明することによって、て んかんの病態解明に繋がる基礎的知見を得たい。

B. 研究方法(倫理面への配慮を含む)

本研究はヒト外科切除脳組織を使用するため、 ヘルシンキ宣言を忠実に遵守し、「臨床研究に関 する倫理指針」に従って進めている。また、愛知 県心身障害者コロニー発達障害研究所「ヒトおよ びヒト材料を対象とする研究」倫理委員会の承認 を得て行っている。

研究には、内側側頭葉てんかん患者で海馬硬化 が認められた症例(グレードIV)と、海馬硬化が 認められなかった症例(グレード0/I)のそれ ぞれから得られた外科切除脳組織を用いた。各症 例から病変対象部位として海馬 CA1 領域、基準部 位として側頭葉新皮質を切り出した。各組織から タンパク質を抽出した。タンパク質酸化損傷の指 標のひとつであるカルボニル化タンパク質の生 成を比較定量し、海馬硬化を呈する海馬組織で強 く酸化損傷を受けるタンパク質を探索した。次い で、飛行時間型質量分析装置を用いて、タンパク 質同定を試みた。

(1)カルボニル化タンパク質の検出

まず、タンパク質の酸化損傷により生成したカ ルボニル基に対して、2,4-ジニトロフェニルヒド ラジン(DNPH)を反応させ、タンパク質結合 2,4-ジニトロフェニルヒドラゾン(DNP)に誘導体化し た。2次元電気泳動法により等電点および分子量 の差に基づいてタンパク質を分離し、PVDF 膜に転 写した後、DNP を特異的に認識する抗 DNP 抗体を 用いたウェスタンブロット法を行って酸化損傷 タンパク質を検出した。

(2) タンパク質発現変動の解析

抽出したタンパク質を蛍光色素で標識し、2次
元電気泳動法にて分離した(2D-DIGE法)。蛍光イメージャーにて電気泳動像を取得し、
ImageMaster 2D Platinum 7.0を用いて各タンパク質の発現量を定量解析した。
(3)タンパク質の同定

(1)、(2)の結果に基づいて絞り込んタンパク質 スポットをゲルから切り出し、トリプシン消化の 後、脱塩・濃縮を行ってサンプルとした。質量分 析(MS)により得られたペプチドピークリストを データベース検索するペプチドマスフィンガー プリント(PMF)法および、MS/MS 解析によるアミノ酸配列決定によりタンパク質を同定した。

### C. 研究結果

昨年度までの結果に新たな症例を加え、グレー ド0/Iを13症例、グレードIVを15症例として 解析を進めた。カルボニル化タンパク質を比較し た結果、海馬硬化が認められる海馬では、3つの タンパク質スポットが強くカルボニル化されて いた。現在、これらのタンパク質の同定を試みて いるが、まだ同定には至っていない。

タンパク質発現変動解析については、グレード 0/I、グレードIVをそれぞれ10症例ずつ用いた。 定量解析の結果、海馬硬化が認められる海馬にお いて発現が有意に増加したタンパク質が8個、減 少したタンパク質が24個検出された。それぞれ についてタンパク質同定を進めている。これまで に、発現が増加したタンパク質として GFAP を、 減少したタンパク質として熱ショックタンパク 質のいくつかを同定した。他のスポットについて も同定を進めている。

#### D. 考察

本研究から、内側側頭葉てんかんにおいて、海 馬硬化が認められる海馬組織で特異的に酸化損 傷を受けるタンパク質の存在が明らかになった。 このような酸化損傷の蓄積はタンパク質の機能 変化をもたらす可能性があり、酸化ストレスがて んかん焦点形成過程において何らかの役割を果 たしていると考えられる。海馬硬化の海馬におい て GFAP の発現が増加したことは、海馬硬化に伴 うグリオーシスを反映していると考えられる。一 方、熱ショックタンパク質は、ストレスに応答し て発現誘導されるが、その発現が減少していたこ とから、海馬硬化組織ではストレスに対する応答 性が低下している可能性がある。

### E. 結論

本研究から、海馬硬化が認められる海馬組織に おいて特異的に酸化されるタンパク質の存在が 明らかになった。タンパク質酸化損傷はリジンを 含む塩基性アミノ酸残基が標的となることから 翻訳後修飾と競合することも考えられる。従って、 酸化ストレスがこれらのタンパク質に何らかの 機能異常を引き起こす可能性が考えられる。今後、 酸化損傷タンパク質を同定することにより、海馬 硬化へと至る病変形成の過程における酸化スト レスの役割が解明できると予想する。

F. 研究発表(上記課題名に関するもの)

1. 論文発表

なし

2. 学会発表

第57回日本新規得病理学会(平成28年6月2日、 弘前市)において発表の予定。

### G. 知的財産権の出願・登録状況(予定を含む)

1. 特許取得

なし

2. 実用新案登録

なし

# optineurin タンパク質の研究

研究代表者 川上 秀史 <sup>1)</sup>

研究分担者 大澤 亮介 1) 柿田 明美 2) 鈴木 秀規 3) 高橋 均 4)

1)広島大学原爆放射線医科学研究所 分子疫学研究分野
 2)新潟大学脳研究所 生命科学リソース研究センター 脳疾患標本資源解析学
 3)広島文教女子大学 人間科学部 人間栄養学科
 4)新潟大学脳研究所 病態神経科学部門 病理学分野

#### 研究要旨

筋萎縮性側索硬化症 ALS の原因遺伝子の一つである Optineurin(OPTN)が、神経変性の発症にどのように機能しているかを明らかにする為に実験を行った。TBK1 と OPTN の遺伝子変異が FTD-ALS の患者で見つかっていることから、TBK1 と OPTN の機能について、神経変性の原因のひ とつとして考えられる傷害ミトコンドリアの除去(マイトファジー)との関わりについて調べた。

# A. 研究目的

ALS 患者と健常者の脳組織で起こっている変 化を生化学的に明らかにし、ALS 患者特異的に生 じる ALS 原因遺伝子 Optineurin タンパクの生化 学的な変化を明らかにすることで ALS の発症機 序を明らかにする。

### B. 研究方法(倫理面への配慮を含む)

HeLa 細胞に Optineurin および TBK1 をトランス フェクションして、CCCP でミトコンドリアに傷害 を与えてから Optineurin タンパクに変化が生じ るかどうかを調べた。

### C. 研究結果

HeLa 細胞に FLAG-OPTN および FLAG-TBK1 をトランスフェクションして、 20µMCCCP 刺激、3 時間および 6 時間後に細胞 を回収して、サンプルを調製した。前年度の報告 にあるようなヒトの脳のサンプルで認められる ような分子量の小さい OPTN 断片は認められな いものの、TBK1存在下でOPTNタンパクの分解 が促進されることが明らかとなった。(図1)



TBK1 と OPTN を共に発現させると 177 番目の セリン残基(S177)をリン酸化すること(図2)、 さらに S177 のリン酸化がオートファジーの際に LC3 との結合を促進することがこれまでに知ら れていることから、S177 のリン酸化がマイトフ ァジーの亢進に関わっていると考えられる。



# WB: anti-pSer177

(図2)

### D. 考察

ALS の発症機序として傷害ミトコンドリアの除 去の異常が可能性の1つとして考えられるが、そ の過程において TBK1 と OPTN が関わっている可能 性が示唆された。TBK1 の欠損もしくは変異体、 OPTN の欠損または変異体を発現した細胞でマイ トファジーの進行に異常があるかどうかを確認 する必要がある。

### E. 結論

細胞内における OPTN、TBK1 の働き、またこ れらの分子のヒト脳組織での状態について更な る解析、検討が必要である。

- F. 研究発表
- 1. 論文発表
- なし
- 2. 学会発表
  - なし

### G. 知的財産権の出願・登録状況

- 1. 特許取得
- なし
- 2. 実用新案登録
- なし
- 3.その他

# 脳小血管病変モデルにおけるスタチンの脳組織保護効果

### 研究代表者 仁藤 智香子 1)

研究分担者 五十嵐 博中 2)

1) 日本医科大学大学院医学研究科 神経内科学分野<sup>2)</sup> 新潟大学脳研究所統合脳機能研究センター

#### 研究要旨

脳小血管病変は脳梗塞ないしは脳出血の大きなリスクファクターとなるのみならず、認知機能や遂 行機能に多大な障害を惹起する。しかし、現時点では、その進展防止法としてはリスクファクターで ある血圧の是正があげられるのみで、これとて一度出現した病理変化、徴候の進展にたいする抑制効 果は小さいのが現状である。一方、臨床に於いて既に汎用されている dyslipidemia を是正する薬剤で あるスタチンが脳血管保護作用を有しており、急性期脳血管障害に対して脳組織保護的に働くという 知見が得られている。本実験では脳微小血管障害モデルである SHR ラットにスタチンを投与し、その 脳組織保護作用を、血液脳関門障害、脳循環障害の観点から磁気共鳴イメージング(MRI)を用いて評 価する。

### A. 研究目的

脳小血管病変に対して、スタチンが病変進展抑制 効果を有するか否かを動物実験において評価する。 具体的には、脳微小血管モデルラットにスタチンを連 続投与し、微小血管病変出現、および小血管病変の マーカーである血液脳関門障害の抑制作用が認め られるかを MRI にて評価する。

B. 研究方法(倫理面への配慮を含む)

矢守らにより開発された高血圧自然発症ラット (Spontaneously Hypertensive Rat: SHR)は若年時よ り体血圧の上昇が認められ、自然経過に於いては脳 に微小出血、微小梗塞、白質病変などの脳微小血 管病変を呈する。

実験にはこのラットを用いる。生後8週齢のSHRを 次の4群に分けて3か月間治療介入を行う。

- 1. Vehicle 投与群(対照群)
- カルシウム拮抗剤投与群:従来、唯一微小血管 病変の進展抑制作用があると考えられる降圧剤 としてカルシウム拮抗剤投与を行う。
- 3. アトルバスタチン投与群:微小血管保護効果があ ると考えられるストロングスタチンの投与群であ

る。

4. カルシウム拮抗剤、アトルバスタチン併用群

上記に関して、治療介入後、日本医科大学設置の 動物用MRI装置を用い、脳循環、血液脳関門障害、 炎症性蛋白、活性酸素、脳血管障害の評価を経時 的に生体にて評価する。

- 脳循環障害:CASL 法による脳血流量絶対値の 算出により各動物の数値比較を行う。
- 血液脳関門障害:DCE 法により、Gd-DTPA の 脳組織への漏出を2 compartment modelを用い て modeling することにより「漏出し易さ」を数値化 し、これをもって血液脳関門障害の指標とする。
- 3. 微小出血・梗塞の出現頻度:T2\* image および T2 image により脳梗塞、脳出血病巣の有無の評 価を行う。
- 活性酸素の産生:ラット脳切片を作成し、ハイドロ エチジン法を用いて活性酸素の産生を評価す る。

得られたデータの画像化および、評価条件の最適

化、数値化は新潟大学脳研究所、統合脳機能研究 センターに設置された画像処理装置にて行う。 得られた数値化データより4群間において微小血管 障害の状態の差異を検討し、アトルバスタチン投与 は脳微小血管障害抑制効果を有するか、および降 圧効果を示さない用量のカルシウム拮抗剤との併用 で相乗効果が認められるかを検証する。

### C. 研究結果

カルシウム拮抗剤群、アトルバスタチン群および両 者併用群では、血管内皮における LOX-1 および MCP1 の発現は対照群に比べて有意に抑制されて いた。また、併用群では、カルシウム拮抗剤群および アトルバスタチン単独群に比して、LOX-1、MCP1 と もに有意な陽性率の低下を認め、活性酸素の産生も 有意に抑制されていた。また、アトルバスタチン投与 は対照群と比較し、有意に脳血管障害(脳梗塞、脳 出血)の発生を抑制し、生存日数を有意に延長させ た。

### D. 考察

脳小血管病変モデルを用いた研究において、自 然経過に於いては、病理組織像にて血管内に投与 した Evans-blue の脳実質内への漏出、小血管の破 綻による微小出血の出現など血液脳関門の障害・破 綻を示唆する病変が月齢を経ると共に増加し、更に は脳梗塞病変が出現することが確認されている。

今回、SHR においては、低用量スタチンに加えて 降圧効果を示さない用量のカルシウム拮抗薬を長期 併用投与することにより、抗酸化および抗炎症作用 が増強され、血管内皮細胞保護に働く可能性が示唆 された。

### E. 結論

ラット脳小血管病変モデルにおいて、スタチンは抗酸化および抗炎症作用により血管障害の発生を有意に抑制した。さらに、カルシウム拮抗薬との併用により血管内皮保護効果が増強され、生存期間を有意に延長させたものと考えられた。

F. 研究発表

1. 論文発表 なし

よし

# 2. 学会発表

なし

G. 知的財産権の出願・登録状況

特記すべきことなし

# 神経変性疾患におけるアクアポリン(AQP)および AQP 関連タンパクの解析

### 研究代表者 星 明彦 1)

研究分担者 角田 綾子1)、宇川 義一1)、他田 真理3)、西澤 正豊2)

研究協力者 柿田 明美 3)

1) 福島県立医科大学神経内科 2) 新潟大学脳研究所神経内科 3) 新潟大学脳研究所病理

### 研究要旨

本年度は多系統萎縮症 (MSA) 剖検脳における AQP および AQP 関連タンパクを含めたアストロサイト の病理学的解析を進めた。MSA 群の線条体では GFAP あるいは S100 β 陽性アストロサイトーシス像と共 に AQP4 および AQP1 の発現は対照群よりも高度であった。一方、AQP 関連タンパクである Kir4.1 およ び GLT-1 は、MSA 群において対照群よりもそれぞれの発現が増強と減弱を示していた。グルタミン代 謝酵素である GS や GDH 発現は MSA 群においていずれも対照群より低下していた。MSA-P 群の方が MSA-C 群より上述の病理学的変化は顕著な傾向であった。今回の結果は、MSA 脳の神経変性プロセスにおい てアストロサイトを介した水・カリウム代謝異常およびグルタミン酸神経毒性が関与する可能性を示 唆するものと考えられる。

### A. 研究目的

我々は、これまでアルツハイマー病(AD)、パ ーキンソン病(PD)、多系統萎縮症(MSA) 剖検脳 で有意な AQP および AQP 関連タンパクの発現変化 を免疫組織学的に確認し、神経変性疾患における これらの役割について考察してきた(Hoshi et al., J Neuropathol Exp Neurol 2012; Hoshi et al., Brain Pathol 2016)。

一方、近年脳内リンパ流を制御する Glymphatic system 仮説 (Nedergaard, Science, 2013)の知 見が集積されつつあり、アストロサイトに発現す る AQP4 がその中心的役割を担うことが判明して きた。我々がこれまで示してきた AQP および AQP 関連タンパクの解析結果は神経変性疾患の Glymphatic system 破綻を示唆し、疾患発症や変 性過程に関与する可能性が考えられる。

本年度は、MSA 剖検脳における AQP および AQP 関連タンパクを含め、アストロサイトに特異的に 発現する分子群の病理学的解析をさらに進めた。

#### B. 研究方法

MSA 群 (n=8) は MSA-C (n=4) と MSA-P (n=4) の 2 群に分類し、線条体 (尾状核、被殻) をター ゲットとした。免疫組織学的に線条体での GFAP、 S100 β、AQP4、AQP1、内向き整流性カリウムチャ ネル Kir4.1、グルタミン酸トランスポーター GLT-1、グルタミン合成酵素 (GS) およびグルタ ミン酸脱水素酵素 (GDH) 発現について対照群 (n=5) と比較し、さらに glial cytoplasmic inclusion (GCI) の多寡との関連性にも着目し 解析を行った。

### C. 研究結果

MSA 群では対照群よりも GFAP あるいは S100 $\beta$ 

陽性アストロサイトーシス像と共に AQP4 と AQP1 発現の増強が認められた。

一方、AQP 関連タンパクである Kir4.1 と GLT-1 免疫染色性については、MSA 群において対照群よ りもそれぞれの発現が増強と減弱を示した。グル タミン代謝酵素である GS や GDH 発現は MSA 群に おいていずれも対照群より低下傾向であった (Figure)。

サブグループの解析結果としては、MSA-P 群の 方が MSA-C 群より上記の変化は顕著であり、また 尾状核よりも被殻でその傾向が強かった。

なお、α-synuclein に標識される GCI は MSA-C 群よりも MSA-P 群の方が、またそれぞれの群にお いて尾状核よりも被殻において高度に蓄積して いた。



#### D. 考察

MSA 脳における詳細なアストロサイト特異的蛋白の解析は検索し得た範囲では渉猟できず、AQPの動態やグルタミン酸代謝、さらにはこれらとGCI 沈着との関連性について注目した研究もない。

今回の結果は、MSA 脳で AQP やその関連タンパ クあるいはグルタミン酸代謝酵素が有意に発現 変化することを示している。我々はこれらの変化 と GCI の蓄積程度の軽重に何らかの相関性がある ことを想定しており、現在定量的なデータ解析を 進めている。これらのアストロサイト特異的蛋白 の病理学的変化は、MSA における GCI 病理を修飾 する可能性があるものと考えられる。

### E. 結論

MSA の神経変性プロセスには水・カリウム代謝 異常およびグルタミン酸神経毒性が関与する可 能性がある。これらを制御するアストロサイトに 特異的に発現する分子群の解析を進めることに よって、新しい観点で MSA の神経変性メカニズム を解明することができるかもしれない。

# F. 研究発表

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G. 知的財産権の出願・登録状況(予定を含む)

- 1. 特許取得
- なし
- 2. 実用新案登録
- なし
- 3. その他
- なし
# 多系統萎縮症剖検脳における TPPP(p25α) 蛋白の発現解析 およびその制御下流遺伝子の探索

研究代表者 石川 欽也 1)

**研究分担者** 柿田明美 2)、他田真理 2)、高橋均 2)、水澤英洋 1)、

横田隆徳1)、太田浄文1)、尾崎心1)

1) 東京医科歯科大学大学院脳神経病態学分野 2) 新潟大学脳研究所脳疾患標本資源解析学分野

#### 研究要旨

多系統萎縮症については、オリゴデンドログリア特異的タンパクである Tubulin polymerization promoting Protein (TPPP)の多系統萎縮症における病態への関与、細胞内局在変化を解析し、それらを 他のオリゴデンドログリア変性を来たす疾患との相違について検討する。また、非中枢神経疾患患者 の標本および凍結脳組織を用いて、対照状態との違いを探索する。加えて、病理学的変化をもたらす 根本原因を解明するためにコントロール例と多系統委縮症例の凍結脳での網羅的遺伝子解析も行った。

#### A. 研究目的

多系統萎縮症はオリゴデンドログリア(以下 ODG)の変性が原因となる孤発性脊髄小脳変性症 である。ODG 特異的蛋白である TPPP が示す TPPP の細胞内局在異常に着目し病理学的解析を用い て多系統萎縮症においての細胞内局在変化と病 態への関与を明らかにすること、多系統萎縮症で 生じている脳白質での網羅的遺伝子発現解析を 行い根本的な多系統萎縮症発症の原因を解明す ることを目的とする。

#### B. 研究方法(倫理面への配慮を含む)

非中枢神経疾患患者(コントロール疾患)の剖 検脳を抗 TPPP 抗体を用いた免疫組織化学にて解 析し脳部位ごとに TPPP 細胞内局在の正常パター ンを分析する。さらに多系統萎縮症患者ではコン トロールと比較してどのような局在変化を来し ているかを解析する。TPPP の局在について、GCI の主たる構成タンパクであるαシヌクレインの 分布との関係についても、リン酸化αシヌクレイ ン抗体と TPPP 抗体との蛍光2重染色を行い、検 討する。また MSA におけるミトコンドリア異常に ついて検討するために TOMM20 抗体を用いて蛍光 染色を行なった。

定量的評価を行うために多系統萎縮症および コントロール凍結脳を用いて脳部位ごとの TPPP 局在の量的変化をイムノブロッティング法とマ イクロアレイ法を用いて評価する。

上記の病理学的変化をもたらす根本原因を明 らかにするために凍結脳を用いた網羅的遺伝子 発現解析(マイクロアレイ)を行い、TPPP 制御下流 遺伝子の探索を行う。

(倫理面への配慮)

