ORIGINAL ARTICLE

Cognitive Impairment and Cortical Degeneration in Neuromyelitis Optica

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Objective: Neuromyelitis optica spectrum disorder (NMOsd) is an inflammatory and demyelinating syndrome characterized by optic neuritis and myelitis. Several magnetization transfer magnetic resonance imaging (MRI) studies have revealed abnormalities in normal-appearing gray matter in NMOsd. The aim of this study is to elucidate the characteristics and pathogenesis of cognitive impairment and neurodegeneration in NMOsd brains.
Methods: Fourteen Japanese patients with serologically verified NMOsd, 17 patients with multiple sclerosis (MS), and 37 healthy controls were assessed with the Rao's Brief Repeatable Battery of Neuropsychological Tests (BRBN). Using 128 tissue blocks from 6 other cases of NMOsd, 3 cases of MS, and 4 controls without central nervous system involvement, we performed quantitative analysis of cortical neuronal loss and layer-specific changes in NMOsd.
Results: In BRBN assessments, 57% of NMOsd patients and 47% of MS patients had impaired performance on at least 3 cognitive tests. Cognitive impairment in NMOsd was common even in the limited form of disease, indicating that NMOsd may progress insidiously from early stages of disease. Neuropathological assessments showed neuronal loss in cortical layers II, III, and IV, with nonlytic reaction of aquaporin-4 (AQP4)-negative astrocytes in layer I, massive activated microglia in layer II, and meningeal inflammation in NMOsd brains. All NMO cases showed no evidence of cortical demyelination.
Interpretation: We demonstrate cognitive impairment and substantial cortical neuronal loss with unique AQP4 dynamics in astrocytes in NMOsd. These data indicate pathological processes consisting not only of inflammatory demyelinating events characterized by pattern-specific loss of AQP4 immunoreactivity but also cortical neurodegeneration in NMOsd brains.

ANN NEUROL 2013;73:65–76

Neuromyelitis optica (NMO) is an inflammatory and demyelinating syndrome of the central nervous system (CNS) that is characterized by optic neuritis and myelitis.[1,2] Detection of NMO immunoglobulin G autoantibody (NMO-IgG), which targets the water channel aquaporin-4 (AQP4; the main channel regulating water homeostasis in the CNS), in the serum of NMO patients distinguishes NMO from other demyelinating disorders with stringent diagnostic accuracy.[3–5] Although the brain is widely recognized to be relatively spared in NMO,[1] most magnetization transfer and diffusion tensor magnetic resonance imaging (MRI) studies of patients with NMO have found abnormalities in normal-appearing gray matter (NAGM) as well as normal findings or minimal changes in normal-appearing white matter, irrespective of abnormalities seen with conventional MRI.[6–9] These findings suggest selective or more severe lesioning of gray matter, which is a site of high AQP4 expression.

In multiple sclerosis (MS) patients, there has been renewed interest in cognitive dysfunction, which affects 45 to 65% of patients sometime during the course of the disease.[10] These processes in MS may primarily arise within gray matter regions and result in cortical subpial demyelination concomitant with meningeal infiltration,[11]

View this article online at wileyonlinelibrary.com. DOI: 10.1002/ana.23721

Received Feb 23, 2012, and in revised form Jul 9, 2012. Accepted for publication Jul 30, 2012.

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TABLE 1: Numbers (%) of Patients Impaired (Below the Fifth Percentile for Normative Values) in Each Measure of Rao's Brief Repeatable Battery of Neuropsychological Tests

Test	NMO Spectrum Disorder Patients, n = 14	MS Patients, n = 17
Attention		
SDMT	7/13 (54)	9/17 (53)
PASAT3	9/14 (64)	8/17 (47)
PASAT2	8/14 (57)	6/17 (35)
Verbal memory		
SRT-LTS	7/14 (50)	7/17 (41)
SRT-CLTR	7/14 (50)	8/17 (47)
SRT-D	5/14 (36)	3/17 (18)
Visual memory		
SPART	2/13 (15)	4/17 (24)
SPART-D	1/13 (8)	1/17 (6)
Expressive language		
WLG	1/14 (7)	4/17 (24)