公表された患者剖検脳を用いた染色結果には 本人を特定できる情報はない。

#### C. 研究結果

これまで TPPP はオリゴデンドログリアの細胞 質にのみ存在するとされていた。東京医科歯科大 学脳神経病態学研究室にて独自に感度、特異度と もに優れた抗 TPPP 抗体を作製した。その抗体を 用いた解析では TPPP はオリゴデンドログリアの 細胞質だけではなく核質および核膜周囲にも免 疫反応が強く認められた。MSA 患者脳を用いた蛍 光 2 重染色ではαシヌクレイン陽性の GCI を形 成する ODG と、GCI が明確でない ODG を計測し 比較したところ、GCI 陽性 ODG では、統計的に有 意に核内 TPPP が消失していた(19.6 ± 10.9 vs 48.63  $\pm$  10.37 %, p<0.05). MSA  $\tilde{C} \alpha$ -synuclein 陽性の GCI を持たない ODG とコントロール患者 脳のODG の核内 TPPP 陽性率を比較すると MSA 患 者で有意に核内 TPPP の減少を認めた(48.63 ± 10.37 % vs 62.4 ± 13.5 %, p<0.05)。MSA では αシヌクレインの沈着よりも早期に核内 TPPP が 減少していることが推測された。ミトコンドリア 関連蛋白 TOMM20、TPPP 抗体、リン酸化 α シヌク レインを用いた蛍光3 重免疫染色では、TPPP は ミトコンドリ蛋白 TOMM20 の充満で膨化した ODG 細胞体に集積し、ほぼ一致して α シヌクレインが 集積する像が見られた。このような、TPPP と TOMM20 の細胞質内集積は、 *α* シヌクレイン沈着 前の細胞にも見られた。

また、マイクロアレイ解析では、大脳白質での 検索で変性が生じる早期の状態の遺伝子発現プ ロファイリングを行った。結果として興味深い遺 伝子を同定した。現在、検体数を増やして意義を 確認中である。そのうえで、培養細胞などを用い て病態への関与を探索する。

#### D. 考察

MSA の ODG において TPPP は細胞質に異常集積 することが報告されているが、その詳細は、まだ 解明されていなかった。今回の研究で我々は、 TPPP が核に多く存在することと、MSA の ODG で は核から辺縁、細胞質の中のミトコンドリアに関 連して集積することを見出した。さらに、この変 化は、GCI 形成より先行することが示唆されたが、 これはこれまでの報告の内容とも一致する。別途 我々が行った研究では、TPPP は本来ミトコンド リアにも存在する蛋白であることが示されてい る。今後この MSA でのミトコンドリア内集積が二 次的な、例えば何らかの防御的機序によって起き ているのか、あるいは、ミトコンドリアへの集積 が病態の悪化因子であるのか、検討する必要があ ると考えた。

マイクロアレイ解析については興味深い遺伝 子発現変動を同定しており、現在解析を続行中で ある。

#### E. 結論

TPPP は ODG 特異的な細胞質内蛋白として知ら れていたが、今回の研究で ODG 核にも豊富に存在 することと、MSA 脳では変性早期から核内から TPPP が減少し、細胞体へ移行しミトコンドリア 蛋白とともに集積することが判明した。

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#### G. 知的財産権の出願・登録状況(予定を含む)

 特許取得 なし
 実用新案登録 なし
 その他 なし

# 悪性脳腫瘍の非コード RNA の機能解析を基盤とした分子標的創薬の展開

# 研究代表者 山中 龍也 1,2)

研究分担者 藤井 幸彦 3)

# 京都府立医科大学・医学部・保健看護学研究科医学講座 京都府立医科大学・医学研究科・腫瘍分子標的治療学分野 3) 本 F レビ アメエウエ アメナ (タイン)

3) 新潟大学·脳研究所·脳神経外科

#### 研究要旨

中枢神経原発悪性リンパ腫(PCNSL)腫瘍組織から DNA/RNA を抽出し、mRNA, SNP アレイ、miRNA 解析を行ってきた。特に PCNSL34 症例の遺伝子発現解析から患者の予後と相関する遺伝子 21 種類を統 計学的方法を用いて選択し、それらの遺伝子の発現レベルから、患者の予後予測式を考案した。さら に、これらの中から 3 種類の遺伝子産物に対する抗体を用いた免疫組織学的解析と年齢、KPS を用い た簡便法で予後が良く予測できることが判明した。現在、高速シーケンス解析から DNA/RNA 解析を進 めているが、今後は腫瘍特異的な遺伝子異常を明らかにし、バイオマーカー開発、創薬に向けた研究 を進める予定である。

## A. 研究目的

中枢神経原発悪性リンパ腫(PCNSL)の腫瘍組 織を、次世代型シーケンサーを用いて RNA シー ケンス解析を行い遺伝子変異・再構成の異常、コ ードおよび非コード RNA の発現を検討する。以 上から疾患特異的な診断治療標的となりうる分 子を選択し、バイオマーカーの開発、および分子 標的創薬に向けた研究を展開する。

## B. 研究方法

PCNSL 対象症例について臨床サンプルと臨床 情報の収集を行った。症例の年齢、性別、 Karnofsky Performance Status (KPS)、画像所見、 髄液所見、治療法、治療効果、副作用、転帰につ いて詳細な検討を行った。PCNSL の凍結腫瘍組 織から DNA/RNA を抽出し、エクソーム, mRNA, SNP アレイ, miRNA 解析を行い、特に患者の予後と相関 する遺伝子を選択し、それらの遺伝子を標的とし た創薬への取り組み、さらには新しい診断方法の 開発に向けた研究を進めている。

2000 年~2010 年に外科的切除ないし生検術を 受けた患者34人から、PCNSL標本を得た。患者の 平均年齢は 65.5 歳(44-76 歳) であり、男性 19 人、女性 15 人であった。手術前の KPS は、80 以 上が10人、60-70が16人、50以下が8人であっ た。病理組織学的には全て瀰漫性大細胞性Bリン パ腫であった。腫瘍の診断後に、患者は高用量メ ソトレキセート単独ないし高用量メソトレキセ ートを含む多剤併用化学療法による第一選択化 学療法を受け、外照射療法(標準線量 20-40 Gy) および再発時に多剤併用化学治療を受けた。初期 治療および維持治療中の腫瘍の再発について、 MRI または CT により観察した。全症例の5年生存 率は 46.37%であった。摘出腫瘍から mRNA を抽出 L, GeneChip Human Genome U133 Plus 2.0 Expression array (Affymetrix, Inc.) (約 47,000 遺伝子を含む)を用いて各 mRNA の発現量を測定 した。これらの発現プロファイルと、追跡調査に より得た各患者の術後の生存期間との相関を Random survival forest model を用いて統計学的 に処理し、発現プロファイルと生存期間との間に 強い相関を示す遺伝子を同定した。さらに、これ

らの遺伝子の発現値を用いた予後予測法を考案 した。

現在、これらの腫瘍 RNA を用いた高速シーケン ス解析を終了し、スーパーコンピューター解析を 行っている。これらの研究は倫理委員会で承認さ れている。

#### C. 研究結果

GeneChip 解析(遺伝子発現解析)から患者の 予後と相関する遺伝子 21 種類を統計学的方法を 用いて選択し、それらの遺伝子の発現レベルか ら患者の予後予測式を考案した。高用量メソト レキセートにより治療された症例および高用量 メソトレキセートを含む多剤併用化学療法によ り治療された症例に拘わらず 21 遺伝子のセット によって予後が良く予測可能であった。21 遺伝 子を用いた予測式により分類された群について、 Kaplan-Meier 曲線を描くと Logrank 検定のため の p 値 (p) は、p<0.0001 であった。この結果 は、21 遺伝子予測式により予後を良く予測可能 であるという事を示す。

さらに、Z 式を算出した方法で、臨床データと 免疫組織化学染色の結果を考慮に入れた、より簡 略化した予後予測式を考案した。

 $\cdot$ Z<sub>2</sub> = 0. 04 × AGE + 1 × DEEP. CONTACT + 0. 82 × PPP3R1 + 0. 75 × BRCA1

 (ここで AGE は年齢値、DEEP. CONTACT とは深部病 変があれば 1,なければ 0、PPP3R1, BRCA1 とは免 疫組織化学染色で1ポイント以上なら 1,0ポイン トなら 0)

・ルール

 $Z_2 > 3.48 \implies Poor$ 

 $Z_2 \leq 3.48 \Rightarrow \text{Good }$ 

このルールに従って生存曲線を描いたものを 図に示す。 $Z_2$ 式でより簡易に予後良好群と不良群 が分けられることがわかる。 $Z_2$ 式の有用性は他の 症例群(validation set)を用いた解析によって も確かめられた。

RNA シーケンス解析は PCNSL30 症例について終 了し、現在、コンピューター解析を行っている。



#### D. 考察

がんの基礎研究の進歩から、同じ病理組織型で あってもその分子病態は個々の腫瘍ごとにかな り相違があることが明らかになってきた。そうし た背景から、がんの薬物療法はバイオマーカーを 用いて治療方法の選択がなされるようになって いる。がん治療はバイオマーカーによって効果の 期待できる症例を選別することによる個別化医 療が主流となると考えられている。

中枢神経系原発悪性リンパ腫(PCNSL)は中枢 神経系に原発する節外性非ホジキン型リンパ腫 で、多くは B 細胞リンパ腫である。PCNSL はあら ゆる年齢層に発生するが、50-60 歳代の高齢者に 好発し、その頻度は最近増加している。本邦では 現在、High dose Methotrexate(HD-MTX) 3.5 g/m<sup>2</sup> 化学療法 3 コース後、全脳放射線治療(30-40 Gy) が広く行われている。その 5 年生存率は約 30%、 生存期間中央値は 33-39.5 か月とされている。

本治療法の問題点として整理してみると、

(1) 生存率の向上が見られたが、全身性非ホジ キン病と比べ治療成績は不良である。

(2)治療効果を予測するバイオマーカーがない ため、画一的な治療が行われている。

(3) 副作用として晩発性の神経毒性がある。

(4)多くは再発し治療抵抗性となり、新たな治 療スケジュールの開発が待たれている。

実臨床では、PCNSL という臨床診断がなされる と、前記にもあるような画一的な治療がなされる が、その予後は1年以内に再発し転帰不良となる 症例から、10年以上にわたって寛解が得られる症 例まで、治療に対する感受性は症例毎でかなりの 相違がある。本研究により治療困難な PCNSL のバ イオマーカーが明らかにされると、予後が不良と 考えられる症例には造血幹細胞移植などを併用 することにより強力な薬物療法を選択すること などから予後の改善が期待できる。このようにバ イオマーカー用いた個別化治療が確立され、治療 成績の向上が期待できる。さらに、シーケンス解 析から腫瘍特異的な遺伝子異常が明らかにされ、 診断マーカーとしての発展、分子標的創薬が展開 されることが期待される。

#### E. 結論

今後、高速シーケンサーを用いた RNA 解析から、 遺伝子変異、遺伝子発現情報解析も行い、体系的 な解析から標的分子を同定し、分子標的創薬を進 める。

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G. 知的財産権の出願・登録状況(予定を含む) 特になし

# UBQLN2 コンディショナルノックアウトマウスの解析に基づく 神経変性機序の解明

- 研究代表者 田中 章景 1) 研究分担者 土井 宏 1) 研究分担者 笹岡 俊邦 2)
- 街浜市立大学大学院医学研究科 神経内科学・脳卒中医学

   新潟大学脳研究所 動物資源開発研究分野

#### 研究要旨

われわれがポリグルタミン病核内凝集体の構成成分として同定した UBQLN2 は家族性 ALS の責任遺伝子 であることが明らかにされたことからも、両疾患の病態に深く関与する機能分子と考えられる。そこで UBQLN2 が神経変性の病態形成に係わる機序を明らかにするために、運動ニューロン特異的なUbqln2 ノックアウトマウ スの作成をめざしている。UBQLN2 Flox マウスを維持し、♂Actb-Cre マウスと♀ Ubqln2<sup>dox/flox</sup>を掛け合わせ、 全細胞でのノックアウトマウスを作成している。これにより、神経変性の病変選択性を明らかにする。また、野生型、変異型の UBQLN2 に結合するタンパクを網羅的に同定することにより、UBQLN2 が神経変性に係わる病 態機序の解明を行った。

#### A. 研究目的

われわれがポリグルタミン病核内凝集体の構成成 分として同定した UBQLN2 は家族性 ALS の責任遺 伝子であることが明らかにされたことからも、両疾患の 病態に深く関与する機能分子と考えられる。 UBQLN2 は、核内凝集体への sequestration により、 また遺伝子変異により、loss of function によって両疾 患の病態を形成することが予想される。そこで、 UBQLN2 のコンディショナルノックアウトマウスを作 成・解析することで、両疾患に共通する神経変性機 序を明らかにする。

#### B. 研究方法(倫理面への配慮を含む)

Ubqln1のショウジョウバエ、オルソログ Dsk2 につい ては過剰発現、ノックアウトともに致死的であることが 知られている。そこで、コンディショナルなノックアウト を行うべく、loxP 配列で UBQLN2 遺伝子を挟み込む UBQLN2 Flox マウスと、細胞群特異的な Cre 発現を おこすマウスを交配し、その表現型と病理像を明らか

にする。まず、運動ニューロンに Cre 発現をおこす VAchT-Cre マウス(Misawa et al, Genesis 2003)との交 配により、運動ニューロン特異的な Ubgln2 ノックアウ トマウスを作成する。Ubgln2 はポリユビキチン化され たタンパク質とプロテアソーム間のアダプターとして 機能しているので、ノックアウトによってユビキチンープ ロテアソーム系による基質の分解障害、蓄積が生じる 可能性が予想される。そこで、マウス樹立後は、運動 機能の観察に加え、ユビキチン陽性封入体、 TDP-43、FUS/TLS の細胞質内蓄積をはじめとする ALS 患者運動ニューロンで観察される各種の病理学 的所見がこのマウスモデルでも見られるかを検討す る。これまでの我々の dynactin1, TDP-43 のコンディ ショナルノックアウトマウス作成の経験(PLos One 2013, Brain 2013)では、Cre 発現の効率から考えると Ubgln2の発現は正常の 50%程度と予想され、完全に ノックアウトされるわけではない。この点から、本モデ ルがALS 病態を反映していることが明らかになれば、 治療戦略として UBQLN2 活性化をめざし、in vitro で スクリーニングを行った薬剤のマウスへの投与を試みる。

これらの各種実験において、新潟大学脳研究所動 物資源開発分野の笹岡教授の助言を得ながら研究 を推進する。また、笹岡教授と共同して、マウスの発 生工学・生殖工学に関する助言をうけ、実験経過の 討論を行い、全ニューロンに Cre 発現をおこすマウス をはじめとした Cre 発現マウスとの交配も同時平行で 行い、特にどのニューロンにおいて神経変性が高度 に生じやすいかを明らかにすることで、神経変性疾 患の病変選択性の形成機序についても検討する。

#### C. 研究結果

われわれは、平成 26 年度より *Ubq1n2* flox マ ウスの作成を開始し、相同組み替えベクターの構 築、組み替え ES 細胞の樹立、キメラマウスの作 成を経てヘテロマウス(Ubq1n2<sup>flox/+</sup>)を取得し、 薬剤耐性遺伝子の除去を行った。平成 27 年度は ♀のホモ接合性マウス(*Ubq1n2<sup>flox/flox</sup>*)マウスと ♂のヘミ接合性マウス(*Ubq1n2<sup>flox/flox</sup>*)を作成し (*Ubq1n2*はX染色体上の遺伝子である。)、恒常的 に flox マウスを維持可能な状態とした。現在は ♂*Actb*-Cre マウスと♀*Ubq1n2<sup>flox/flox</sup>*を掛け合わ せ、全細胞でのノックアウトマウスを作成してい る。

#### D. 考察

コンディショナルノックアウトマウスについ てはまだ作成中であるため、これに直接関与する 研究成果はないが、質量解析により UBQLN2 結合 タンパクの網羅的同定を行い、野生型 UBQLN2 と変 異型 UBQLN2 で異なる結合性を示す結合タンパク 質の同定に成功した。この結合タンパクは UBQLN2 に特異的なドメインであり、変異が集中している PXX ドメインを欠失させると結合性が低下することを確認 している。

平成 28 年度には、全ニューロンに Cre 発現をお こすマウスをはじめとした Cre 発現マウスとの交配を 行うことで、神経変性の病変選択性を明らかにする。

また、野生型、変異型の UBQLN2 に結合するタン パクを網羅的に同定することにより、UBQLN2 が神経 変性に係わる病態機序を解明する。

#### E. 結論

UBQLN2 Flox マウスの作成が終了し、今後コン ディショナルノックアウトマウスの解析を行い 病態解析を進めていく。

F. 研究発表(上記課題名に関するもの)

1. 論文発表

- なし
- 2. 学会発表

なし

G. 知的財産権の出願・登録状況(予定を含む) なし

# 免疫不全を伴わない患者に発生する Epstein-Barr virus 関連の

# 中枢神経原発悪性リンパ腫の臨床病理学的検討

研究代表者 杉田 保雄 1) 研究分担者 柿田 明美 2)

1) 久留米大学医学部病理学講座 2) 新潟大学脳研究所

#### 研究要旨

EBV 関連陽性の PCNS リンパ腫の臨床事項および EBV 関連の PCNS リンパ腫の進展と宿主の免疫機能との相関関係を検討した。今回の研究では EBV 関連の PCNS リンパ腫症例群が EBV 陰性の PCNS リンパ腫群 に比較して統計学的には予後に有意差は得られなかった。しかし、EBV 関連の PCNS リンパ腫症例群と 比較して EBV 陰性群では長期生存例がみられ、両者に差がある可能性は否定できない。本腫瘍と進展と宿 主の免疫機能について PD-1/PD-L1 発現で検討した。EBV 陰性の PCNS リンパ腫群では高頻度に PD-1 発現が腫瘍浸潤リンパ球にみられ、一方、PD-L1 発現が腫瘍、組織球にみられた。この結果から宿主と 腫瘍との間に免疫寛容の状態が成立していることが考えられる。一方、EBV 関連の PCNS リンパ腫症例 群では PD-L1 の発現は陰性例と大差がないが、PD-1 については 83%の症例で PD-1 陽性リンパ球はほ とんどみられなかった。この現象は腫瘍と宿主間における免疫寛容が不成立いうよりも、むしろ宿主 側の免疫応答の減弱を示唆してる所見と考えられた。

#### A. 研究目的

中枢神経原発悪性リンパ腫(PCNSリンパ腫)の脳 内浸潤機構、腫瘍発生についてのEpstein-Barr Virus (EBV)の役割を明らかにする。本腫瘍の臨 床事項の解析を行い、さらにEBV関連のPCNSリンパ 腫の進展と宿主の免疫機能との相関関係を明らかに してPCNSリンパ腫の臨床病理像を解明する。

#### B. 研究方法(倫理面への配慮を含む)

- 前年度 in situ hybridization (ISH)で検出 した EBV 陽性例の臨床事項を検討する。
- 前年度 in situ hybridization (ISH)で検出 した EBV 陽性例とほぼ年齢、性別が一致する EBV 陰性例と臨床事項を照合させて EBV 関連の

PCNS リンパ腫の臨床上の特徴を明らかにする。

- 前年度と同様に PCNS リンパ腫症例を対象として腫瘍の免疫回避機構に関わっている免疫 チェックポイント分子である Programmed death 1(PD-1)およびそのリガンドである PD-L1 検討する。
- ④ EBV 陽性 PCNS リンパ腫症例で PD-1/ PD-L1 の
   発現を検討して EBV 陽性 PCNS リンパ腫と宿主
   の間の免疫応答の状態を検討する。
- ⑤ 臨床病理学的な検討を行い、①~④で得られ たデータとリンパ腫症例の予後、生物学的態 度との相関関係を明らかにする。
- ⑥ 本研究の実施にあたっては久留米大学倫理 委員会での承認の上で行われた。

## C. 研究結果

- 今回の研究で予後調査可能であった EBV 関連 の PCNS リンパ腫症例は ISH で検出された 23 例 中 20 例であった。性別では男性:11 例、女性:9 例であった。年齢は 56 歳から 96 歳(平均 67<u>+</u>18 歳)であった。治療内容としては生検(BP)およ び外科手術(SR)のみの症例は7 例であり、BP お よび SR に放射腺・化学療法(RA, CH)を受けた症 例は 13 例であった。BP, SR のみの症例の生存期 間あるいは追跡期間は平均 5.4<u>+</u>7.0 ヵ月であり、 BP, SR, RA, CH の治療を受けた症例では平均 27.7<u>+</u>28.2 ヵ月であった。EBV 関連の PCNS リン パ腫症例においても BP, SR, RA, CH の治療群の方 が BP, SR のみの治療群に比べて生存期間あるい は追跡期間は長い傾向がみられた(P=0.02)。
- 今回の研究で予後調査可能であった EBV 関連 陽性の PCNS リンパ腫 19 症例と今回の研究で予 後調査可能であった EBV 陰性の PCNS リンパ腫 20 症例との比較検討を Kaplan Meier 生存曲線に より行った(下図)。



A 群は EBV 関連陽性の PCNS リンパ腫であり、B 群は EBV 陰性の PCNS リンパ腫である。長軸方向 は生存月数を示している。EBV 陰性の PCNS リンパ 腫の方が予後が良い傾向が伺をれたが、 Log-rank test による検定では有意差は得られ なかった (P=0.48)。

③ EBV 陰性の PCNS リンパ腫での PD-1/PD-L1 の 検討可能例は 21 例であり、腫瘍細胞が PD-L1 陽性を示した例は 21 例中 11 例であり、 陰性 10 例中5 例で介在組織球が陽性であった。PD-L1 陽性 16 例では全例で TIL が PD-1 陽性を示した。 PD-1, TIA-1, FOXP3 陽性の TIL 数の平均値はそ れぞれ 39±53, 81±82, 5.90±6.80 であり、 標識数において PD-1 は TIA-1 は統計学的に相 関を示したが (r=0.71)、 FOXP3 とは相関しなか った (r=0.23)。 TIL における TIA-1 標識数は FOX 標識数よりも有意に高かった (p<0.001)

一方、EBV 陽性の PCNS リンパ腫での PD-1/PD-L1 の検討可能例は 12 例であり、腫瘍 細胞が PD-L1 陽性を示した例は 12 例中 4 例で あり、陰性 8 例中 4 例で介在組織球が陽性であ った。PD-1 については EBV 陰性例の PD-1 陽性 リンパ球の平均値を示した症例は 2 例のみであ り、残り 10 例(83%)では PD-1 陽性リンパ球は ほとんどみられなかった。また腫瘍細胞からの PD-L1 陽性 4 例では全例でリンパ球は PD-1 陰性 を示した。

#### D. 考察

① Oyama らは中枢神経以外で発生した EBV 関連 陽性の B 細胞型リンパ腫の予後は陰性のもの に比較して高齢者に好発し、生物学的に悪性 度が高く予後不良であったことを報告して いる(1)。Utsuki らは EBV が ISH で強く発現 した症例ほど予後不良であったことを報告 している(2)。一方、Jamal らは予後は症例に よって様々であり、一定の傾向はみられなか ったと報告している(3)。

今回の私共の研究では EBV 関連陽性の PCNS リンパ腫症例群の方が EBV 陰性の PCNS リンパ 腫群に比較して予後が悪い傾向が伺われたが、 Log-rank test による検定では有意差は得ら れなかった (P=0.48)。その理由として 1)比 較対象とした EBV 陰性の PCNS リンパ腫群も 高齢者例が多くて放射線、化学療法などが完 遂できなかった例が多かったこと。2) 両群共 に症例数が少なかったことの2点が考えられ る。しかし、

EBV 関連陽性の PCNS リンパ腫症例群では長期 生存例は皆無であり、今後、症例数を増やし て再検討する必要がある。

② Programmed death 1(PD-1)は免疫チェックポ イント分子であり、そのリガンドである PD-L1 と共に腫瘍細胞の免疫回避機構に重要 な役割を担っている。今回の研究では EBV 陰 性の PCNS リンパ腫群では高頻度に PD-1 の発 現が腫瘍浸潤リンパ球、PD-L1 の発現が腫瘍、 介在する組織球にみられて宿主と腫瘍との 間に免疫寛容の状態が成立していることが 明らかになった。

一方、EBV 関連陽性の PCNS リンパ腫症例群で は12例中4例で腫瘍細胞に PD-L1 が発現し、 他の4例においても介在組織球に PD-L1 発現 がみられた。しかし、PD-1 については EBV 陰 性例の PD-1 陽性リンパ球の平均値を示した 症例は2例のみであり、残り10例では PD-1 陽性リンパ球はほとんどみられなかった。ま た腫瘍細胞からの PD-L1 陽性4例では全例で リンパ球は PD-1 陰性を示した。これは宿主 と腫瘍との間に免疫寛容の状態が成立して いないというよりも、むしろ宿主側の免疫応 答の減弱を示唆してる所見と考えられる。し たがって EBV 関連陽性の PCNS リンパ腫症例 と陰性症例では腫瘍発生の機序に差がある ことが示唆される。

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# E. 結論

 EBV 関連陽性の PCNS リンパ腫症例群の方が EBV 陰性の PCNS リンパ腫群に比較して予後が悪 い傾向が伺われたが、Log-rank test による検 定では有意差は得られなかった(P=0.48)。

② EBV 関連陽性の PCNS リンパ腫症例群では、腫

瘍細胞に PD-L1 が発現し、他の4例においても 介在組織球に PD-L1 発現がみられた。しかし、 PD-1 については EBV 陰性例の PD-1 陽性リンパ 球の平均値を示した症例は2例のみであり、残 り10例では PD-1 陽性リンパ球はほとんどみら れなかった。また腫瘍細胞からの PD-L1 陽性4 例では全例でリンパ球は PD-1 陰性を示した。 これは宿主と腫瘍との間に免疫寛容の状態が 成立していないということではなくて、むしろ 宿主側の免疫応答の減弱を示唆してる所見と 考えられる。

#### F. 研究発表(上記課題名に関するもの)

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第 33 回日本脳腫瘍病理学会・2015 年 5 月 29 日・ 高松

#### G. 知的財産権の出願・登録状況(予定を含む)

 特許取得 無し
 実用新案登録
 無し
 その他
 無し

# 脳アミロイドアンギオパチー関連炎症の発症機構の解明

# 研究代表者 坂井 健二1)

研究分担者 柿田 明美 2)

1) 金沢大学附属病院 神経内科 2) 新潟大学脳研究所 デジタル医学分野

#### 研究要旨

脳アミロイドアンギオパチー (CAA) は脳や髄膜内の血管へのアミロイドの沈着により生じる疾患で ある。CAA では沈着した Aβに対する免疫反応によって生じる炎症・血管炎 (CAA 関連炎症) を引き起 こすことが知られているが、高度な炎症を生じる機序の詳細は不明である。本研究では CAA 関連炎症 の発症機構を明らかにするため、炎症や免疫系の活動に関連するサイトカインや蛋白についての定量 的な解析や遺伝子多型に関する検討を行い、CAA 関連炎症の発症との関連を明らかにする。方法につ いて、新潟大学脳研究所で病理診断され、Boston criteria の possible CAA 以上を満たす症例につい てパラフィンブロックおよび凍結組織を収集する。TREM2 および TGF-β1 の定量的な解析について、免 疫組織化学染色やリアルタイム PCR 法を用いた mRNA の定量を行う予定である。また、凍結組織を利用 して DNA を抽出し、TREM2、TGF-β1 や apolipoprotein E の遺伝子多型の解析も行う。これらの結果に ついて CAA 群と CAA 関連炎症群を比較検討する。本年度は研究を行うための倫理申請と解析を行う症 例の選択、条件検討のための予備実験を行った。

#### A. 研究目的

脳アミロイドアンギオパチー(CAA)は脳や髄 膜内の血管へのアミロイドの沈着により生じる 疾患であり、アミロイドβ蛋白(Aβ)の沈着によ る CAA は 60 歳以上の約半数に認められる。CAA で は沈着した Aβに対する免疫反応によって生じる 炎症・血管炎(CAA 関連炎症)を引き起こすこと が知られているが、高度な炎症を生じる機序の詳 細は不明である。本研究では CAA 関連炎症の発症 に関連する危険因子を明らかにするため、炎症や 免疫系の活動に関連するサイトカインや蛋白に ついて定量的な解析を行い、CAA 関連炎症との関 連を明らかにする。CAA 関連炎症の発症に関連す る危険因子を明らかにし、発症予防や新規治療法 開発の端緒とすることが目的である。 B. 研究方法(倫理面への配慮を含む)

CAA のみおよび CAA 関連炎症を生じた症例につ いて、孤発性 Aβ型アミロイドアンギオパチー (CAA)の遺伝的危険因子で、炎症に関連するサ イトカインである transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1)および脳内の免疫系を担当す る細胞であるミクログリアの活動に関連する蛋 白であり、アルツハイマー病との関連が報告され た triggering receptor expressed on myeloid cells (TREM2)の脳内における発現量を解析し、 CAA 関連炎症との関連を明らかにする。また、凍 結組織より DNA を抽出し、TGF- $\beta$ 1 や TREM2、 apolipoprotein Eの遺伝子多型についての解析も 行う。

対象とする症例について、新潟大学脳研究所で 病理診断され、Boston criteriaの possible CAA 以上を満たす症例とし、それらのパラフィンブロ ックおよび凍結組織を収集する。

脳組織における TREM2 および TGF-β1 の定量的 な解析について、TREM2 については、パラフィン ブロックを用いて免疫組織化学染色を行う。 TGF-β1 の解析については凍結脳を用いてリアル タイム PCR 法を用いた mRNA の定量にて行う。こ れらの結果について CAA 群と CAA 関連炎症群を比 較検討する。

研究に利用する検体については匿名化が行われている。また、研究計画について、金沢大学医 学倫理委員会と新潟大学医学部倫理委員会において承認済みである。

#### C. 研究結果

平成 27 年度では、新潟大学脳研究所における 保存検体利用のための倫理委員会への申請およ び承認、必要な検体の準備と金沢大学における予 備実験を行った。

対象とする症例について、脳出血を伴う CAA の 剖検例では 12 症例、生検例では 31 症例、CAA 関 連炎症は剖検例で 2 例、生検例では 7 例の検体が 利用可能であることを確認した。

金沢大学神経内科では遺伝子多型解析のため のプライマーの設定と条件の検討を行った。

#### D. 考察

研究の遂行に必要な倫理申請は終了し、すでに 承認が得られている。今後は提供を受けた検体を 利用し、金沢大学において予定している解析を行 っていく。

#### E. 結論

研究の遂行に必要な倫理委員会の承認と症例 の抽出はほぼ終了しており、今後は金沢大学神経 内科において、新潟大学脳研究所より提供された 検体を使用した解析を行っていく。

# **F. 研究発表**(上記課題名に関するもの) 1. 論文発表

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#### G. 知的財産権の出願・登録状況(予定を含む)

 特許取得 なし
 実用新案登録 なし
 その他 なし

# 筋線維メインテナンスに果たす WWP1 ユビキチンリガーゼの機能の解析

# 研究代表者 今村 道博<sup>1)</sup>

# 研究分担者 武田 伸一<sup>1)</sup> 笹岡 俊邦<sup>2)</sup>

<sup>1)</sup> 国立精神・神経医療研究センター神経研究所 <sup>2)</sup> 新潟大学脳研究所生命科学リソース研究センター

#### 研究要旨

ヒト筋ジストロフィーの病因解明及び治療法の開発においては筋ジストロフィーモデル動物を用いた研究から極めて重要な知見を得ることができるため、我々はNH-413と呼ばれる筋ジストロフィーモデルニワトリに注目し、分子病態の解析を進めている。この筋ジストロフィーの原因はWWP1E3 ユビキチンリガーゼ遺伝子のミスセンス変異に因るとされているが、その分子機構は不明である。我々はWWP1に対する特異抗体を作製し、NH-413ニワトリの骨格筋に発現する変異型WWP1タンパク質の発現と局在が正常型分子とは異なることを明らかにすると同時に、これと同じ変異を導入したWWP1分子を発現するトランスジェニックマウス(Tgマウス)を作製し、表現型を解析した。その結果、ミスセンス変異による1アミノ酸置換がWWP1を不安定化し、E3 ユビキチンリガーゼ活性を消失させることを明らかにした。

#### A. 研究目的

本研究はNH-413 筋ジストロフィーニワトリに 着目し、その分子機構を明らかにすることを目的 としている。

デュシェンヌ型筋ジストロフィーの原因遺伝 子(ジストロフィン遺伝子)とその遺伝子産物が同 定されて以来、様々な種類の筋ジストロフィーの 原因遺伝子が明らかにされている。これと並行し て自然発生により系統維持されていた筋疾患実 験動物の原因遺伝子も明らかにされ、ヒトと同じ 原因遺伝子を有する動物モデルはヒト筋ジスト ロフィーの病態の解明や治療法開発に大きな役 割を果たしてきた。一方、筋ジストロフィーニワ トリについては、その原因が WWP1 遺伝子のミ スセンス変異に因るものと報告されたが、これに 類似するヒト筋疾患は見出されていない。本研究 はこの変異型 WWP1 による筋変性の分子機構を 解明することで、これに関わる分子群を同定し、 未だ原因が明らかにされていないヒト筋疾患と の関連を検証することも目標としている。

#### B. 研究方法(倫理面への配慮を含む)

NH-413 ニワトリはヘテロでも軽度の筋ジスト ロフィー症状を呈するため、N-413 で同定された ものと同じ変異(R436Q)を持つ WWP1を CAG プロモ ーターにより発現する Tg マウスを作製して、解 析に用いた。マウスの骨格筋における WWP1 の局 在とタンパク質分子の安定性は蛍光組織染色法 とイムノブロット法によって解析した。WWP1分解 断片の構造と酵素活性に関する解析のため、 R436Q変異を特異的に認識する抗体と、E2分子の 共有結合に必須な 890 番目のシステイン残基 (890C)のアミノ基端側を認識する抗体を作製し た。また通常の WWP1 検出においてはマウス WWP1(全918アミノ酸)の199番目から346番目ま でのアミノ酸配列を抗原として得られたアフィ ニティー精製抗体を用いた。R436Q Tg マウスの骨 格筋における α-DG の糖鎖修飾と機能について はイムノブロット法とラミニンオーバーレイ法 を用いて行った。尚、マウスを用いた本研究は国 立精神・神経医療研究センター神経研究所の小型 実験動物倫理問題検討委員会による審査と承認 を得て行われた。

#### C. 研究結果

正常マウス組織におけるWWP1の発現は、骨格筋、 心臓、肺、肝臓、脾臓、腎臓、腸、脳で 130 kDa のタ ンパク質として発現していたが、R436Q WWP1 Tgマ ウスにおいてはこの 130-kDa 分子に加え、骨格筋と 心筋のみに約90 kDa の分解断片が検出された。こ の分解物はWWP1のR436Q変異を特異的に認識す る抗体と反応するのに対し、130-kDa(未分解) WWP1 とは反応しなかったことから、R436 変異型 WWP1 のほとんどが分解され 90-kDa 断片となったこ とを示していた。これとは逆に 890C の直前を認識す る抗体は130-kDa分子と反応し、90-kDa断片とは反 応せず、分解断片がユビキチンリガーゼ活性を有さ ないことが示された。一方、Tg マウス骨格筋の免疫 組織染色では、NH-413 ニワトリでの結果とは異なり、 筋形質膜への局在は消失せず、むしろより強くなると いう現象が認められた。Tg マウスの骨格筋における α-DG の糖鎖修飾については、イムノブロットでもオ ーバーレイ法によるラミニン分子との結合能評価にお いても野生型マウスとの間に差は認められなかった。 また、免疫沈降法による解析では変異型或いは正常 型WWP1とDG分子との結合を検出することはできな かった。

#### D. 考察

我々の研究から、ニワトリではミスセンス変異による 1アミノ酸置換が WWP1 の分解と筋形質膜からの消 失を誘起することが示唆されていたが、本研究により、 特定のアミノ酸の変化がWWP1の分解を横紋筋特異 的に生じさせることを明らかにすることができた。 NH-413 とは異なり、90-kDa 消化断片は安定であっ たが、E3 ユビキチンリガーゼとしての酵素機能を失っ ていることが明らかとなった。また、この消化断片が筋 形質膜への局在をより強固にしていることが示唆され たが、このニワトリとの違いについては不明である。マ ウスでは正常型 WWP1 が野生型マウスと同じレベル で発現しており、これが NH-413 ニワトリとの違いであ ることから、正常型 WWP1 の非存在下での変異型 WWP1を発現させ解析する必要がある。α-DG につ いては R436Q Tg マウスにおいて、糖鎖修飾とラミニ ン結合能について特筆すべき変化は認められず、ま た、免疫沈降でもDGとWWP1との結合は示されなか ったが、これについても正常型 WWP1 の非存在下で の再度解析を行う必要があると考えられた。

#### E. 結論

本研究により我々は NH-436 筋ジストロフィー ニワトリに見出された WWP1 遺伝子のミスセンス 変異が横紋筋特異的な WWP1 分解を生じ、E3 ユビ キチンリガーゼの酵素活性を消失させることを 明らかにした。また、変異型 WWP1 の発現はα-DG の糖鎖修飾異常に直接関与しないことが示唆さ れた。しかしながら、正常型 WWP1 が残存してい ることの影響を検証する必要があり、今後は WWP1 遺伝子のノックアウトマウス、或いは変異型 WWP1 のノックインマウスを用いた解析の必要性が確 認された。

# **F.研究発表**(上記課題名に関するもの) 1.論文発表

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#### 2. 学会発表

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#### G. 知的財産権の出願・登録状況 なし

# ゲノム編集技術と生殖工学技術を用いた効率的な遺伝子改変マウス作製

# 研究代表者 中潟 直己 1)

研究分担者 中川佳子1) 笹岡俊邦2)

1) 熊本大学生命資源研究·支援センター 2) 新潟大学脳化学研究所

#### 研究要旨

これまでのノックアウトマウス作製では、ES細胞を介したキメラマウスの樹立が主流であり、作製までにかなりの 時間とコストを要してきた。しかし、近年、ゲノム編集技術を用いた遺伝子改変マウス作製の報告が数多くなされ、 短時間、低コストな遺伝子改変マウスの作製が可能となった。ゲノム編集技術の開発、改良はかなりのスピード で進んでおり、この技術の発展に同調して遺伝子改変マウスの作製を進めるには、生殖工学技術を活かした効 率的な遺伝子改変マウス作製法を確立していくことが不可欠である。本研究では、ゲノム編集技術と生殖工学 技術を用いて、効率的な脳疾患関連遺伝子改変マウス作製法を確立する。

#### A. 研究目的

近年、TALEN (transcription activator-like effector nuclease)やclustered regulatory interspaced short palindromic repeat (CRISPR)-CRISPR-associated protein 9 (Cas9)を利用したゲノム編集技術の発展 は目覚ましく、これらのプラスミドベクターやmRNA (CRISPR-Cas9 ではgRNAとmRNA)を受精卵~イン ジェクションすることにより、短時間、低コストな遺伝子 改変マウスの作製が可能となった。ゲノム編集技術の 開発、改良はかなりのスピードで進んでおり、新規の ベクター開発や効率的な遺伝子改変マウス作製法 についての研究が盛んに行われている。本研究では、 新規のゲノム編集ベクターや体外受精および受精 卵の凍結保存などの生殖工学技術を用いた、より 効率的な脳疾患関連遺伝子改変マウス作製法の 開発、改良を目的とする。

# B. 研究方法(倫理面への配慮を含む)

私達は、体外受精により作製した凍結融解受精卵 を用いて、TALEN による効率的な遺伝子破壊マウス の作製が可能であることを報告した(Nakagawa *et al.*, 2014)。しかしながら、C57BL/6 マウスを用いた体外 受精により作製した凍結融解受精卵が CRISPR-Cas システムによる遺伝子破壊マウスの作製に利用可能 か否か明らかとなっていなかった。また、 CRISPR-Cas システムを用いたゲノム編集では、標的 遺伝子への特異性の低さから、しばしばオフターゲッ ト問題が言及される。そのため、野生型 Cas9 ベクタ ーの他、標的配列への特異性を上げるよう開発され たニッカーゼ型 Cas9 ベクター、FokI 融合型 dCas9 ベクターを用いて、同一遺伝子座での遺伝子破壊マ ウス作製を試みた。

CRISPR-Cas 9 ベクターは、1)ガイド RNA と Cas9 ヌクレアーゼ、2)2 つのガイド RNA と Cas9 ニッカーゼ、 3)2 つのガイド RNA および FokI ヌクレアーゼと不活 性化 Cas9 の融合タンパク質(FokI-dCas9)を発現す るベクターを構築した。これらの環状 DNA を体外受 精により作製した凍結融解受精卵の前核内へインジ ェクションした。生存卵を偽妊娠雌マウスへ移植し、 産子への発生率および変異導入効率の確認を行っ た。

#### C. 研究結果

全ての実験区から標的遺伝子破壊マウスが得られ

たことにより、体外受精により作製した凍結受精卵は CRISPR-Cas システムによる遺伝子破壊マウスの作 製にも利用可能であることが明らかとなった。Cas9 ヌ クレアーゼの利用では、変異導入効率が最も高く、 産子への発生率は最も低かった。一方、Cas9 ニッカ ーゼの利用では、その逆に、産子への発生率が高い ものの、変異導入効率は最も低かった。FokI-dCas9 の利用では、変異導入効率、産子への発生率ともに バランスのとれた成績であった(Nakagawa *et al.*, 2015)。本研究ではCRISPR-Casシステムを用いて遺 伝子破壊マウスを作製するための有用な知見を提供 することができた。