MS = multiple sclerosis; NMO = neuromyelitis optica; PASAT = Paced Auditory Serial Addition Test (3 = 3s/digit; 2 = 2s/digit); SDMT = Symbol Digit Modality Test; SPART = 10 of 36 Spatial Recall; SPART-D = SPART-Delayed Recall; SRT = Selective Reminding Test; SRT-CLTR = SRT–Consistent Long-Term Retrieval; SRT-D = SRT–Delayed Recall; SRT-LTS = SRT–Long-Term Storage; WLG = Word List Generation.

or they could be secondary pathological changes in the gray matter regions resulting from continuing damage in the cerebral white matter.[12] Meanwhile, little is known about the characteristic features of cognitive decline in patients with NMO and its detailed pathogenesis.[6,13] We investigated the clinical relevance of the cognitive impairment occurring in NMO spectrum disorder (NMOsd) from the early to the terminal stage, and demonstrated cognitive impairment and substantial cortical neuronal loss with unique AQP4 dynamics in astrocytes in NMOsd.

Materials and Methods

Neuropsychological Assessments

All patients in this series were Japanese. We reviewed the medical records of 14 patients with NMOsd (5 with the definite form and 9 with the limited form) and 17 patients with MS who visited the MS/NMO clinic of the Department of Neurology, Niigata University Hospital in 2009 and gave informed consent. Thirty-seven healthy controls were recruited for neuropsychological assessments. We stringently defined the definite form of NMO as fulfilling all items of the 2006 NMO criteria,[2] and limited NMO to either: (1) optic neuritis with seropositivity for AQP4 autoantibody but without brain, brainstem, or spinal cord lesions; or (2) myelitis with seropositivity for AQP4 autoantibody but without optic nerve involvement. As a disease control, MS was defined as clinically definite MS according to the International Panel criteria for MS,[14] excluding definite and limited NMO. All patients with NMOsd were seropositive for the AQP4 autoantibody, whereas MS patients were not. Cognitive batteries were always tested during the remission phase in all NMOsd patients. At the time of evaluation of cognitive functions, patients with NMOsd and MS were relapse free for at least 12 weeks, and 93% of NMOsd patients had received steroids at a mean dose (standard deviation) of 16.5 (10.2) mg/day, whereas 76% of MS patients had received interferon-β treatment. Healthy controls had no neurological or major psychiatric illnesses (Supplementary Table 1).

Neuropsychological assessment was performed using the Rao's Brief Repeatable Battery of Neuropsychological Tests (BRBN), version A.[10] This battery includes tests of verbal immediate and delayed recall memory (Selective Reminding Test [SRT], SRT–Long-Term Storage [SRT-LTS], SRT–Consistent Long-Term Retrieval [SRT-CLTR], and SRT–Delayed Recall [SRT-D]), spatial immediate and delayed recall memory (10 of 36 Spatial Recall Test [SPART] and 10 of 36 SPART–Delayed Recall [SPART-D]), sustained attention, concentration, speed of information processing (Paced Auditory Serial Addition Test [PASAT] at 3 seconds and 2 seconds, Symbol Digit Modality Test [SDMT]), and verbal fluency on semantic stimulus (Word List Generation [WLG]). 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 and healthy controls participating in the study.

TABLE 2: Numbers (%) of Patients with Cognitive Impairment

	NMO Spectrum Disorder Patients		MS Patients
	8/14 (57)		8/17 (47)
Cognitive impairment	Limited Form	Definite Form	
	6/9 (67)	2/5 (40)	

For each patient, impaired performance was defined as below the fifth percentile for the normative values for each cognitive test, as established in a healthy population of the present study. Patients with impaired performance on at least 3 cognitive tests were considered to have cognitive impairment.[20]
MS = multiple sclerosis; NMO = neuromyelitis optica.

TABLE 3: Correlation Coefficients between Demographic and Clinical Characteristics and Measures of BRBN of Neuromyelitis Optica Patients and MS Patients (Spearman Rank Order Correlations for Age, Education, Duration, EDSS, and Brain Lesion Burden on Magnetic Resonance Imaging Findings)

Characteristic	Age	Education	Duration	EDSS	White Matter Lesion Burden[a]	Gray Matter Lesion Burden[a]
NMOsd patients						
Education	−0.446	1				
Duration	−0.314	0.564	1			
EDSS	0.479	0.053	0.047	1		
BRBN index[b]	0.044	−0.191	0.161	−0.210	−0.228	−0.264
SDMT	−0.906[c]	0.541	0.243	−0.488	0.044	−0.220
PASAT3	−0.633[d]	0.337	−0.040	−0.395	−0.240	−0.155
PASAT2	−0.729[e]	0.572	0.289	−0.189	−0.293	−0.155
SRT-LTS	−0.490	0.254	0.375	0.002	0.048	−0.465
SRT-CLTR	−0.493	0.383	0.366	0.000	0.177	−0.386
SRT-D	−0.471	0.319	0.358	0.165	0.110	−0.350
SPART	−0.555[d]	0.171	0.356	−0.570[d]	−0.278	−0.132
SPART-D	−0.657[d]	0.512	0.159	−0.337	−0.159	0.222
WLG	−0.082	0.508	0.018	0.153	0.269	0.350
MSNQ-P	−0.191	0.465	0.155	−0.027	0.192	0.500
ESS	−0.005	0.230	0.460	−0.155	−0.265	−0.202
HDRS	−0.240	0.150	0.055	0.104	−0.038	0.051
MS patients						
Education	−0.169	1				
Duration	0.214	0.198	1			
EDSS	0.408	−0.288	0.218	1		
BRBN index[b]	0.111	−0.323	0.173	0.169	0.384	−0.171
SDMT	−0.270	0.231	−0.412	−0.604[d]	−0.385	−0.143
PASAT3	−0.026	0.344	−0.247	−0.317	−0.521[d]	0.093
PASAT2	0.115	0.256	−0.320	−0.244	−0.489	−0.078
SRT-LTS	−0.053	−0.151	−0.155	0.016	−0.034	0.228
SRT-CLTR	−0.110	0.111	−0.294	−0.141	0.019	0.233
SRT-D	−0.035	−0.005	−0.363	−0.011	0.085	0.418
SPART	0.094	0.328	−0.005	−0.025	−0.421	0.028
SPART-D	0.004	0.391	−0.015	−0.099	−0.418	0.055
WLG	−0.145	0.206	−0.389	−0.394	−0.510[d]	0.139
MSNQ-P	0.017	0.311	0.099	0.244	−0.044	0.325
ESS	0.632[e]	0.040	0.472	0.630[e]	0.356	0.098
HDRS	0.244	−0.566[d]	0.148	0.347	0.401	−0.266

[a]Number of white matter lesions or gray mater burden was defined as T2 lesions >3mm.
[b]A cognitive impairment index was constructed using the mean and standard deviation from a normative sample for Rao's battery.[48]
[c]Correlation is significant at the 0.001 level (2 tailed).
[d]Correlation is significant at the 0.05 level (2 tailed).
[e]Correlation is significant at the 0.01 level (2 tailed).
BRBN = Rao's Brief Repeatable Battery of Neuropsychological Tests; EDSS = Expanded Disability Status Scale of Kurtzke; ESS = Environmental Status Scale; HDRS = Hamilton Depression Rating Scale; MS = multiple sclerosis; MSNQ-P = the patient-reported version of the Multiple Sclerosis Neuropsychological Screening Questionnaire; NMOsd = neuromyelitis optica spectrum disorder; PASAT = Paced Auditory Serial Addition Test (3 = 3s/digit; 2 = 2s/digit); SDMT = Symbol Digit Modality Test; SPART = 10 of 36 Spatial Recall; SPART-D = SPART-Delayed Recall; SRT = Selective Reminding Test; SRT-CLTR = SRT–Consistent Long-Term Retrieval; SRT-D = SRT–Delayed Recall; SRT-LTS = SRT–Long-Term Storage; WLG = Word List Generation.

Neuropathological Assessments

The study was performed on brain materials from 6 patients with NMOsd (5 with the definite form and 1 with the limited form; they are different from cases evaluated for cognitive dysfunction using the BRBN), 3 patients with MS, and 4 controls without CNS involvement (1 each with Lambert–Eaton syndrome, congenital myopathy, rhabdomyolysis, and abdominal hemorrhage) at autopsy (Supplementary Table 2). All cases with NMOsd had typical pathological findings of NMO in the spinal cord or optic nerves: pattern-specific loss of AQP4 immunoreactivity in inflammatory demyelinating lesions.[5,15–17] Confounding pathologies were excluded, including Alzheimerlike changes.