# D. 考察

体外受精により作製した凍結融解受精卵が、様々 なゲノム編集技術を用いた遺伝子破壊マウスの作製 に利用可能であることが明らかとなった。そのため、 今後もC57BL/6マウスを用いて体外受精を行い、凍 結保存した受精卵を使用することにより、交配後に採 卵する新鮮受精卵の使用よりも、効率的に遺伝子改 変マウス作製作業を進める。

ゲノム編集技術を利用した受精卵へのインジェク ション法では、遺伝子破壊マウスは効率良く作製可 能であるが、条件的ノックアウトマウスやノックインマウ スの作製については、産子への発生率や目的とする 変異マウス作製効率がいまだ低く、改善されるべき課 題である。そのため、今後は条件的ノックアウトマウス やノックインマウスの効率的な作製法についても検討 を行い、効率的に脳疾患関連遺伝子改変マウスを作 製するための研究を進める。

#### E. 結論

当センターでは、これまで、様々な生殖工学技術 の開発、改良のための研究を行ってきた。今回、研 究課題としたゲノム編集技術は、遺伝子改変マウス 作製のスタンダード技術となりつつあり、生殖工学技 術を有効利用できれば、更なるゲノム編集技術の発 展および効率的な脳疾患関連遺伝子改変マウスの 作製に貢献できると考える。

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- 凍結受精卵を用いた CRISPR/Cas9 システムによ る遺伝子破壊マウスの作製

第62回日本実験動物学会総会2015年 京都

G. 知的財産権の出願・登録状況(予定を含む) 該当なし

# ALS/FTLD における TDP-43 関連軸索内 mRNA 輸送障害の解析

# 研究代表者 長野 清一1)

研究分担者 小野寺 理<sup>2)</sup>、西澤 正豊<sup>3)</sup>、崎村 建司<sup>4)</sup>、荒木 敏之<sup>1)</sup>

1) 国立精神・神経医療研究センター神経研究所疾病研究第五部

2) 新潟大学脳研究所脳科学リソース研究部門分子神経疾患資源解析学分野

3) 同臨床神経科学部門神経内科学分野

4) 同基礎神経科学部門分子細胞神経生物学分野

#### 研究要旨

ALS/FTLD-Uで神経細胞内に異常沈着する TDP-43 は RNA 結合蛋白質であり、mRNA の輸送に関与する ことが推測されている。我々はこの TDP-43 による軸索内での特定の mRNA の輸送機能が ALS/FTLD-U では障害され神経変性を生じると考え、培養神経細胞での検討により同蛋白質の標的 mRNA 候補として リボソーム蛋白質 mRNA を同定した。本研究では神経特異的 TDP-43 遺伝子欠損マウス由来の培養大脳 皮質神経細胞を用いて解析を行い、軸索の伸長障害および軸索でのリボソームによる蛋白質合成能の 障害を認めた。これらより TDP-43 はリボソーム蛋白質 mRNA の輸送を介した軸索での蛋白質合成能の 維持により神経細胞機能を保っており、その障害が ALS/FTLD-U の発症要因となっている可能性が考え られた。

# A. 研究目的

筋萎縮性側索硬化症(ALS)およびユビキチン 陽性封入体を伴う前頭側頭葉変性症(FTLD-U)で は RNA 結合蛋白質である TAR-DNA binding protein (TDP) -43 が病変部位の神経細胞内に異常凝集、 沈着することが知られている。TDP-43 は核内での 遺伝子の転写や pre-mRNA のスプライシングの制 御に加え、核外での mRNA の輸送に関与すること が推測されている。我々は TDP-43 の異常沈着に よりその軸索への特定の mRNA の輸送機能が障害 され ALS/FTLD-U における神経変性が生じる可能 性を考え、培養神経細胞を用いた検討により TDP-43 の輸送標的 mRNA 候補としてリボソーム蛋 白質 mRNA を同定した。そこで本研究では TDP-43 の発現低下が軸索の形成やそこでのリボソーム の機能に影響を与えるかを明らかにするため、新 潟大学脳研究所で作成された神経特異的 TDP-43 遺伝子欠損マウスを用いた解析を行った。

#### B. 研究方法(倫理面への配慮を含む)

神経特異的TDP-43遺伝子欠損マウスはTDP-43 floxマウスとEnolase 2-Creマウスとの交配によ り得られたものを用いた。胎生15日齢の胎仔より 大脳を摘出して皮質神経細胞の培養を行い、培養 開始2日後に抗βⅢ-チューブリン抗体を用いた 免疫組織染色を行って最も長い神経突起長の計 測により軸索長を評価した。さらに培養開始5日 後に培地中にピューロマイシン(最終濃度10μ g/mL)を1時間添加して新規合成蛋白質の標識を 行い、抗ピューロマイシン抗体を用いた免疫組織 染色を行って軸索中における蛋白質合成能を評 価した。本動物実験は国立精神・神経医療研究セ ンター神経研究所小型動物実験倫理問題検討委 員会で承認を受けた計画に基づいて実施した

## C. 研究結果

神経特異的 TDP-43 遺伝子欠損 (TDP-43 flox/flox, Eno2-Cre(+)) マウス由来の大脳皮質 神経細胞はコントロール (TDP-43 flox/flox, Eno2-Cre(-)および TDP-43 flox/+, Eno2-Cre(+)) マウス由来の神経細胞と比較して軸索の伸長が 不良であった。また神経特異的 TDP-43 遺伝子欠 損マウス由来の大脳皮質神経細胞ではコントロ ールマウスに比し軸索でのピューロマイシンの 取り込みが低下しており、同部位でのリボソーム による蛋白質合成能の低下が認められた。

#### D. 考察

我々が同定した TDP-43 によるリボソーム蛋白 質 mRNA の軸索への輸送機能が神経特異的 TDP-43 遺伝子欠損マウスの神経細胞では障害され、それ により軸索での局所蛋白質合成能が低下して軸 索の伸長不全が生じることが示唆された。今後さ らに TDP-43 によるリボソーム蛋白質 mRNA の詳細 な輸送機構や軸索局所でのリボソーム機能障害 と神経変性との関連につき、明らかにする必要が ある。

#### E. 結論

ALS/FTLD-Uにおいて TDP-43 の細胞内異常沈着 に伴う神経変性の機序として、同蛋白質によるリ ボソーム蛋白質 mRNA 輸送の障害を介した軸索で の局所蛋白質合成能の低下が関与する可能性が 考えられた。

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# 筋萎縮性側索硬化症脊髄における VGF の局在に関する研究

# 研究代表者 嶋澤 雅光 1)

研究分担者 野田 泰裕<sup>1</sup>、原 英彰<sup>1</sup>、柿田 明美<sup>2</sup>)

1) 岐阜薬科大学 薬効解析学研究室 <sup>2)</sup> 新潟大学脳研究所 脳疾患標本資源解析学分野

#### 研究要旨

我々は、VGFの発現を増加させる薬剤が ALS(筋萎縮性側索硬化症)モデルマウスにおいて発症時 期および生存期間を延長することを明らかにした。また、ALS患者の脊髄における VGF 陽性細胞数 が減少していることを明らかにした。VGF は prohormone convertase などの酵素により切断され、生成 したペプチドが生理活性を持つ。我々の過去の検討より、ALS病態に対して VGF 由来ペプチド AQEE-30が保護作用をもつ可能性が示唆されている。しかし、VGFの産生細胞や産生組織は特定され ておらず、これらを明らかにすることで、VGFの病態に対する関与を明らかにすることができると考 える。そこで今回、ヒト ALS患者のサンプルにおいて *in situ* hybridization 法を用いて解析を行う。

#### A.研究目的

筋萎縮性側索硬化症 (Amyotrophic lateral sclerosis: ALS) は上位および下位運動ニューロン が選択的かつ進行性に変性、脱落する神経変性疾 患である。多くの症例は孤発例 (約 90%) であり、 約10%は家族性に発症すると考えられている。家 族性の遺伝子変異として、Cu/Zn superoxide dismutase1 (SOD1) 遺伝子のミスセンス変異など が報告されており、ヒト変異 SOD1 (SOD1<sup>G93A</sup>)を 過剰発現させたトランスジェニックマウスが研 究に用いられている。治療薬としては、グルタミ ン酸放出抑制薬であるリルゾールが用いられて いるが、病態の進行抑制効果は約3ヶ月程度であ り、より有効な新規治療薬の開発が望まれている。 過去の報告において、孤発性 ALS の患者および ALS モデルマウスである SOD1<sup>G93A</sup>マウスの脳脊 髄液において、分泌ポリペプチドである VGF nerve growth factor inducible (VGF) の発現量が減 少しており、SOD1<sup>G93A</sup>マウスにおいて VGF の減 少は ALS の主要評価項目である筋力低下に先行

して起こることが示唆されている(1)。

VGF は、中枢あるいは抹消の神経細胞や膵臓、 下垂体前葉、副腎髄質、消化管等の内分泌細胞に 発現している分泌ポリペプチドであり、プロセッ シングによって生じる様々なペプチドが生理活 性を有する。VGF は摂食行動やエネルギー恒常性 の維持に関与することや、うつ病の改善や海馬シ ナプスの可塑性増進、海馬歯状回の神経新生に関 与することが報告されている。過去の研究により、 パーキンソン病、N-methyl-D-aspartat 誘発高眼圧網 膜障害などの神経変性を来す病態モデルマウス に対し、VGF の発現誘導剤は神経保護作用を示す ことが明らかになり、神経保護作用を有する VGF 由来ペプチドの存在が示唆された。また、ハンチ ントン病モデル細胞において、VGF 前駆タンパク 質のC末端30アミノ酸からなるAOEE-30が神経 保護作用を示すことを明らかにしている (2)。さ らに、VGF の発現誘導剤は ALS モデルマウスに 対して神経保護作用を示すことが報告されてい る (3)。これらより、VGF 由来ペプチドは ALS に

対して保護作用を持つ可能性が示唆される。しか し、ALS 病態における内在性の VGF 発現は脳脊 髄液中でしか検討されておらず、その産生細胞な どは明らかにされていない。本検討では *in situ* hybridization 法等を用いて ALS 患者組織切片にお いて、VGF の発現部位や発現量の変化などを詳細 に検討する。

#### B.研究方法

RNA プローブの作製を岐阜薬科大学において 行う。標的配列および設計したプライマーを示す。



Blend taq plus (Toyobo) を用いて、PCR 反応を行い、 目的配列を増幅する。Template DNA には VGF 過 剰発現プラスミドベクターを用いた。PCR 産物を 1%アガロースゲルを用いて電気泳動し、トラン スイルミネーターで観察しながら切り取ったゲ ル小片を NucleoSpin® Gel and PCR Clean-up (TAKARA Bio)を用いて精製する。

pGEM-T (Easy) vector systems を用いて、PCR 産物 を vector へ導入し、コンピテントセル (Nippon gene) ヘトランスフォーメーションを行い、Amp (+) 寒天 LB 培地で培養する。翌日に数コロニー を選択し、液体 LB 培地 5mL 中で培養を行った後、 PureYield<sup>™</sup> Plasmid Miniprep System (Promega)を用 いてプラスミド DNA を回収する。M13 プライマ ーおよび target sequence 3'プライマーを用いて PCR を行い、導入遺伝子の挿入方向を確認した後、 制限酵素 Sal I を用いてプラスミド DNA を1本鎖 化する。得られた 1 本鎖 DNA を template とし、 DIG RNA Labeling Kit (SP6/T7) (Sigma) を用いて DIG 標識 RNA プローブを得る。 サンプルが届き 次第染色操作を行う。

本研究は新潟大学脳研究所で「病理解剖ならび に病理検体保存とその使用に関する承諾」が得ら れている症例を用い、新潟大学医学部倫理委員会 の承認(申請中)および岐阜薬科大学生命倫理委 員会の承認(承認番号:岐阜市薬会第334号)を 得た上で行う。

#### C.研究結果

2015年10月23日(金)に共同研究の方針につい て、新潟大学脳研究所脳疾患標本資源解析学分野 教授 柿田 明美先生と打ち合わせを行なった。 その会議で、本研究課題についての今後の方針に ついて議論した。その打ち合わせにより、古典的 ALS では脊髄と並行して大脳皮質運動野も障害 されるため、サンプルが限られている脊髄で検討 を行う前に、運動野パラフィン切片で検討を行っ てはどうかとご提案していただき合意した。現在、 岐阜薬科大学生命倫理委員会の許可を得ており、 新潟大学の倫理申請審査中である。岐阜薬科大学 において *in situ* hybridization のプローブ作製を進 めている。両倫理委員会の許可を受けた上で、新 潟大学よりサンプルを受領次第染色を開始する。

#### D.考察

ヒト VGF に対する RNA プローブに関しては過 去に使用実績が無いため、独自に設計を行った。

#### E.結論

サンプルが届き次第実験を開始し、2016年6月 頃を目途に結果を報告する予定である。

#### F.研究発表(上記課題名に関するもの)

1.論文発表

# なし

2.学会発表

なし

G.知的財産権の出願・登録状況(予定を含む)

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# 神経回路の興奮性に対する CB2 受容体の役割の解明

研究代表者	菅谷	佑樹 1)
研究分担者	狩野	<sub>方伸</sub> 1)
研究分担者	崎村	建司 2)

1) 東京大学大学院医学系研究科 2) 新潟大学脳研究所

#### 研究要旨

近年、カンナビノイドCB<sub>2</sub>受容体が中枢神経系においても発現し、何らかの機能を持っている可能 性が示唆されている。本研究では、遺伝子改変技術により作出したCB<sub>2</sub>受容体コンディショナルノッ クアウトマウスやCB<sub>2</sub>受容体の薬理学的阻害を用いて、神経回路の興奮性におけるCB<sub>2</sub>受容体の役割 を明らかにすることを目的とした。野生型マウスにおけるCB<sub>2</sub>受容体単独の薬理学的阻害では、カイ ニン酸誘発発作に影響が認められなかったが、CB<sub>1</sub>ノックアウトマウスではCB<sub>2</sub>受容体の薬理学的阻 害によって発作が重篤化した。また、野生型マウスにおいてCB<sub>1</sub>受容体との共阻害によりてんかん焦 点の形成を促進する効果が認められた。この結果から、CB<sub>2</sub>受容体が神経回路の興奮性を抑制する可 能性が示唆された。現在、CB<sub>2</sub>受容体の中枢神経系における機能をより詳細に明らかにするためにC B<sub>2</sub>コンディショナルノックアウトマウスを作成中である。

## A. 研究目的

内因性カンナビノイドは生体において合成さ れる脂質メディエーターであり主に神経系、免疫 系において重要な役割を担っていると考えられ ている。神経系に強く発現しているカンナビノイ ドCB1受容体はシナプス伝達を逆行性に抑制し 学習や情動、食行動、神経細胞保護に重要な役割 を果たしていることが報告されている。もう一つ のカンナビノイド受容体であるCB2受容体は免 疫系細胞に発現し、免疫反応を抑制していること が報告されている。しかし近年、CB2受容体が 中枢神経系に発現し、神経細胞の機能を調節して いる可能性を示す報告が多くされている。ただ、 CB。受容体の抗体染色は世界的にいまだ成功し ておらず、神経細胞特異的ノックアウトマウスを 用いた研究もないことから、神経細胞における発 現と機能は明らかになっていない。本研究はカン ナビノイドCB2受容体について、新潟大学脳研 究所の崎村教授と共同で神経細胞特異的ノック アウトマウスを作出し、行動学的、生理学的、解 剖学的解析を行い、CB2受容体の中枢神経系に おける役割を明らかにすることを目的とする。

#### B. 研究方法(倫理面への配慮を含む)

興奮性細胞特異的にCB<sub>2</sub>受容体を欠損したマ ウスを作出する目的で、CB<sub>2</sub>floxedマウ スをC57BL/6系統で作出し、興奮性細胞で Creを発現するEmx1-Creマウスと交 配する。これらのマウスを用いて電気生理学的、 行動学的に解析する。特にCB<sub>2</sub>受容体の活性化 が回路の異常興奮やてんかん発作を抑制する可 能性があるとの結果が得られていることから、カ イニン酸や電気刺激によるてんかん誘発試験を 行う。並行して、CB<sub>2</sub>受容体シグナルの急性阻 害と長期阻害では異なる影響があることが報告 されていることから、薬理学的な急性阻害を行い、 同様に解析する。

#### C. 研究結果

現在、CB<sub>2</sub>floxedマウスを樹立に成功 し、薬剤耐性カセットの除去を行っている。今後、 Creマウスと交配し、解析対象の動物を作出し て解析に供する。

ノックアウトマウスの作成と並行して、CB。 受容体の薬理学的な阻害によるCB2受容体の役 割の検討を行った。その結果、野生型C57BL /6NマウスではCB。受容体阻害薬を投与して もカイニン酸誘発性てんかん発作に影響は認め られなかったが、СВ1受容体ノックアウトマウ スではCB。受容体阻害薬の投与によりカイニン 酸誘発性てんかん発作開始までの潜時が短縮し、 死亡率が有意に上昇した。内因性カンナビノイド 2 アラキドノイルグリセロールを産生できないマ ウス (DGL α / ックアウトマウス) でもCB<sub>1</sub>受容体ノックアウトマウスより発作潜時が短く 高い死亡率が認められた。したがって、2 アラキ ドノイルグリセロールによるСВ。受容体の活性 化がてんかん発作の悪化を阻止している可能性 が示唆された。また、長期間の発作誘発によるて んかん回路の形成もCB1受容体とCB2受容体 の共阻害によって促進した。

#### D. 考察

以上の結果から、CB2受容体シグナリングは 神経回路の興奮性を抑制し、てんかん発作重積に よる死亡率を低下させる働きがあると考えられ た。さらにてんかんの進行に伴う回路変化の過程 にも影響する可能性が示唆された。本実験では全 身投与による薬理学的阻害を用いており、作用を 発現している細胞種の特定ができなかった。これ までの報告によると中枢神経系の神経細胞での CB<sub>2</sub>受容体mRNAの発現が認められているが、 抗体染色による受容体の発現パターンの解析に 関しては信頼性の高い報告がない。今後は、ノッ クアウトマウスをもちいた抗体の作成などを通 じて、解剖学的な発現パターンを明らかにすると ともに、コンディショナルノックアウトマウスを 用いてスライス電気生理による詳細な機能解析 が必要である。

#### E. 結論

CB<sub>2</sub>受容体の活性化は神経回路の異常興奮を 抑える可能性が示唆された。今後はコンディショ ナルノックアウトマウスを用いた細胞特異的な CB<sub>2</sub>受容体の欠失による機能解析が必要である。

#### F. 研究発表(上記課題名に関するもの)

1. 論文発表

なし

#### 2. 学会発表

Gordon Research Conference: Cannabinoid Function in the CNS・2015 年 5 月 24-29 日・ル ッカ、イタリア

G. 知的財産権の出願・登録状況(予定を含む) なし

# タウオパチーにおける運動ニューロン障害の病理学的研究

# 研究代表者 吉田 眞理 1)

# 研究分担者 三室マヤ1)、岩崎靖1)、柿田明美2)、桑野良三3)

1) 愛知医科大学加齢医科学研究所 2) 新潟大学脳研究所病理学分野

3) 同附属生命科学リソース研究センター遺伝子機能解析学分野

#### 研究要旨

臨床的に運動ニューロン疾患との鑑別が問題となったタウオパチーを抽出し、病理学的特徴を解析した。 剖検5400例中、ALS、FTLD-MND、PLSとの鑑別が問題となった3症例が存在し、2例は上位および下位運 動ニューロン系などに4リピートタウ陽性封入体を形成するglobular glial tauopathy (GGT)と診断され た。GGT症例は中心前回と錐体路に高度な変性を示し、小球状タウ陽性オリゴデンドログリア封入体 が多数出現した。脊髄錐体路では小径線維の脱落が強く、大径線維は残存する傾向を認めた。脊髄前 角などの下位運動ニューロンにはタウ陽性封入体が出現するものの、細胞脱落は比較的軽度に留まり、 脊髄前根の大径軸索の脱落は軽度であった。GGTでは、TDP-43 proteinopathy type B の病理像に比し て、上位と下位運動ニューロン系の軸索障害の選択性や程度に相違がある可能性が示唆された。