All cases underwent assessment of 8 up to 12 blocks per autopsy case. Four micrometer-thick, paraffin-embedded sections were obtained and stained with hematoxylin and eosin and Klüver–Barrera. Selected sections were immunostained with polyclonal or monoclonal antibodies (listed in Supplementary Table 3) as described previously.[5] Antibody binding was visualized using the avidin–biotin–peroxidase complex (ABC) method (Vectastain ABC kit; Vector Laboratories, Burlingame, CA) or the tyramide signal amplification (TSA) method (TSA Plus Cyanine3/Fluorescein System; Perkin Elmer Life Sciences, Boston, MA; see Supplementary Table 3).[18]

Statistical Analyses

Data analyses were performed using Prism 5 (GraphPad Software, San Diego, CA) and SPSS 19 (IBM, Chicago, IL) software. Statistical analyses between the 2 subgroups of NMOsd and controls were performed using the Mann–Whitney *U* test. Statistical analyses between 3 subgroups of NMOsd, MS, and healthy controls were performed using analysis of variance, the Kruskal–Wallis *H* test, or the chi-square test. When significant results were obtained, multiple comparisons among subgroups were performed using the Bonferroni, Bonferroni–Dunn, or Tukey multiple comparison tests. Spearman rank correlation coefficient was estimated to assess the strength of the linear relationship between cognitive test performance, clinical parameters, and brain lesions on MRI. All statistical analyses were considered significant at p values of <0.05.

Results

Cognitive Impairment in Patients with NMOsd and MS

NMOsd and MS patients who were recruited for BRBN assessments did not differ from healthy controls in age, gender, or education. No significant differences were observed between the mean scores of these NMOsd and MS patients for disease duration, annual relapse rates, and Kurtzke Expanded Disability Status Scale (EDSS; see Supplementary Table 1),[19] because 64% of NMOsd patients had the limited form in the study of neuropsychological assessments in our series. No NMOsd patients converted to a secondary progressive course. Figure 1 shows the performance of NMOsd patients, MS patients, and healthy controls on all BRBN measures and the cognitive impairment index (BRBN index). NMOsd patients performed significantly worse than healthy controls on the SDMT, PASAT3, PASAT2, SRT-LTS, SRT-CLTR, and SRT-D, whereas MS patients performed significantly worse than healthy controls on the SDMT, PASAT3, PASAT2, SRT-LTS, SRT-CLTR, SRT-D, and WLG (see Fig 1A). The BRBN index scores of patients with NMOsd or MS were significantly worse than those of healthy controls (see Fig 1B). Impaired performance (below the fifth percentile for normative values) on at least 3 cognitive tests[20] was found in 57% of NMOsd patients and 47% of MS patients (Tables 1 and 2). Cognitive decline in patients with limited NMO tended to be similar to that in patients with definite NMO.

Correlations between Cognitive Test Performance and Several Clinical Parameters

In NMOsd patients, age had a negative effect on most measures (SDMT, PASAT3, PASAT2, SPART, and SPART-D; Table 3). In MS patients, EDSS was negatively correlated with SDMT, and white matter lesion burden in the brain was also negatively correlated with PASAT3 and WLG (see Table 3). Therefore, age had a significant impact on cognitive function in NMOsd patients, and EDSS and white matter lesion burden had a significant effect in MS patients.

In general, depression, fatigue, pain, apathy, and medications may potentially worsen cognitive test performance and should be considered as covariables.[21] In NMOsd patients, depression, fatigue, and apathy were not correlated with performances in any BRBN measures, but pain had a slightly negative effect on SRT-LTS (see Supplementary Tables 1 and 4). NMOsd patients had received low doses of steroids at evaluation, and the steroid dosage was not correlated with performances in any BRBN measures (data not shown), although corticosteroid therapy influences mood and cognitive symptoms.[22] These data suggest that age and pain may potentially influence cognitive impairment in NMOsd patients. However, when cognitive test performance was controlled for age and pain using logistic regression analysis, NMOsd patients were 32× as likely to have cognitive impairment compared with healthy controls (Supplementary Table 5).