#### A. 研究目的

筋萎縮性側索硬化症 (amyotrophic lateral sclerosis, ALS)、あるいは前頭側頭型認知症を 伴う運動ニューロン疾患(frontotemporal lobar degeneration with motor neuron disease, FTLD-MND) では 95%以上の症例で通常 TDP-43 proteinopathy による神経細胞封入体と細胞障害 を認める。一方、臨床的に ALS、FTLD-MND、ある いは原発性側索硬化症 (primary lateral sclerosis、PLS)との鑑別が問題となる症例の中 に、タウオパチーが存在し、中心前回や脊髄前角 などの運動ニューロン系に封入体を形成する。 TDP-43 proteinopathy とタウオパチーにおける運 動ニューロン障害の類似点と相違点を比較し、タ ウオパチーにおける運動ニューロン系障害の特 徴を病理学的に検討する。この相違点は、臨床像 や検査所見に反映され、生前により正確な診断に 到達できる可能性が考えられる。

B. 研究方法(倫理面への配慮を含む)

愛知医科大学加齢医科学研究所に蓄積された 5400 例の剖検例中、運動ニューロン疾患との鑑別 が問題となったタウオパチーの症例を抽出し、上 位および下位運動ニューロン障害の特徴を検討 した。また新潟大学脳研究所内で、運動ニューロ ン障害を呈したタウオパチーの症例の病理像を 参照した。

病理学的検索は、中心前回を含む前頭葉、側頭 葉、頭頂葉、後頭葉、基底核、視床、中脳、橋、 延髄、小脳、脊髄の各髄節、HE、KB 染色、 Gallyas-Braak 染色、AT8, RD3, RD4 免疫染色、CD68, リン酸化ニューロフィラメント、リン酸化 TDP-43 の免疫染色を施行し、上位および下位運動ニュー ロン系の変性を評価した。また剖検時にグルター ル固定した頚髄、胸髄、腰髄の錐体路と前根を観 察した。

#### C. 研究結果

5400 例中アルツハイマー病を除くタウオパチ ーは、ピック病 5 例、進行性核上性麻痺 110 例、 大脳皮質基底核変性症 33 例、嗜銀顆粒病 86 例、 石灰化を伴う神経原線維変化病 5 例、分類不能の タウオパチー 14 例であり、この中で ALS/FTLD-MND/PLS との鑑別が問題となった症例 が分類不能のタウオパチー14 例中 3 例にあった。 3 例中 2 例は病理学的に globular glial tauopathy (GGT)の病理像を示し、1 例は 3 リピ ートと 4 リピートのタウオパチーを示した。GGT の 2 例中 1 例の臨床病理像を報告する。

【症例】82歳女性。74歳から右手の書字障害と、 階段の上りにくさを自覚した。76歳右手で箸を持 てず歩行障害が進行したため ALS を疑われ神経内 科に紹介された。会話は緩徐で言葉が出にくく、 仮面様顔貌を認めた。眼球運動と挺舌はほぼ正常、 舌の萎縮はなかった。頚部は固縮し、右上肢の肘 関節は屈曲位で自動運動がほとんどなく、ジスト ニアを認めた。利き手は右で書字は困難、左手は 観念運動失行を認めた。両下肢は右優位の筋力低 下と固縮があり起立は困難、四肢深部反射は亢進、 筋萎縮や線維束収縮はなく、感覚障害はなかった。 HDS-R 26/30。 頭部 MRI では左前頭から頭頂の萎 縮、SPECT では左中心前回の血流低下を認めた。 以上から臨床的に大脳皮質基底核変性症、原発性 側索硬化症を疑った。78歳から四肢は屈曲拘縮で 無動、言語はあいさつのみで、挺舌や眼球運動は 従命不可、強制泣きを認めた。79歳時には、意思 疎通困難となり、80歳嚥下障害のため経管栄養と なった。声をかけると視線を向けた。82歳肺炎で 死亡した。

【病理所見】肉眼的所見:脳重 1090g。左優位 の両側の中心前回、運動前野の高度萎縮、割面で は前頭葉皮質、白質、脳幹の萎縮、黒質、青斑核 の褪色を認めた。小脳歯状核は保たれ、脊髄前根 は肉眼的に保たれていた。組織学的所見:中心前 回、運動前野皮質は強い細胞脱落とグリオーシス を認め、皮質下白質はU-fiber を含めて萎縮淡明 化を示した。基底核では被殻、淡蒼球、視床下核、 視床の軽度の細胞脱落とグリオーシスを認めた。 黒質、青斑核の細胞脱落を認め、動眼神経核はよ く残存しNFT はみられなった。小脳歯状核、上小 脳脚はよく保たれていた。脊髄は前側索の萎縮、 淡明化を認め前角は萎縮しグリオーシスを認め たが、大型の運動ニューロンの細胞は萎縮してい るものが多いものの、細胞脱落の程度は比較的軽 度に留まっていた。Bunina 小体の出現はみられず、 ニッスル小体は比較的保たれるものが散見され た。錐体路は中心前回から皮質下白質、内包後脚、 大脳脚錐体路、橋縦束、延髄錐体、脊髄錐体路と 連続した変性を認めた。

Gallyas-Braak, AT8、RD4 免疫染色では皮質、 白質、基底核、脳幹部などに oligodendroglia 優位に globular inclusion (GOI) を多数認め、 太い coiled body の形態も多くみられた。 Astrocyte の封入体は少数で、tufted-astrocyte や astrocytic plaque はなかった。大脳皮質の神 経細胞には少数の pretangle を認めた。脊髄前角 には GOI とともに大型運動ニューロンにも G-B, AT8, RD4 陽性封入体を認めた。GOI などのグ リア封入体の出現は中心前回、運動前野に多数認 め、それ以外の前頭側頭葉では出現量はかなり減 少した。

リン酸化ニューロフィラメントの免疫染色で は、脊髄錐体路の軸索脱落は大径小径ともに高度 であったが、高度な脱落であるにもかかわらず大 径線維の残存傾向を認めた。脊髄前根の大径軸索 は頚髄、胸髄、腰髄のいずれのレベルでも残存を 認めた。グルタール固定した脊髄錐体路は有髄線 維の強い脱落を認めるが、大径線維の一部残存を 認め、前根においても大径有髄線維の残存を認め た。リン酸化 TDP-43 の免疫染色では、海馬、海 馬傍回、前頭葉の神経細胞やグリア細胞に dot 状 の陽性像を認め、脊髄前角にも少数の dot 状の陽 性像を認めた。

老人性変化はNFT/AT8 stage III, argyrophilic grain は Saito stage III。以上の所見から本例 は GGT と病理診断した。

## 【遺伝子検索】

凍結保存脳から解析したタウの遺伝子には MAPT遺伝子変異は確認されなかった。APOEタ イピングは 3/3 であった。

#### D. 考察

GGT は、従来分類不能とされていた 4 リピート (4R) タウオパチーの中から、グリアに小球状の タウ封入体が蓄積する一群として Kovac らにより 提唱された疾患である<sup>1,2)</sup>. GGT は多彩な症状を 示し、前頭側頭型認知症 (frontotemporal dementia: FTD)、進行性非流暢性失語症

(progressive non-fluent aphasia: PNFA), 進 行性核上性麻痺 (progressive supranuclear palsy: PSP), 大脳皮質基底核変性症

(corticobasal degeneration: CBD), PLS など と臨床診断される. GGT は病理学的に, 封入体が 前頭側頭葉に分布するタイプ I, 運動野と錐体路 に分布するタイプ II, 前頭側頭葉と錐体路の両方 に分布するタイプ III の 3 型に分類される。本例 は運動野と錐体路に高度の変性、前頭側頭葉と脊 髄前角にも GOI などの広がりがあることからタイ プ III に相当すると考えられた。GGT には PLS や ALS との鑑別が問題となる症例が報告されている。 3, 4)

GGTにおける上位および下位運動ニューロン系 の障害が、異なる蛋白である TDP-43 proteinopathy による運動ニューロン系の障害 と類似しているのかあるいは相違があるのか、相 違があるとするとどの様な相違がありこれが臨 床像にどのように反映するのかが問題である。今 回の少数例の検討では、GGT では、大径軸索の選 択性障害はやや弱く、小径線維の障害がより優位 である可能性が示唆された。通常の ALS では錐体 路は大径優位に大径線維と小径線維が脱落する。 このため、TDP-43 proteinopathy による ALS より、 筋萎縮や筋力低下、あるいは筋電図での神経原性 変化などの臨床的な LMN の症候はより軽く、タウ と TDP-43 では異なる細胞障害機序が推測された。

#### E. 結論

タウオパチーの中で、新たな 4R タウオパチー の一群として確立された GGT では、TDP-43 proteinopathy に比して、大径軸索の障害がより 軽く、上位と下位運動ニューロン系の軸索障害の 選択性や程度に相違がある可能性が示唆された。 参考文献

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# F. 研究発表

## 1. 論文発表

佐々木良元他. 認知症を伴う筋萎縮性側索硬化症 の臨床像を呈した globular glial tauopathy の 1 剖検例(現在投稿中)

#### 2. 学会発表

吉田眞理他. 第 56 回日本神経病理学会総会学術 研究会 2015 年 6 月福岡市

#### G. 知的財産権の出願・登録状況

なし

# 内在性 TDP-43 遺伝子改変と筋萎縮性側索硬化症モデルへの応用

# 研究代表者 佐藤 俊哉 1)

#### 研究分担者 大久保 直1)、東 貞宏1)、小野寺 理2)

1) 北里大学医学部実験動物学 2) 新潟大学脳研究所

#### 研究要旨

Zinc-Finger Nuclease (ZFN) を用い、内在性 TDP-43 C 末領域の部分欠損マウス8系統を樹立した。 この系統は特性により3種に大別され(A~C系統)、150アミノ酸残基からなるC 末領域の中央部6 アミノ酸までの欠損(A系統)では、ホモマウスにおいても明らかな異常を指摘できなかったが、C 末領域の約半分程度を欠損(B・C系統)させると致死となり、TDP-43機能が失われた。蛋白レベルで 検討すると、前半部分欠損(B系統)では36kDaの変異蛋白が確認されたが、核から細胞質に局在が 変化し、核蛋白としての TDP-43機能が失われた。一方、後半部分欠損(C系統)では僅かなバンドが 確認されるのみであり、変異蛋白が何らかの品質管理を受けていると考えられた。これらの結果から、 TDP-43C末領域の前半部分は機能維持に重要であり、後半部分は蛋白の安定性維持に重要な領域であ ることが明らかとなった。

#### A. 研究目的

筋萎縮性側索硬化症 (ALS) は、運動神経特異 的な変性を特徴とする神経難病である。2006年秋、 孤発性 ALS に認められるユビキチン陽性封入体 (skein-like inclusion) の主要な構成蛋白とし て TAR DNA-binding protein 43 kDa (TDP-43) が 発見された。さらに研究分担者の小野寺らは、常 染色体性優性遺伝形式を示す家族性 ALS の原因遺 伝子として TDP-43 を再発見し、ALS の発症機序に おいて TDP-43 が一次的な役割を担うことを明ら かにした。しかし現在までに多くの TDP-43 変異 が報告されているが、その変異が TDP-43 の C 末 領域に集中している理由は不明である。そこで人 工制限酵素技術の先駆けとなった Zinc-Finger Nuclease (ZFN) を用い、内在性 TDP-43 C 末領域 欠損マウスの作成を計画した。この研究により、 C 末領域の生理的機能を理解するとともに、その 理解に基づき、全く新しい ALS モデルの開発を進 めることを目的とする。

B. 研究方法(倫理面への配慮を含む)

第1世代のゲノム編集技術である ZFN を用い、 TDP-43C末領域の中央部を標的とした部分欠損マ ウスを作成し、その変異の効果を検討した。 TDP-43の残存活性に関しては、既に TDP-43ノッ クアウトマウスが着床早期に死亡することが報 告されていることから、各系統のホモ接合体マウ スを作成し、その死亡時期により評価した。次に ヘテロ接合体マウスの大脳から mRNA を抽出し、 qPCR 法を用いて TDP-43 mRNA 発現量を測定するこ とにより、オートレギュレーション機構の関与を 検討した。また変異 TDP-43 蛋白の細胞内局在変 化などを検討するため、細胞分画とウェスタンブ ロット、あるいは病理学的解析により解析した。

なお、本研究における遺伝子組換え実験は、北 里大学医学部遺伝子組換え実験安全管理委員会、 および北里大学遺伝子組換え実験安全委員会の 審査と学長の承認を受けて実施した。また動物実 験は、北里大学医学部動物実験委員会の審査と医 学部長の承認を受けて実施した。

#### C. 研究結果

ZFN を用いて内在性 TDP-43 C 末領域の部分欠損 マウス 8 系統を樹立した。この 8 系統を塩基配列 から推定される蛋白構造により分類すると、150 アミノ酸残基からなる TDP-43 C 末領域の(A) 6 アミノ酸までの欠損、(B) 前半部分欠損、(C)後 半部分欠損の 3 種に大別され、グループ毎に異な る特性が認められた。

6 アミノ酸までの欠損(A 系統)では、ホモマ ウスは正常に生まれ、明らかな表現型の異常も認 められなかった。ウェスタンブロットでは、アミ ノ酸欠損に応じたバンドシフトが認められたが、 TDP-43 mRNA 量は野生型と変わらず、TDP-43 機能 には著明な変化は無いものと推定された。

一方、TDP-43C末領域の約半分程度を欠損(B・ C 系統) させると、ホモマウスが主に着床前後に 致死となり、TDP-43機能が失われた。両系統のへ テロマウスは、体格の小さい1系統を除き、正常 に生まれた。全ての系統で TDP-43 mRNA 量は野生 型に比して増加し、TDP-43機能の低下を補正する オートレギュレーション機構の関与が示唆され た。しかしヘテロマウスの脳を用いて蛋白レベル で検討すると、両系統に大きな相違があった。前 半部分欠損(B系統)では、36 kDaの変異蛋白が 確認されたが、核から細胞質に局在が変化し、核 蛋白としての TDP-43 機能が失われることが明ら かとなった。他方、後半部分欠損(C系統)では 僅かなバンドが確認されるのみであり、変異蛋白 が何らかの品質管理を受けている可能性が高い と考えられた。

最後に、B系統に認められた 36 kDa の変異蛋白 の挙動を病理学的に解析したが、変異蛋白は細胞 質内に比較的均一に分布し、明らかな凝集体形成 は認められなかった。

#### D. 考察

TDP-43C末領域は、グリシンリッチ領域(GRR)、 Q/N 領域、C 末の終端領域に分類することができ るが、その3領域全てに ALS 変異が認められる。 しかし、この3領域の機能的差異は明らかではな く、領域内に生じた変異による機能的変化を推定 することも難しい。本研究から明らかにされた点 は、GRR と Q/N 領域の約半分を含む TDP-43C 末領 域の前半部分が機能維持に重要であること、Q/N 領域の残りとC末の終端領域を含む後半部分が蛋白の安定性維持に重要であることが明らかにされた。特に強調すべきは、36 kDa の前半部分欠損 蛋白の挙動で、核移行シグナル(NLS)が残っているにも関わらず核内に移行しない現象で、この 原因を検索することが、TDP-43の核内外シャトル 機構の解明、さらに細胞質内での凝集体形成機構 の究明につながる可能性がある。

本研究は。新潟大学脳研究所で樹立したマウス を用いた結果であるが、既に報告された培養細胞 による結果とは大きな乖離がある(Ayala et al. *J Cell Sci* 2008 121: 3778-85)。これが意味す ることは、TDP-43の生理的機能を正確に理解する ためには個体レベルでの解析が必須であり、新潟 大学脳研究所のリソースを用いた共同研究を継 続することが重要であると考えている。

#### E. 結論

TDP-43C末領域の前半部分は機能維持に重要で あり、後半部分は蛋白の安定性維持に重要な領域 であることが明らかとなった。

F. 研究発表(上記課題名に関するもの) 1. 論文発表 該当なし

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#### 3. 学会発表

該当なし

G. 知的財産権の出願・登録状況(予定を含む) 該当なし

# アメリカ平原ハタネズミ(Prairie Vole)からの ES 細胞樹立条件の検討、 及び CRISPR/Cas9 法による遺伝子 KO ハタネズミ作出の試み

研究代表者 西森 克彦 1) 研究分担者 崎村 建司 2)

1) 東北大学農学研究科·応用生命科学専攻 2) 新潟大学脳研究所細胞神経生物学分野

#### 研究要旨

高度な社会性を持つ北米原産平原ハタネズミ(ハタネズミ)は、社会性低下を伴う神経疾患研究のモ デル動物として注目されるが、遺伝子変換系が整備確立されていない大きな弱点を持つ。本共同研究 で我々は遺伝子 KO ハタネズミ作成を目指し、ハタネズミ ES 細胞,又は同 iPS 細胞の樹立と、生殖工 学確立による胚盤胞への当該細胞導入によるキメラ作成より、遺伝子操作ハタネズミを作成する技術 開発を計画した。並行し、受精卵への CRISPR/Cas9 法適用によるオキシトシン受容体(OXTR)遺伝子 KO ハタネズミ作成を計画した。ES/iPS 細胞樹立に関しては、正常初代繊維芽細胞へ6 因子を PiggyBac Transposon により導入し、三胚葉分化能等を保持しマウス ES 細胞の性質と極めて近いハタネズミ iPS 細胞を樹立した。また、CRISPR/Cas9 法により、受精卵より OXTR 遺伝子 KO ハタネズミを取得した。

#### A. 研究目的

社会性の低下を伴う神経疾患のモデル動物と して高度な社会性を持ちながら齧歯類として扱 いが容易であるアメリカ平原ハタネズミ (Prairie vole) より、その遺伝子を破壊・操作 したモデル動物を作成し、社会性を支える遺伝子 基盤、神経回路基盤などの研究を進めることは、 自閉症を始め、統合失調症や鬱病、認知症など、 社会性の低下を伴う様々な神経疾患の基礎的研 究や、こうした症状の回復を狙った精神医薬開発 などに大きな可能性を与える事となる。この為、 (1) ハタネズミ ES 細胞(又は iPS 細胞)を樹立し、 ES 細胞での相同組み替えによる遺伝子変換と、キ メラハタネズミ作成を経由した、遺伝子変換ハタ ネズミ作成の可能性、及び、(2)ハタネズミ生殖 工学を樹立し、これにより得たハタネズミ受精卵 を利用、CRISPR/Cas9 技術のハタネズミ受精卵へ の直接適用により、ES細胞やiPS細胞、そして胚 盤胞キメラ等を経由せずに、遺伝子 KO ハタネズ ミ作成の技術を開発する事、を本研究の研究目的 とした。その為、①ハタネズミ iPS 細胞の樹立を 試み、並行して②ハタネズミ受精卵へガイド RNA と Cas9mRNA を導入する事で、OXTR 遺伝子の破壊 を試みる事とした。

#### B. 研究方法(倫理面への配慮を含む)

27 年度は、iPS 細胞樹立の為の最新技術をハタ ネズミ胚より得た初代繊維芽細胞に適用し、 Piggybac transposon 骨格に 0ct3/4、Sox2、Klf4、 c-myc、Lin28、Nanog の 6 種のリプログラミング 遺伝子を搭載した PB-6F ベクターを作製、これを 用いてリプログラミング遺伝子をハタネズミ繊 維芽細胞に導入した。

一方、ハタネズミへの過排卵処置と、受精卵の 取得法の確立に努め、また過排卵処置後の未受精 卵を得て、ハタネズミ精子を利用した in vitro fertilization を行うなど、ハタネズミ受精卵の 取得方法を検討、確立することとした。さらに、 この受精卵へ、CRISPR/Cas9 法を適用し、ハタネ ズミオキシトシン受容体(OXTR)遺伝子を直接破 壊する事を試みることとした。

#### C. 研究結果

ハタネズミ胚より得た初代繊維芽細胞に
Piggybac transposon 骨格に Oct3/4、Sox2、Klf4、
c-myc、Lin28、Nanog の6種のリプログラミング
遺伝子を搭載した PB-6Fベクターを導入したところ、三胚葉分化能を持ち、核型が正常で、諸性質がマウス ES 細胞に酷似したハタネズミ iPS 細胞を樹立した。

一方、ハタネズミに対する過排卵処理法、ハタ ネズミ精子の凍結保存法、ハタネズミ卵子の in vitro fertilization 法、ハタネズミ受精卵のハ タネズミ卵管、又は子宮への移植による,後代ハ タネズミの取得等に成功し、ハタネズミ生殖工学 を確立した。また過排卵処置後のハタネズミ受精 卵へ CRISPR/Cas9 法を適用し、OXTR 遺伝子の破壊 されたハタネズミ (OXTR 遺伝子 KO ハタネズミ) の作製に成功した。

#### D. 考察

CRISPR/Cas9 法は、ハタネズミ受精卵に対して 極めて有効であり、遺伝子組 KO ハタネズミ作成 に成功した。しかし、現在得ているハタネズミ作成 殖工学は、決して十分なレベルの受精卵、又は未 受精卵を得られるレベルとは言い難く、季節やそ の他未知の因子・条件によりその受精卵や、未受 精卵の収量は著しく変動する。従って、その誘導 方法や取得条件についての検討を更に進めてい く必要がある。

今回樹立したハタネズミ iPS 細胞は、マウス ES 細胞に類似した性質を示し、今後ハタネズミ胚 盤胞への導入によるキメラハタネズミ作成を経 由した、遺伝子操作ハタネズミ作製法が確立され る可能性を示した。

また、CRISPR/Cas9 法により、単純な遺伝子 K0 だけでなく、短い DNA 配列(FLAG や LoxP など)、 或いは長い DNA 配列(EGFP や Td-Tomato などの蛍 光マーカーなど)の特定遺伝子領域への正確な導 入についての試行も、充分高いその可能性を示し ている。

#### E. 結論

ハタネズミ生殖工学樹立とCRISPR/Cas9法により、OXTR遺伝子KOハタネズミを作製した。また、 良好な性質を持つハタネズミ iPS 細胞を樹立した。

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# G. 知的財産権の出願・登録状況(予定を含む)

- 1. 特許取得
  - なし
- 2. 実用新案登録
- なし
- 3.その他
  - なし

# パーキンソン病治療における標的タンパク質としての Inhibitory PAS Domain

# Protein の検証

研究代表者 十川 和博 1) 研究分担者 葛西 秋宅 1)、柿田 明美 2)

1) 東北大学大学院生命科学研究科 2) 新潟大学脳研究所

#### 研究要旨

Inhibitory PAS Domain(IPAS)は低酸素遺伝子応答を抑制する転写因子であり、ミトコンドリアでのアポト ーシスを促進する因子としても機能する。われわれは IPAS のアポトーシス活性がパーキンソン病(PD) での神経細胞死に関与することを解明した。さらに培養細胞系で DNA トランスフェクションによって 一過性に発現した IPAS が紫外線照射によって活性化した p38 MAPK 経路の MKII によってリン酸化さ れること、このリン酸化は IPAS の C 末端領域で起きること、さらにこのリン酸化によってミトコンド リアのクラスター化とアポトーシス活性が増加することを見出した。パーキンソン病でキーとなる酸化 ストレスは、NF-κB を介して IPAS を転写活性化し、さらに翻訳後修飾によって制御していることが明 らかになった。

# A. 研究目的

パーキンソン病は酸化ストレスの亢進によっ て、引き起こされることが報告されている。酸化 ストレスは MAPK の活性化を誘導することが知 られているので、IPAS が MAPK によってリン酸 化される可能性を検討した。

#### B. 研究方法(倫理面への配慮を含む)

HEK293T 細胞などの培養細胞に JNK と p38 の 活性化を引き起こす紫外線を照射し、IPAS による ミトコンドリアのクラスタリングとカスパーゼ 3 の活性化を、抗体染色によって解析した。また、 関与するプロテインキナーゼを特異的な阻害剤、 siRNA によるノックダウンによって同定した。 IPAS の欠失変異体を用いて、リン酸化領域を推定 した。

#### C. 研究結果

紫外線照射により、HEK293T 細胞にトランスフ ェクションにより発現した IPAS の C 末端領域が 修飾されることが欠失変異体を用いて判明した。 特異的な阻害剤を用いてリン酸化酵素を特定し たところ、JNK の阻害剤は阻害せず、p38 MAPK の阻害剤と、それによるリン酸化で活性化する MKII の特異的阻害剤が IPAS によるミトコンドリ アのクラスター化や、アポトーシスをほぼ完全に 阻害し、MKII が直接のリン酸化酵素であること が判明した。また、MKII の siRNA によるノック ダウンも紫外線の効果を阻害した。

#### D. 考察

本研究を通じて、パーキンソン病に関与する酸 化ストレスは、IPASの転写活性化を促進するほか に、同ストレスで活性化する p38 MAPK 経路を介 して、IPASのアポトーシス活性を促進することが 判明した。酸化ストレスは2重に IPAS 活性を調 節していることになる。現在リン酸化部位を特定 中であり、特定後、リン酸化抗体を作成し、ヒト パーキンソン病患者脳において、IPAS リン酸化が コントロール脳に比較して増強していることを 解明する。

## E. 結論

IPAS は酸化ストレスで活性化する MKII によって C 末端領域がリン酸化された。この修飾によって IPAS のアポトーシス活性は亢進した。

#### **F. 研究発表**(上記課題名に関するもの) **1. 論文発表**

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# 2. 学会発表

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- G. 知的財産権の出願・登録状況(予定を含む)
- 1. 特許取得
- なし
- 2. 実用新案登録
- なし
- 3. その他
- なし

# APP 細胞内ドメインの神経毒性の解析

研究代表者	中山	耕造1)
研究分担者	長瀬	<sub>尚志</sub> 2)
	笹岡	俊邦 <b>3)</b>

1) 信州大学医学部医学科・人体構造学講座、2) 信州大学医学部医学科・病理学講座、3) 新潟大学 脳研究所附属生命科学リソース研究センター・バイオリソース研究部門・動物資源開発研究分野

#### 研究要旨

我々は Notch や Delta の解析から、 $\gamma$ -セクレターゼ本来の生理機能は1型膜蛋白質のシグナル伝達の 調節にあるという仮説を提唱している。さらに、amyloid precursor protein (APP) も Notch に類似し た  $\gamma$ -セクレターゼによって制御されるシグナル伝達機構を持ち、それがアルツハイマー病(AD)の発 症に関係していると考えている。実際、 $\gamma$ -セクレターゼによって APP が切断されるときに Aβと同時 に生じる細胞内ドメイン (AICD) が核へ移行し、遺伝子発現を変化させ、神経細胞選択的にアポトー シスを誘導することを細胞レベルで明らかにしている。これらの結果は、AD の原因だと考えられて いる Aβ以外にも、APP を介した AD 発症に関与する機構が存在することを示唆している。

AICD によって引き起こされる神経細胞選択的アポトーシスの機序を解明することを目標に、本年 度は AICD 核移行検出系の構築、及び核移行を制御していると考えられる AICD 脱リン酸化酵素の解 析をおこなった。

#### A.研究目的

我々は、Notch や Delta の解析から、γ-セクレタ ーゼ本来の生理機能は amyloid precursor protein (APP)を含む1型膜蛋白質のシグナル伝達の制 御にあり、それが APP のシグナル伝達を通してア ルツハイマー病(AD)の発症に関係していると推 測している(総説: Nakayama et al., Cell Mol. Neurobiol., **31**,887,2011)。実際に我々は、γ-セクレ ターゼで細胞膜から細胞質へと切り出された APP の細胞内ドメイン (AICD)が核へ移行し、 遺伝子発現を大きく変化させ、神経細胞選択的に アポトーシスを引き起こす事を示している(総 説: Nakayama et al., Current Psychopharmacology, **1**,155,2012)。

これらの結果を基に、本研究の目的は、AD に おける新規創薬ターゲット分子の検索を念頭に、 AICD の神経毒性の分子機序、特に細胞質から核 への移行の過程を解明することにある。

AICD の核移行には、アダプター蛋白質 Fe65 との結合が必須であることが知られている。また 我々は、AICD と Fe65 との結合には AICD の脱リ ン酸化が必要ではないかと考えている。本年度は 将来的に必要となる AICD 核移行阻害物質のスク リーニング系の確立を目指して(1) AICD 核移 行検出系の構築を行った。加えて、前年度から続 けている(2)AICD 脱リン酸化酵素の解析を行っ た。

B.研究方法(倫理面への配慮を含む)。

(1) AICD 核移行検出系の構築

Fe65に依存した AICD の核移行を検出する系を 構築するために、AICD の前に Gal4 を繋いだ発現 ベクターを構築した。このベクターの場合、γ-セ クレターゼによる切断がなくてもフュージョン 蛋白質(Gal4-AICD)が細胞質に存在することに なる。つまり通常シグナルが伝わるときに起こる、 γ-セクレターゼによる AICD の細胞膜から細胞質 への切り出しを必要とせず、構成的にシグナルが 伝わることになる。

Gal4 依存性のプロモーターとルシフェラーゼ cDNA を持つレポータープラスミドを核内に持つ 細胞に、この発現ベクターを導入した場合、 Gal4-AICD が核に移行するとルシフェラーゼが発 現する事になる。

#### (2) AICD 脱リン酸化酵素の同定

各分画の脱リン酸化活性の測定に用いる基質は、 大腸菌で産生したリコンビナント AICD を [<sup>32</sup>P]-γ-ATPを用いてin vitro で<sup>32</sup>P ラベルすること によって作製する予定であったが、この方法では 効率よくラベルする事ができなかった。そこで、 <sup>32</sup>P を用いてメタボリックラベルを行なった。具 体的には、HEK293T 細胞に、FLAG タグを持つ AICD の発現ベクターを導入して FLAG-AICD を 発現させた。遺伝子導入1日後、リン酸を含まな い培養液に、5µg/mlのNocodazoleと1mCi/mlの<sup>32</sup>P リン酸を添加した培養液に置換してさらに一日 培養を行なった。培養の後、抗FLAG 抗体カラム を用いて細胞のライセートから FLAG-AICD を精 製し、脱リン酸化アッセイのための基質として用 いた。

脱リン酸化酵素の精製は、1回の実験について 約50匹のマウスの脳(約25g)をホモジェネート し、遠心して約20mlのライセートを得て、これ を10mlのDEAE sepharoseカラムに添加した。カ ラムからの溶出は、NaClの濃度勾配によっておこ なった。さらに、AICD 脱リン酸化酵素活性を持 つDEAE sepharoseフラクションを20mM リン酸 カリウムバッファーに対して透析し、2mlの Creamic Hydroxyapatiteカラムに添加した。カラム からの溶出は、リン酸の濃度勾配によっておこな った。

各フラクションの脱リン酸化酵素活性は、各 フラクションに前述の<sup>32</sup>P ラベルした AICD を加 え測定した。

なお、動物を用いる実験は、信州大学動物実験 等実施規程に基づいておこなった。

#### C.研究結果

#### (1)AICD 核移行検出系の構築

サルの腎臓由来の Cos7 細胞と、マウスの神経 由来の N2a 細胞に Gal4-AICD を発現させ、Fe65 の共発現の有無でルシフェラーゼ活性を比較し た。なお、インターナルコントロールとしては、 CMV プロモーターとクラゲルシフェラーゼを持 つベクターを用いた。

AICD を持たない Gal4 のみ発現させた場合、 Fe65 の有無にかかわらず、どちらの細胞でもルシ フェラーゼ活性は検出できなかった。

Gal4-AICD を発現させても、両方の細胞におい て、Fe65 を共発現させなければ極めて低いルシフ ェラーゼ活性しか示さなかった。しかしながら、 どちらの細胞においても、Fe65 を共発現させると 10 倍以上に活性の増強が見られた。

#### (2) AICD 脱リン酸化酵素の同定

マウス脳のライセートを DEAE sepharose カラ ムで分画し場合、225-300mM の NaCl で溶出した フラクションに AICD 脱リン酸化酵素活性が認め られた。

これらのフラクションをさらに Hydroxyapatite カラムに吸着させ、リン酸濃度を上げる事により 蛋白質を溶出した。その結果、幅広い範囲(150mM から 300mM)のフラクションが AICD 脱リン酸化 活性を示した。

溶出されたフラクションの幅が広いために、 Hydroxyapatite で分画できていない可能性が考え られた。しかしながら、各フラクションを SDS 電 気泳動して銀染色をおこなったところ、フラクシ ョンでバンドのパターンは異なっており、分画自 体はうまくいっていた。

電気泳動では、AICD 脱リン酸化酵素活性と相 関するバンドは検出できず、さらなる精製が必要 でありゲルろ過を行ったところ、活性を検出でき なくなった。現在、その原因を調べている。

#### D.考察

#### (1) AICD 核移行検出系の構築

Gal4-AICD を発現させても Fe65 を共発現させ ないとルシフェラーゼ活性が検出できないとい う結果は、構築した系において AICD の核移行に Fe65 が必須である事を示している。つまり、ここ で検出されたルシフェラーゼ活性は、細胞質に存 在する Gal4-AICD に Fe65 が結合したために Gal4-AICD が核に移行して、レポーターが持つ Gal4 依存プロモーターを活性化させ、ルシフェラ ーゼの発現を誘導したと考えられる。

現在のところ我々は、AICD は構成的にリン酸 化されており、AICD と Fe65 の結合には AICD 自 体の脱リン酸化が必要だと考えられる結果を得 ている。Phos-tag による電気泳動を行ったところ、 このアッセイ系では AICD を高発現させたために、 大半の AICD はリン酸化されていなかった。その 結果、脱リン酸化なしに AICD が Fe65 と結合で きたのではないかと考えている。従ってこの系は、 Fe65 に依存した AICD の核移行を反映しており、 AICD 核移行阻害物質のスクリーニング等に有用 だと考えられる。

(2) AICD 脱リン酸化酵素の同定

前述のように我々は、AICD は構成的にリン酸 化されており、AICD と Fe65 の結合には AICD 自 体の脱リン酸化が必要だと考えている。本研究に おいて、マウスの脳のライセート中に AICD 脱リ ン酸化活性を検出した。このライセートを DEAE sepharose カラムで分画したところ、225-300mM の NaClで溶出したフラクションに高い AICD 脱リン 酸化活性が認められた。これらの結果は、AICD 脱リン酸化酵素が実際に存在することを示して おり、この酵素が AICD の核移行を調節している という仮説が正しいことを示唆していると考え ている。

AICD 脱リン酸化活性が認められた DEAE フラ クションを Hydroxyapatite カラムを用いて分画し たところ、150mM から 300mM のリン酸で溶出し たフラクションに AICD 脱リン酸化活性が認めら れた。電気泳動では、AICD 脱リン酸化酵素活性 と相関するバンドは検出できず、さらなる精製が 必要であり、ゲルろ過により精製を進めたところ、 活性が検出できなくなった。現在、活性が検出で きなくなった原因を調べている。

なお、カルシニューリンは脳で極めて多い脱リ ン酸化酵素である。この酵素が AICD 脱リン酸化 酵素である可能性を検討するためにリコンビナ ントカルシニューリンを用いて検討したが、 AICD 脱リン酸化活性は検出できなかった。従っ て、我々が同定しようとしている酵素は、カルシ ニューリンではないと考えられた。

#### E.結論

(1) AICD 核移行検出系の構築

Fe65 に依存した AICD の核移行を、ルシフェラ ーゼを測定することにより高感度で検出する細 胞系を構築した。この系は、AICD 核移行阻害物 質のスクリーニング等に有用だと考えられた。

#### (2) AICD 脱リン酸化酵素の同定

AICD 脱リン酸化酵素の同定を目標に、蛋白質 の精製を進めている。この酵素を同定できた場合、 実際に AICD の核移行及び神経細胞特異的な細胞 死に関わっていることを確認するために、精製し た蛋白質の情報を基に cDNA をクローニングし、 それを培養細胞に発現させて検証する予定であ る。

# F.研究発表(上記課題名に関するもの) 1.論文発表

なし

#### 2.学会発表

なし

#### G.知的財産権の出願・登録状況(予定を含む)

1.特許取得なし
 2.実用新案登録なし
 3.その他なし
# 意思伝達不能状態(Stage V、TLS)の筋萎縮性側索硬化症の 臨床病理学的検討

# 研究代表者 望月葉子1)2)

**研究分担者** 林健太郎 3)、竹内亮子 4) 5)、高橋 均 5)、柿田明美 5)、渡部和彦 6)、小柳清光 7)、 小森隆司 1)、磯崎英治 3)

都立北療育医療センター内科・神経内科 2)都立神病院検査科 3)都立神病院脳神経内科
 4)新潟大学脳研究所神経内科 5)新潟大学脳研究所病理学分野

· 利為人子脑研先別怦徑的科 · 利為人子脑研先別柄理子分野

6) 都医学研運動・感覚システム研究分野 7) 信州大学医学部神経難病学

### 研究要旨

意思伝達不能(Stage V)になった ALS 例の臨床病理学的な特徴について大脳皮質病変を中心に検討した。対象は TDP-43 陽性封入体を有する 6 例、SOD1 陽性封入体を有する 3 例と FUS 陽性封入体 を有する 2 例。大脳萎縮の有無は蓄積蛋白の違いでは説明できなかった。TDP-43 陽性例では海馬歯状 回に NCI のない例では軽度な変性が前頭葉に限局していたが、NCI のある例では大脳皮質に変性と多数の NCI が出現していた。FUS 陽性例では、逆に大脳皮質の変性が高度な例で海馬歯状回顆粒細胞に NCI はなかった。SOD1 陽性例の 3 例の大脳皮質は比較的保たれていた。したがって、Stage V に至る 病理学的背景は、大脳病変よりも、全例に共通していた高度な運動ニューロン変性に加えた黒質、淡 蒼球、視床下核、脳幹網様体、小脳出力系の変性と考えられた。

### A.研究目的

筋萎縮性側索硬化症(ALS)例のなかで、人工 呼吸器を装着して外眼筋も含めた全随意筋が完 全麻痺し、現在の補助手段では意思伝達不能にな った(意思伝達能力 Stage V、完全閉じ込め症状状 態: totally locked-in state; TLS) 症例の臨床経過、 神経病理所見を多数例で検討し、このような症例 の臨床病理学的な特徴とその多様性の有無を明 らかにする。平成26年度には、StageVの症例に は、発症2年以内に呼吸器を装着した例が多く、 家族性/遺伝子変異 ALS のほか、TDP-43 陽性封入 体を有する孤発例があること、高度な運動ニュー ロン変性と運動ニューロン系を超えた広汎な変 性を全例で呈し、その病変分布には共通性と多様 性があり、蓄積蛋白の違いでは分類できないこと を明らかにした。今年度は、蓄積蛋白ごとの違い をさらに検討し、神経病理学的特徴を明らかにす ることを目的とした。

#### B.研究方法

昨年度抽出した意思伝達能力 Stage V の 11 例に ついて、神経細胞・神経線維の脱落、グリオーシ スの程度と免疫染色標本により、TDP-43、SOD1、 または FUS による神経細胞質内封入体(NCI)の 出現について検索した。

### C.研究結果

対象は 11 例で、発症から呼吸器装着までの期間は平均 17.1 ヶ月であった。早期から行動障害、 感情障害、言語障害を生じた前頭側頭型認知症例 はなかった。

神経病理学的には、全例で上位及び下位運動ニ ューロンの非常に高度な変性に加えて黒質、淡蒼 球、視床下核、脳幹網様体に中等度以上変性があ った。小脳歯状核、上小脳脚の変性も全例に見られたが、軽度な症例はStage Vに至ってから死亡 までの期間が短かった。変性が高度な部位では神 経細胞脱落が高度なために、NCIの評価をできな かった。

大脳萎縮が高度であったのは TDP-43 陽性の 3 例と FUS 陽性の 1 例で、その他の例では大脳は比較的保たれていた。

大脳皮質病変については、TDP-43 陽性例では 海馬歯状回に NCI がない例で変性は前頭葉に限 局して脳重は保たれていた。これに対し、NCI が ある例では側頭葉、海馬支脚と大脳皮質に変性が あり、NCI は大脳皮質にも広汎に確認され、この うちの 3 例は高度な大脳萎縮を呈していた。FUS 陽性例では、p.P525L 変異例は前頭葉に強い高度 な大脳萎縮があり、compact NCI が大脳皮質に多 数出現していたが、海馬歯状回顆粒細胞に NCI は なかった。大脳萎縮が目立たなかった p.K510M 変 異例は、小脳入力系の変性も伴っていた点が特徴 で、non-compact NCI が海馬歯状回顆粒細胞にも 出現していた。SOD1 陽性例の 3 例の大脳皮質の 神経細胞脱落はなく、1 例で少数の NCI が側頭葉 に見られた。

### D.考察

病理学的には、全例で上位及び下位運動ニュー ロンの非常に高度な変性に加えて黒質、淡蒼球、 視床下核、脳幹網様体に中等度以上変性があった。 小脳歯状核、上小脳脚といった小脳出力系にも全 例で変性があり、これらの変性が軽度な例はStage Vから死亡までの期間が短かったことから、病変 により出現する時期が異なる可能性があると考 えられた。以上の病変は、大脳病変の強弱や蓄積 蛋白の違いにかかわらず、共通していたので、こ れらの共通病変が、Stage V に至る病理学的背景と 考えられた。

Nishihira ら(2008) は TDP-43 陽性 NCI を持つ例 の ALS の病変を運動ニューロン系以外に変性の 乏しい type 1 と、運動ニューロン以外の変性も目 立つ type 2 に分類し、海馬歯状回の NCI は type 2 の指標となる事を報告している。TDP-43 陽性例 の大脳皮質病変も、海馬歯状回の NCI の有無で分 類可能であることが明らかになった。そして、海 馬歯状回に NCI のない例も運動ニューロンを超 えて広汎な変性を来し、Stage V となりうる事を明 らかにした。一方、FUS 陽性例では、大脳病変が 高度な症例で海馬歯状回に NCI がなく、大脳病変 が軽度な例で歯状回に NCI があった。これは、 TDP-43 例と逆の傾向であり、FUS 陽性例では、 海馬歯状回顆粒細胞の NCI が大脳の広範囲な病 変の指標にはならないことが明らかになった。 SOD1 例の大脳皮質は保たれる傾向がみられた。 SOD1 変異例に前頭側頭型認知症例の報告は少数 であり、SOD1 陽性例では大脳皮質病変が軽度な 例が多い可能性がある。

#### E.結論

Stage V に至った症例は、大脳皮質病変には多様 性があったが、高度な運動ニューロン変性に加え て、黒質、淡蒼球、視床下核、脳幹網様体、小脳 出力系の変性が共通しており、Stage V に至る病理 学的背景と考えられた。このことから、発症から 呼吸器装着までが早い症例では特に、運動ニュー ロン以外の症状の出現、画像による拡大病変の出 現に注意することが意思伝達能力の低下を予測 するうえで重要と考えられた。

### **F.研究発表**(上記課題名に関するもの) 1.論文発表 準備中。

#### 2.学会発表

林 健太郎、望月葉子、竹内亮子、小森隆司、高 橋 均、柿田明美、渡部和彦、関絵里香、新井信 隆、小柳清光、清水俊夫、長尾雅裕、磯崎英治: 意思伝達不能状態(stage V)となった筋萎縮性側 索硬化症(ALS)における大脳萎縮の免疫組織学 的検討. 第 56 回日本神経病理学会学術研究会、 2015 年 6 月 5 日、福岡。

### G知的財産権の出願・登録状況(予定を含む)

1.特許取得なし。
 2.実用新案登録なし。
 3.その他なし。

# 酸化ストレスによる神経細胞機能の障害と細胞死に関する研究

研究代表者	柴田	亮行 1)
研究分担者	野口	範子 <sup>2)</sup>
研究分担者	柿田	明美 3)

# <sup>1)</sup>東京女子医科大学病理学第一講座 <sup>2)</sup>同志社大学生命医科学部医薬分子機能学 <sup>3)</sup>新潟大学脳研究所脳疾患標本資源解析学分野

#### 研究要旨

アルツハイマー病(AD) 脳海馬におけるコレステロール-24-水酸化酵素(C240Hase)、カルモジュリ ンキナーゼ II (CaMKII)、リン酸化 CaMKII (p-CaMKII)、切断型カスパーゼ 8 (C-Casp8) およびリン 酸化 RIPK (p-RIPK)の局在と筋萎縮性側索硬化症(ALS)におけるセルロプラスミン(Cp) およびア コニターゼ 1 (ACO1)の局在を免疫組織化学的に解析した。AD 脳海馬では、CaMKII と RIPK のリン酸 化活性化が観察された。ALS 脊髄では、アストロサイトにおける Cp 発現亢進とミクログリアにおける ACO1の発現維持が観察された。AD、ALS のいずれの病変の主座においても、酸化ストレス亢進の関与 が示唆された。

### A. 研究目的

本研究の目的は、神経変性疾患の細胞死の様式 と細胞死における酸化ストレスの関与を明らか にすることにある。

### B. 研究方法(倫理面への配慮を含む)

本研究は、新潟脳研究所と東京女子医科大学の 各倫理委員会で、あらかじめヒト剖検材料を用い た研究計画書を提出して承認を得た後、開始され た。AD 脳海馬(5例)のホルマリン固定パラフィ ン包埋切片に対し、C240Hase、CaMKII、p-CaMKII、 C-Casp8、p-RIPKの各々に対する特異抗体を反応 させ、免疫反応産物をポリマー免疫複合体法で可 視化した。ブニナ小体の出現する孤発性筋萎縮性 側索硬化症(ALS)脊髄(5例)のホルマリン固 定パラフィン包埋切片に対し、Cp、AC01の各々に 対する特異抗体を反応させ、免疫反応産物をポリ マー免疫複合体法で可視化した。免疫反応産物の 局在は、連続切片における各種細胞マーカーの免 疫組織化学染色標本との比較により同定した。

### C. 研究結果

AD 脳海馬では、C240Hase と C-Casp8 に対する 有意な免疫活性は検出されなかったが、CaMKII 免 疫活性は全ての錐体細胞の細胞質に均一に分布 しており、p-CaMKII 免疫活性は約3分の2程度の 錐体細胞の細胞質に粗大顆粒状の分布を示し、 p-RIPK 免疫活性は少数の錐体細胞の細胞質に粗 大顆粒状の分布を示した。

ALS 脊髄では、Cp と ACO1 の発現様式に違いが みられた。Cp 免疫活性は脊髄前角の反応性アスト ロサイトに局在し、染色性が増強していた。これ に対し、ACO1 免疫活性は脊髄前角の活性化ミクロ グリアに局在していたが、染色性は増強していな かった。

### D. 考察

AD 脳海馬標本の観察から、一部の錐体細胞内部

でカルシウム流入の亢進を反映する CaMKII のリ ン酸化とネクロトーシスの実行部隊である RIPK のリン酸化が起こっているものの、ネクロトーシ スの秘儀金と目されている酸化コレステロール の産生酵素 C240Hase の発現ならびにデスシグナ ル起因性アポトーシス仲介因子 Casp8 の切断が起 こっていないことが判明した。p-CaMKII と p-RIPK のいずれに対する免疫活性も錐体細胞の細胞質 に粗大顆粒状構造物を形成していた意義づけは 現時点において困難である。

ALS 脊髄標本の観察結果から、中枢神経系の主 なCp発現の場であるアストロサイトがCpを過剰 発現する一方、ミクログリアにおける ACO1 の発 現量に増減がないことが判明した。近年、Cp は炎 症促進性サイトカイン IL-1βの刺激を受けた網膜 ミュラー細胞で発現レベルの増加が報告されて いる。これまでに報告された多くの ALS 研究では、 脊髄における IL-1β 産生を含む炎症活動亢進が指 摘されている。ミュラー細胞とアストロサイトの 類似性を考慮すると、脊髄内炎症反応がアストロ サイトの Cp 発現レベルを高めた可能性は十分あ りうる。一方、我々は最近、培養ミクログリアに 可溶性 Cp を添加すると培養上清中のグルタミン 酸が増加することを発見した。これは、細胞外か らの Cp 刺激を契機にミクログリア内部でグルタ ミン酸が産生され、細胞外へ放出されたことを意 味している。細胞内でグルタミン酸を産生する酵 素として近年、ACO1 が注目されている。ACO1 は 細胞質内の鉄イオン濃度が低いと不活性型であ る iron-regulatory protein (IRP) として存在す るが、鉄イオン濃度が上昇すると活性型となるこ とが知られている。一方、Cp は細胞外鉄イオンを 細胞内へ導く性質を有する。従って、細胞外 Cp の増加はミクログリア内部への鉄イオン流入を 介して ACO1 の酵素活性をもたらすことが予想さ れる。その際、ACO1の発現レベルに変動が起こら ないという実験結果が報告されている。従って、 本研究で AC01 の染色性に変動がみられなかった ことは頷ける結果である。

### E. 結論

AD 脳海馬における細胞内カルシウムイオン流入とネクロトーシスの証拠となる結果が観察された。

ALS 脊髄における Cp 発現亢進と AC01 活性上昇の関与が示唆された。

今回、パーキンソン病剖検脳を用いた解析が不十 分であったため、次年度の課題としたい。

### F. 研究発表(上記課題名に関するもの)

1. 論文発表

なし

### 2. 学会発表

なし

#### G. 知的財産権の出願・登録状況(予定を含む)

- 1. 特許取得: なし
- 2. 実用新案登録:なし
- 3. その他:なし

# ドパミン-D1R シグナルが心不全に果たす役割の解明

研究代表者	小室	一成 1)
研究分担者	笹岡	俊邦 2)
研究分担者	山口	敏弘 1)

1) 東京大学医学部循環器内科 2) 新潟大学脳研究所 生命科学リソース研究センター

### 研究要旨

神経伝達物質ドパミンは運動調節や情動に関わり、パーキンソン病等神経疾患の発症と治療に重要 な役割を持っている。一方、ヒトの心不全において、ドパミンアナログ、D1ドパミン受容体(D1R)ア ゴニストの長期投与はその予後を悪化させることが報告されているが、心臓におけるドパミンの生理 的作用や心不全における役割はほとんど明らかにされていない。本研究は、ドパミン-D1Rシグナルが 心不全の発症・進展に関与するという仮説を検証することで、心不全発症および進展における新たな 分子機構の解明に取り組むものである。我々は、心不全の進展とともに顕著に変化する遺伝子として D1Rを見いだし、その発現が主に心筋細胞に多く認められることから、現在、心筋細胞特異的 D1R 欠 損マウスやテトラサイクリン制御による D1R 発現マウスに心不全を誘導し、表現型を解析中である。

### A. 研究目的

本研究では、マウス心不全モデルを用い、不全 心においてドパミンが果たす役割を解析するこ とで、心不全発症・進展において働く分子細胞機 序の解明に取り組む。心臓における自律神経活性 化の重要性は以前から知られているが、本研究は 今まで解析されることが少なかった、自律神経に おいて産生されるドパミンとその受容体である D1R の役割について注目して解析することで心不 全における新たな病態生理を見いだすことを目 的とする。

### B. 研究方法(倫理面への配慮を含む)

申請者らは、心不全モデルマウスの心臓におい て D1R の発現が病態の進行とともに増加すること や、心臓におけるドパミンの spill over が増加 していることを示唆する予備的データを得てい る。これらの予備的データをもとに以下の点に注 目して研究を進めた。

① 不全心の D1R 発現心筋細胞の局在の同定

心不全の進行とともに mRNA レベルで心臓 D1R の 発現が増加するというデータを得てきたが、心臓 における D1R 高発現細胞の局在は同定しきれてい なかった。これまでイムノブロッティングや免疫 染色法を用いて D1R の局在の証明を図ってきたが、 心臓における D1R 発現量が多くないことから完全 にその局在を同定できるには至っていなかった。 そこで、申請者らは分担者より提供された LacZ ラベルされた D1R 強制発現マウス心臓組織を Xgal 染色することで D1R の発現細胞の局在同定を図っ た。

② 心筋一細胞レベルでの D1R の発現量解析

申請者らはランゲンドルフ法を用いて単離した 不全心筋細胞においても D1R が mRNA レベルで上 昇していることを確認している。申請者らの研究 室ではランゲンドルフ法で単離した心筋一細胞 毎の mRNA の発現量の違いを同定するシステムも 既に構築済みである。同システムを用いて心不全 時に D1R を高発現する細胞を解析することでその 特徴や意義に迫った。

③ In vivo における D1R シグナルの重要性

心筋特異的 D1R コンディショナルノックアウト (CK0) マウスを作成し、同マウスにおいて心不 全を誘導することで D1R が心不全の進行にどのよ うな影響を及ぼすのかを検証する。更には、分担 研究者の協力により得た、テトラサイクリン制御 による D1R 発現マウスに圧負荷心不全モデルを作 成し、(1)生理的機能解析、(2)分子生物学的解析、 (3)生存解析を行うことでドパミン-D1R シグナル が心不全の病態形成において果たす役割を検討 する。

### C. 研究結果

分担者の協力により、D1R 強制発現マウスを作 成し、同マウスに心不全を誘導の上、心臓 Xgal 染色を行ったところ、D1R を発現している細胞が び漫性・散在性に増加していることを見出した。 また、心筋一細胞レベルの RNA 解析でも、心不全 時に D1R を高発現している細胞は一部の心筋細胞 のみであることが明らかとなり、両者の結果は矛 盾しないものであった。現在、心筋細胞特異的 D1R ノックアウトマウスに心不全を誘導し解析を行 っている。

### D. 考察

心不全の進展に伴い発現が増加する遺伝子と して D1R を見いだし、その発現増加は限局的な心 筋細胞にのみ認められることを明らかにした。ま た、心筋細胞における D1R 刺激は CREB シグナル の活性化を誘導していることも明らかにしてい る。神経細胞や近位尿細管細胞においては D1R シ グナルは Na-K ATPase を制御していることが知ら れており、心臓においては心不全のみならず不整 脈発症に影響を与える可能性も考えられる。In vivo で心筋細胞におけるドパミン-D1R シグナル の阻害効果を明らかにするために心筋特異的 D1R ノックアウトマウスに現在心不全を誘導し、その 効果を解析中である。

今後は、ドパミン-D1R シグナルを活性化させた 心筋細胞がどの様な表現型を示すのかを in vitro で解析していく必要もある。現在アデノウイルス を用いて D1R を強制発現した心筋細胞を作成中で ある。同時に、分担者が進めているテトラサイク リン誘導型 D1R レスキューマウスの D1R 発現様式 と運動機能の解析の研究成果とあわせて検討す ることで、ドパミン-D1R シグナルの更なる役割の 理解を深めることにつなげていく

### E. 結論

心不全の進展とともにD1Rの発現が限局的な心 筋細胞において増加し、心筋細胞におけるドパミ ン-D1R シグナルは CREB シグナルの活性化を誘導 することを見いだした。今後の課題として、心筋 細胞特異的D1R ノックアウトマウスに対する心不 全誘導時の表現型解析を行うとともに、D1R の高 発現心筋細胞の in vitro における機能解析も必 要となってくると考える。

# F.研究発表(上記課題名に関するもの) 1.論文発表

なし

### 2. 学会発表

平成 27 年度 新潟大学脳研究所共同利用共同研 究 合同セミナー・平成 27 年 12 月 22 日・新潟

### G. 知的財産権の出願・登録状況(予定を含む)

特許取得
 該当なし
 実用新案登録
 該当なし
 その他
 特記事項なし

# 胎仔期および発達期の脳におけるドーパミン受容体 D1R の機能解析

研究代表者	大久得	₹ 直 <sup>1)</sup>	
研究分担者	佐藤	俊哉1)	
研究分担者	笹岡	俊邦 <sup>2)</sup>	

1)北里大学医学部実験動物学
 2)新潟大学脳研究所動物資源開発研究分野

#### 研究要旨

ドーパミン D1 受容体(D1R) は、運動を促進する役割があると考えられている。これまでに、コン ディショナル D1R 発現マウスを用いることにより、成熟マウスにおいて D1R の働きを抑制すると運動量 の減少が起こることが判明した。しかし、通常の D1R ノックアウトマウス(K0)では、運動の亢進が認め られる。このような行動発現の差は、胎仔期からの D1R の欠損が脳機能の正常な構築に影響を及ぼすこ とを示唆している。そこで本研究では、まず D1R を生後発達期において時期特異的にノックダウン(KD) し、その脳に及ぼす影響を形態学的・生化学的・行動学的に解析することを目的とした。生後母乳を介 してドキシサイクリンを飲水させた D1R KD マウスを用いて各種行動解析を行った。その結果、D1R がノ ックダウンされる時期により行動の表現型に違いが見られたことから、発生発達期と成体期における D1R の機能が異なる可能性が示唆された。

### A. 研究目的

ドーパミン D1 受容体(D1R) は、運動を促進 する役割があり、成熟マウスにおいてコンディシ ョナルに D1R の働きを抑制すると運動量の減少が 起こる。しかし、通常の D1R ノックアウト(KO)マ ウスでは、運動の亢進が認められる。このような 行動発現の違いは、胎仔期から D1R が欠損してい るか、あるいは成熟期になって欠損するかによっ て、脳機能や脳組織の構造に異なる影響を与えて いると示唆される。そこで本研究では、D1R を発 達期、および成体期に特異的にノックダウン(KD) して運動機能を行動レベルで解析し、D1R の機能 を明らかにすることを目的とした。

### B. 研究方法(倫理面への配慮を含む)

Tet-Off システムを導入したコンディショナ ル D1R 発現マウスの受精卵を仮親マウス(ICR系統)に移植後、ドキシサイクリンを飲水投与する ことにより、発達期および成体期のD1Rの発現を ノックダウンした。コントロールとして、野生型 マウス、D1R K0マウス、同時期に水のみを与えた コンディショナルD1R 発現マウスを用い、各種行 動解析を行った。KDマウスは自力での摂餌が困難 になり衰弱する可能性があるので、その場合は実 験をやめ安楽死とした。

### C. 研究結果

平成26年度に検討した、発達期におけるD1RKO

の条件に沿って、生後0日からドキシサイクリン を母乳を介してあるいは直接飲水によって投与 した。D1Rの発現が検出不可能なレベルに達した 時期に、D1RKDマウスと他のコントロールマウス の行動の表現型を比較した。行動解析として、オ ープンフィールドテストによる新奇環境での活 動性、さらにバランスビームテストによる協調運 動、平衡感覚を評価した結果、発達期の D1RKD マウスでは、成体期のKDマウスと比べ行動異常 の現れ方が異なることが判明した。 該当無し 3. その他

該当無し

### D. 考察

発達期と成体期では、正常な行動発現に D1R が 必要であるが、その機能は異なる可能性があると 考えられた。D1R K0 マウスのように、受精卵の段 階から D1R がないと、他のドーパミン受容体によ る何らかの機能的補償作用が働く可能性もある と考えられた。

### E. 結論

生後発達期および成体期に D1R の発現をノッ クダウンしたところ、各行動テストにおいて異な る表現型が現れたことから、発達期と成体期では D1R が異なる機能を持つことが示唆された。

F. 研究発表(上記課題名に関するもの) 1. 論文発表 なし

#### 2. 学会発表

笹岡俊邦、佐藤朝子、知見聡美、大久保直、前島 純、新井慧、砂山智子、小田佳奈子、酒井清子、 前田宜俊、神保幸弘、馬川恵梨子、佐藤俊哉、藤 澤信義、横山峯介、南部篤 D1 dopamine receptor-mediated signal is required to maintain normal motor activity 第 38 回日本分子生物学会 総会 2015 年 12 月 3 日 神戸

G.知的財産権の出願・登録状況(予定を含む)
 1.特許取得
 該当無し
 2.実用新案登録

### Astroblastoma の網羅的遺伝子解析

研究代表者 信澤 純人 1)

研究分担者 横尾 英明 1) 柿田 明美 2)

1) 群馬大学大学院医学系研究科病態病理学分野 2) 新潟大学脳研究所病理学分野

#### 研究要旨

Astroblastoma は小児〜若年成人に発生する非常に稀な原発性脳腫瘍である。病理組織学的に、 astroblastic rosettes と呼ばれる血管周囲性偽ロゼット構造を特徴とするが、この構造は浸潤性グ リオーマ等にも見られることがあり、また、特異性の高い免疫組織化学的マーカーを欠くことから診 断に難渋することがある。本研究では、astroblastoma に特異性の高い分子マーカーを検索するため、 網羅的遺伝子解析を行った。高解像度の arrayCGH 法を用いた解析では、ある染色体全体に渡って chromothripsisを思わせるような大小の欠失が無数に認められた。また、鑑別を要する腫瘍に特異的 として最近報告された遺伝子異常は見られなかった。今回の検索で認められた異常がどのように腫瘍 発生に関わっているかは不明であるが、本腫瘍は分子遺伝学的に独立性の高い疾患群であると考えら れる。

### A. 研究目的

Astroblastoma は非常に稀な原発性脳腫瘍の一 つであり、その組織診断基準は明確とは言えない。 他のグリオーマや髄膜腫と類似した組織所見を 呈するが、特異性の高い免疫組織化学的マーカー はいまだ知られていない。また、遺伝子異常に関 する報告はほとんど存在していない。今回の研究 では、高解像度の arrayCGH 法を用いて、 astroblastoma におけるゲノム全体に亘るコピー 数の異常を網羅的かつ高精度に検出し、遺伝子異 常の全体像の解明と、診断に有用な特異性の高い 分子マーカー、治療の標的となる可能性のある分 子の探索を行う。

### B. 研究方法(倫理面への配慮を含む)

・脳腫瘍を専門とした複数の病理医の診断が一致 した astroblastoma 症例の収集を行う。

・収集した症例のパラフィン包埋切片から

genomic DNA を抽出し、arrayCGH 法によりコピー 数異常を検索する。

本研究は群馬大学医学部ヒトゲノム・遺伝子解 析研究に関する倫理委員会での審査を受け、承認 を得ている。

### C. 研究結果

全国の複数の施設から astroblastoma と診断さ れた症例、およびそれが疑われた症例を 20 例程 集め、1. 境界明瞭であること、2. 偽乳頭状構造 を示すこと、3. 血管周囲に比較的均質な硝子化 (線維化)を認めること、4. 血管指向性を示し ていること、5. 上皮様の細胞形態を有している ことに重点を置き、脳腫瘍を専門とした複数の病 理医にて検討した結果、典型的な astroblastoma として意見が一致した症例を8 例収集することが できた。そのうち6 例のパラフィン包埋切片から 質の高い genomic DNA を抽出することができ、 arrayCGH 法を行った。結果、解析した全例におい て、ある染色体全体に渡って chromothripsis を 思わせるような大小の欠失が無数に認められた。 その他、2 例で recurrent に見られる異常が 3 個 認められた。また、astroblastoma と同様に、星 細胞や上衣細胞への分化を示す angiocentric glioma や ependymoma などの腫瘍に特異的として 最近報告された遺伝子異常の存在を示唆する結 果は得られなかった。

### D. 考察

網羅的な遺伝子解析において全例に認められ た異常は、他の疾患群において recurrent に見ら れるものとして現在までに報告されていないた め、astroblastoma に特異的と考えられる。また、 astroblastoma に類似した腫瘍に特異的な遺伝子 異常も認められていないため、本腫瘍は分子遺伝 学的に独立性が高いと考えられる。

### E. 結論

Astroblastoma は、病理組織学的に類似した腫 瘍が複数あるが、分子遺伝学的には独立性の高い 疾患群であると考えられる。

F. 研究発表(上記課題名に関するもの) 1. 論文発表 無し。

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# G. 知的財産権の出願・登録状況(予定を含む) 無し。

# 筋萎縮性側索硬化症脊髄における GPNMB 凝集体に関する研究

### 研究代表者 原 英彰 1)

研究分担者 長原 悠樹 1)、嶋澤 雅光 1)、柿田 明美 2)

1) 岐阜薬科大学 薬効解析学研究室 2) 新潟大学脳研究所 脳疾患標本資源解析学分野

#### 研究要旨

我々は筋萎縮性側索硬化症 (ALS) 患者腰髄で GPNMB (Glycoprotein nonmetastatic melanoma protein B) 凝集体が認められることを報告した。しかしながら GPNMB 凝集体の正体は明らかになっていな い。そこで ALS 患者および対照疾患患者の脊髄パラフィン切片を作製し、ALS 患者における GPNMB 凝集体の局在を検討した。GPNMB 凝集体は ALS 患者の頸髄・胸髄・腰髄の灰白質および白質で観察 された。GPNMB は GFAP (Glial fibrillary acidic protein) と共局在せず、MAP2 (Microtubule-associated protein 2) や SMI32 (Neurofilament H non-phosphorylated) と共局在していた。また、GPNMB は TDP-43 [Transactive response (TAR)-DNA-binding protein 43] と共局在していた。以上より、GPNMB 凝集体は ALS 患者脊髄の神経細胞に局在し、TDP-43 とも何らかの関連があることが示唆された。

### A.研究目的

筋萎縮性側索硬化症 (ALS) は筋肉の萎縮や変 性、運動機能障害等が生じる進行性の神経変性疾 患であり、有病率は 2-7 人/10 万人である。患者の 約半数が発症後 3-5 年以内に死亡する非常に重篤 な疾患であるにもかかわらず、唯一の治療薬であ るリルゾールも生存期間を約3ヶ月延長するのみ であり、病態解明や新規治療薬の開発が切望され ている。GPNMB (Glycoprotein nonmetastatic melanoma B; Osteoactivin, DC-HIL) は悪性黒色腫 細胞から発見された一回膜貫通型糖タンパク質 であり、種々の癌の転移に関与する他、骨吸収・ 骨形成、緑内障等にも関与する。

我々は DNA マイクロアレイにより、ALS モデ ルマウスである SOD1<sup>G93A</sup> [superoxide dismutase 1 (glycine 93 changed to alanine)] マウス脊髄におい て、野生型マウスと比較して GPNMB が上昇して いることを明らかにした。また、ALS 患者の脊髄 や血清、脳脊髄液で発現上昇すること、GPNMB の過剰発現によって SOD1<sup>G93A</sup> マウスの生存期間 が延長することを報告した。さらに、GPNMB が ALS の *in vitro* モデルである SOD1<sup>G93A</sup> 誘発運動神 経細胞死を抑制することも報告した。さらに ALS 患者の脊髄 (腰髄灰白質) で、GPNMB の異常な 凝集体が見られることを明らかにした (参考文献 1)。しかしながら GPNMB 凝集体がどこに局在す るか、どのような物質と結合しているか等は明ら かにされていない。

TDP-43 [Transactive response (TAR)-DNA-binding protein 43] は、ALS や FTLD (前頭側頭葉変性症) 等の疾患との関連が指摘されているタンパク質 である。TDP-43 は正常な神経細胞では主に核に 存在しているが、ALS 等の疾患では細胞質中に凝 集・蓄積し、核から消失することが知られている。

平成 26 年度、ALS 患者 (2 例) および対照疾患 患者 (2 例) の検討を行ったところ、ALS 患者脊 髄 で 観 察 された GPNMB 凝集体 が MAP2 (Microtubule-associated protein 2) と共局在する可 能性が示唆された。そこで本年度、ALS 患者 (10 例) および対照疾患患者 (10 例) において、同様 に検討を行うとともに、GFAP (Glial fibrillary acidic protein) や SMI32 (Neurofilament H non-phosphorylated) と共局在するか検討した。さ らに、ALS 患者脊髄における GPNMB 凝集体が TDP-43 と共局在するか検討した。

### **B.研究方法**(倫理面への配慮を含む)

新潟大学脳研究所において孤発性 ALS 患者 10 例および対照疾患 (非神経変性疾患) 患者 10例の 頸髄・胸髄・腰髄のパラフィン切片 (厚さ 4µm) を 作製した。

岐阜薬科大学において、脱パラフィン操作 (キ シレン15分×3回、無水エタノール10秒×2回、 99%エタノール 10 秒×2 回、90%エタノール 10 秒×1回、70%エタノール10秒×1回、蒸留水10 秒×1回)を行った。クエン酸緩衝液 (pH 6.2)を 用いて 120℃ で 15 分間オートクレーブすること により賦活化した。PBS で Wash 後、10%Horse serum によりブロッキング (室温、1時間) を行っ た。PBS で Wash 後、Goat anti-human GPNMB polyclonal antibody (1/20; #AF2550, R&D) を4°C で一晩反応させた。PBS で Wash 後、Alexa 546-conjugate donkey anti-goat IgG (1/1,000; #A11056, Invitrogen) を室温で1時間反応させた。 PBS で Wash 後、Mouse anti-MAP2 monoclonal antibody (1/500; #ab11267, abcam), Mouse anti-SMI32 (1/1,000;#NE1023, Millipore Corporation)、または Rabbit anti-TDP-43 polyclonal antibody (1/100; #10782-2-AP, Proteintech) を室温 で1時間反応させた。PBS で Wash 後、Alexa Fluor 488-conjugate rabbi anti-mouse IgG (1/1,000; #A21204, Invitrogen)、または Alexa Fluor 488-conjugated chicken anti-rabbit IgG antibody (1/1,000; #A21441, Invitrogen) を室温で1時間反応 させた。PBS で Wash 後、Hoechst33342 (1/5,000) 又 は DAPI (1/1,000) を室温で 30 分反応させること で核染色を行った。PBS で Wash 後、フルオロマ ウントを用いて封入した。Olympus IX50 inverted epifluorescence microscope (Olympus) を用いて、頸 髄、胸髄、腰髄それぞれについて、灰白質と白質 を撮影し、評価を行った。

本研究は新潟大学脳研究所で「病理解剖ならび

に病理検体保存とその使用に関する承諾」が得ら れている症例を用い、新潟大学医学部倫理委員会 の承認 (承認番号: 2428) および岐阜薬科大学生 命倫理委員会の承認 (承認番号: 岐阜市薬会第 353-1号) を得た上で行った。

### C.研究結果

GPNMB 凝集体は ALS 患者の頸髄・胸髄・腰髄 の灰白質および白質において、対照疾患と比較し て有意に増加していた。

GPNMB 凝集体は、神経細胞 (細胞体・樹状突 起) のマーカーである MAP2 や、軸索のマーカー である SMI32 と共局在していた。その一方で、ア ストロサイトのマーカーである GFAP とは共局在 していなかった (Fig.1)。

TDP-43 は正常な神経細胞では主に核に存在し ているが、ALS 等の疾患では細胞質中に凝集・蓄 積し、核から消失することが知られている。ALS 患者脊髄の神経細胞の細胞質において TDP-43 凝 集体が生じていた。また、二重免疫染色を行った ところ、GPNMB 凝集体は TDP-43 と共局在して いることが明らかになった (Fig.2)。



Fig.1 ALS 患者腰髄灰白質における二重免疫染色



Fig.2 GPNMB と TDP-43 の二重免疫染色

### D.考察

GPNMB 凝集体は、神経細胞(細胞体・樹状突起)のマーカーである MAP2 や、軸索のマーカー である SMI32 と共局在していた。その一方で、ア ストロサイトのマーカーである GFAP とは共局在 していなかった。よって、GPNMB 凝集体は ALS 患者脊髄の神経細胞において形成されているこ とが明らかになった。

また、GPNMB 凝集体は TDP-43 と共局在して いた。我々のこれまでの研究で、GPNMB が in vivo および in vitro の ALS モデルに対して保護作用を 示すことが示唆されている (参考文献 1-3)。従っ て GPNMB は TDP-43 凝集に起因した細胞障害を 抑制するために、ALS 患者脊髄の神経細胞におい て発現増加するのかもしれない。GPNMB 凝集体 がどうして生じるのか、どのような作用をしてい るか等は今後の検討課題である。

### E.結論

ALS 患者の頸髄・胸髄・腰髄で GPNMB 凝集体 が生じることが明らかになった。また、GPNMB 凝集体はアストロサイトには局在しておらず、神 経細胞の細胞体や軸索に局在する可能性が示唆 された。よって、本研究により ALS 患者脊髄の GPNMB 凝集体の局在が明らかになった。

また、GPNMB 凝集体は ALS 患者脊髄において TDP-43 と共局在していた。さらなる検討が必要 ではあるが、GPNMB 凝集体が TDP-43 と関連が ある可能性が示唆された。本研究をさらに進める ことで、ALS の病態解明や治療薬の開発につなが ると考えられる。 **F.研究発表**(上記課題名に関するもの) 1.論文発表 準備中

### 2.学会発表

なし

G.知的財産権の出願・登録状況(予定を含む)
1.特許取得なし
2.実用新案登録なし
3.その他なし

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### ヒト神経疾患脳における DAP12 発現の病理学的検討

**研究代表者**:佐々木 惇 1) 研究分担者:柿田 明美 2)

1) 埼玉医科大学病理学, 2) 新潟大学脳研究所

### 研究要旨

Nasu-Hakola病(以下、NHDと略す)では近年, *DAP12* (DNAX-activation protein 1), also known as *TYROBP* (TYRO protein tyrosine kinase-binding protein)遺伝子およびDAP12のアダプター分子である *TREM2* (triggering receptor expressed on myeloid cell 2)の変異ないし欠損が見出され, 脳内の蛋白発 現消失が脳病変の病態に関与する可能性が示唆された. DAP12 (DNAX activating protein of 12 kDa) はI型膜貫通糖タンパクであり, 染色体19q13に位置する遺伝子でコードされている.本研究では、NHD などの神経疾患でのNHD12の役割を明らかにするために, 剖検および生検脳組織を用いて, DAP12蛋白/ 遺伝子発現を検討した.

平成26年度までの結果について、佐々木が筆頭著者となり、共同研究者の柿田をはじめとする共著者 と英文論文を作成した。その論文はNeurogenetics誌に受理され、2015年度に掲載された。

NHD以外の疾患として、悪性脳腫瘍である膠芽腫を検討したところ、一部の症例で多数のtumor associated macrophage (TAM)がDAP12強陽性となり、腫瘍周囲では、activated microglia, resting microglia, perivascular cellも陽性となった. さらにTAMは膠芽腫組織内の微小血管増殖部に増加していることが示された。

#### A. 研究目的

本研究の目的は NHD などのヒト神経疾患での DAP12 の役割の解明であり,研究期間内に以下の 点を明らかにする:

1) DAP12 遺伝時異常

2) DAP12 蛋白発現

3) 脳内 DAP12 蛋白発現のミクログリア特異性・ 活性化との関係

# B.研究方法(倫理面への配慮を含む)

新潟大学脳研究所に保存された NHD2 例と他院

NHD2 例、埼玉医科大学国際医療センターで摘出された膠芽腫 30 例と非神経疾患剖検例 6 例を対象 とした。いずれの症例とも遺族への説明は臨床担 当医師からなされており,文書により研究に使用 することに関する同意が得られていた.また.す でに診断された後の保存資料であり,資料提供者 本人や遺族への不利益や危険性は全く生じてい ない.

DAP12 遺伝子検索は、NHD 凍結脳を用いて、シ ークエンス、RT-PCR、real time PCR により行っ た。 DAP12 蛋白解析は、Western blot と免疫組織化 学(IH) により行った。IH では、ホルマリン固定 脳パラフィン切片を作製し, LSAB 法ないし EnVision FLEX 法を行った.一次抗体として, Iba1, glucose transporter 5 (Glut5), MHC class II (CR3.43), CD68 (PG.M1), CD204 (SRA-E5), CD163 (10D6), 抗 DAP12 抗体(FL-113 と ab11017), を用 いた.

### C. 研究結果

今年度は、従来の結果にもとづき、activated microglia や TAM での DAP12 発現を解析した。抗 DAP12 抗体 (ラビット, FL-113) を用いた免疫染色 では、非神経疾患脳において, DAP12 は resting microglia, perivascular cells, meningeal macrophages, choroid plexus macrophage に認め られた, 一方, 抗 DAP12 抗体 (goat, ab11017) では、明らかな陽性像は得られなかった.

一方, 脳腫瘍手術摘出組織においては, 多数の TAM が DAP12 強陽性となり, 腫瘍周囲では, activated microglia, resting microglia, perivascular cell も陽性となった. さらに膠芽 腫の MVP 部に M2 型を含む多数の TAM がみられ、 血管増生での役割が示唆された。

### D. 考察

NHD 脳における DAP12 蛋白異常は、蛋白レベル での発現低下だけでなく、蛋白の機能異常がある 可能性が示された。NHD は、DAP12 ないし TREM2 分子の異常による、"microgliopathy"の可能性 が高いが、その発症メカニズムは未だ未解決であ る。今後は、microglia と axon との関係、ミクロ グリアとオリゴデンドログリア・ミエリンとの関 係についての詳細な解析が必要と考える。

従来の検討で, 我々は, DAP12 が perivascular cell を含む脳内の macrophage 系細胞にも発現す ることを示した. さらに, 悪性グリオーマ組織内 の TAM にも DAP12 が発現することを初めて示した. 今後は DAP12 分子の発現細胞数や発現レベルの解 析が必要と考えられた。具体的には悪性 glioma においても, 免疫組織化学だけでなく, western blot 法, real-time PCR 法による mRNA および蛋 白発現量の検討が必要であり、DAP12 とともに macrophage 受容体分子である TREM2 や CSF1-R の 発現との比較解析も望まれる.

### E. 結論

今回、我々は、NHD 症例の中には通常の症例と 同様の臨床・病理像を示し、*DAP12* 遺伝子変異を 持つにも拘わらず、DAP12 の mRNA や蛋白が高発現 される症例があることを初めて論文発表した。今 後は、DAP12 ないし TREM2 の DNA、mRNA、蛋白の詳 細な解析、疾患特異性、および他の受容体分子発 現との比較検討が必要であることが示唆された.

### F. 研究発表

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### G. 知的財産権の出願・登録状況(予定を含む)

 特許取得 なし
 実用新案登録 なし
 その他
 なし

時間的空間的特異的 Scrapper ノックアウトマウスの作製と解析

研究代表者 矢尾 育子 1) 2)
 研究分担者 伊藤 誠二 1)
 研究分担者 片野 泰代 1)
 研究分担者 崎村 建司 3)

1) 関西医科大学 2) 浜松医科大学 3) 新潟大学

#### 研究要旨

SCRAPPER は神経シナプスに局在し、神経伝達物質放出の制御にかかわるユビキチン E3 リガーゼであ る。Scrapper 遺伝子ノックアウト (SCR-KO) マウスが生後致死であるため、成体での SCRAPPER 分子の 機能を十分に解析できなかった。そこで部位特異的なコンディショナル KO (cKO) マウスを作製し、そ の詳細な機能解析を行うことを計画した。前年度までに、コンディショナルノックアウトマウス作製の ための floxed 型のベクターを構築し、組換え ES 細胞株よりキメラマウスを作出している。生殖系列に ES 細胞が分化した個体より標的 floxed 型マウスを樹立し、F1 世代のマウスが得られており、ドライバ ーマウスとの交配により時間的空間的特異的 SCR-cKO マウスを作製しようとしている。今後ドライバー マウスとの交配により部位手特異的 SCR-cKO マウスを作製するとともに、以前に解析した null ノック アウトマウスと同じ表現型が得られるかも合わせて検討するため、共同研究を継続していく予定である。

### A. 研究目的

Scrapper 欠損マウス(SCR-K0 マウス)は体が 小さい上に寿命が短く、恐怖記憶形成の異常、脳 の海綿状変性や神経細胞の萎縮といった老化現 象が見られる。SCR-K0マウス脳において変動して いる分子の同定が神経変性疾患病態解明の1つの 鍵になると考えられる。

SCR-KOマウスは、多くの個体が生後半年程度で 死亡する。また、産仔数もメンデルの法則に従わ ず少ないことから、SCRAPPER が発生段階において も機能していることが予想される。本研究では、 致死の表現型を回避し脳に限定した機能を解析 することができる、部位特異的なコンディショナ ルKOマウスを作製し解析をおこなう。

### B. 研究方法

floxed 型のベクターを構築し、このベクターを

新潟大学崎村研究室で開発された RENKA 株 ES 細胞に導入し、相同組み換え体を選別する。これらの組換え ES 細胞株よりキメラマウスを作出し、 生殖系列に ES 細胞が分化した個体より標的 floxed型マウスを樹立する。さらに、脳部位特異 的な Cre 発現マウスを利用して、部位特異的なノ ックアウトマウスを作製して解析に供する。

動物実験計画は大学の内規に準じて行う。実験 時には適切に麻酔処理を行い、動物への苦痛を可 能な限り除く。

### C. 研究結果

現在までに、コンディショナルノックアウトマ ウス作製のための floxed 型のベクターを構築し、 このベクターを新潟大学崎村研究室で開発され た RENKA 株 ES 細胞に導入し、相同組み換え体を 選別した。これらの組換え ES 細胞株よりキメラ マウスを作出し、生殖系列に ES 細胞が分化した 個体より標的 floxed 型マウスを樹立し、F1 世代 のマウスを得ている。今年度、浜松医大への導入 を完了し、FLP マウスとの交配を進めた。現在、 ドライバーマウスとの交配により、時期あるいは 部位特異的なノックアウトマウスを作製する段 階である。

### D. 考察

脳部位特異的な Cre 発現マウスを利用して、部 位特異的なノックアウトマウスを作製して解析 に供する計画であることから、今後、交配を行い ノックアウトマウスラインの樹立が必要である。 また、解析に適するドライバーマウスの選択およ び交配、その後の解析を行う必要がある。現存す るノックアウトマウスはバックグラウンド系統 が複数の雑種となっていることから、新たに得ら れるマウスを用いた解析はバックグラウンド系 統の影響を消去できる点でも有用であると考え る。

#### E. 結論

SCR-cKOマウス作製のためのfloxed型マウスを 樹立し、F1世代のマウスが得られた。今後さらに 繁殖、ドライバーマウスとの交配により部位手特 異的 SCR-cKO マウスを作製する。

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なし

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### G. 知的財産権の出願・登録状況(予定を含む)

- 1. 特許取得
- なし
- 2. 実用新案登録
- なし
- 3.その他

なし