Increased Meningeal Inflammation but Absence of Cortical Demyelination in NMOsd Brains

All of 64 blocks from 6 NMOsd cases showed significantly increased meningeal inflammation, including CD45RO-positive T cells, CD20-positive B cells, and major histocompatibility complex class II-positive cells,

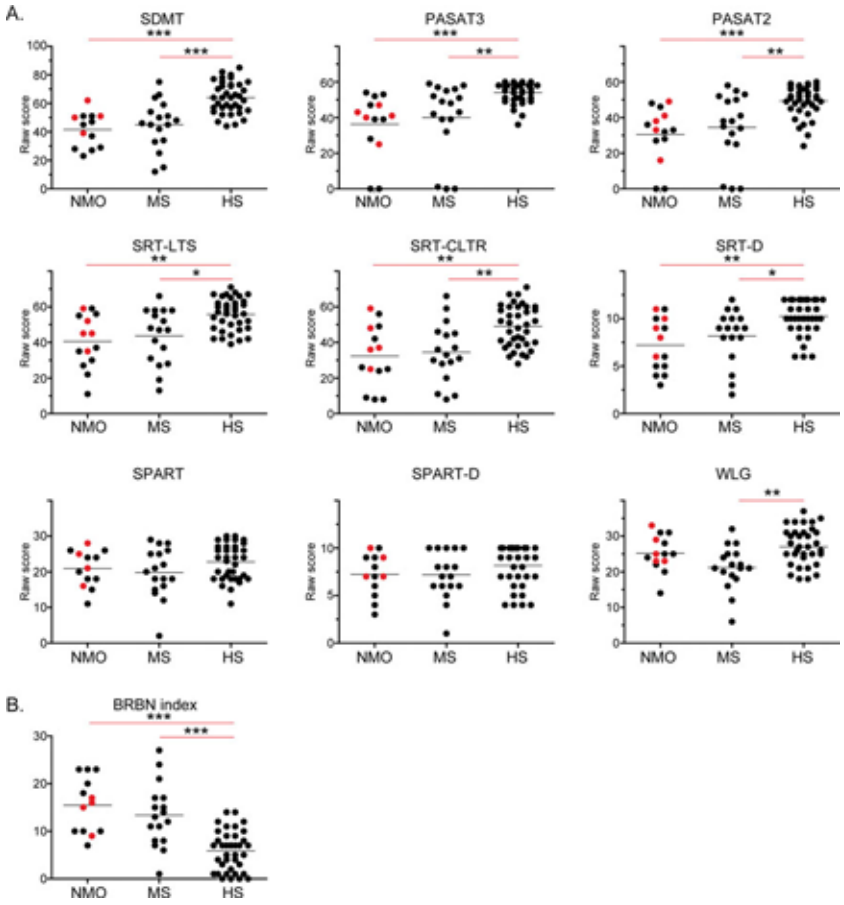

FIGURE 1: Cognitive decline in patients with neuromyelitis optica (NMO) spectrum disorder (NMOsd) and multiple sclerosis (MS). (A) Patients with NMOsd performed significantly worse than healthy controls on the Symbol Digit Modality Test (SDMT), Paced Auditory Serial Addition Test (PASAT) 3 (3s/digit), PASAT2 (2s/digit), Selective Reminding Test-Long-Term Storage (SRT-LTS), SRT–Consistent Long-Term Retrieval (SRT-CLTR), and SRT-Delayed Recall (SRT-D), whereas MS patients performed significantly worse than healthy controls on the SDMT, PASAT3, PASAT2, SRT-LTS, SRT-CLTR, SRT-D, and Word List Generation (WLG). The cognitive decline in limited NMO patients tended to be similar to that in definite NMO patients *(red dots)*. Bars indicate the mean for each group. (B) The cognitive impairment index (Brief Repeatable Battery of Neuropsychological Tests [BRBN] index) scores in patients with NMOsd or MS were significantly worse than those of healthy controls. The BRBN index score[48] is a continuous variable obtained by applying a grading system to each patient's score on every cognitive test and depends on the number of standard deviations (SDs) below the mean normative value. The patient was given a grade of 0 if he or she scored at or above the mean score of the control participants. Grade 1 was assigned if the patient scored below the control participants' mean score but at or above 1 SD below that mean. Grade 2 was assigned if the patient achieved a score >1 SD but ≤2 SDs below the control participants' mean. Finally, grade 3 was assigned if the patient scored >2 SDs below the control participants' mean. These grades were then summed across all variables to give an overall measure of cognitive dysfunction for each patient. HS = healthy subjects; SPART = 10 of 36 Spatial Recall; SPART-D = SPART–delayed recall. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

in contrast to control brains (Fig 2). Meningeal inflammation was abundant in NMOsd brains, but B-cell follicle-like structures, which are a prominent feature in MS brains,[11,23,24] were not detected in NMOsd meninges. All NMOsd cases showed preservation of myelin basic protein immunoreactivity (Supplementary Fig 1) and Nogo-A–positive cells (data not shown) within the cerebral cortex and no evidence of cortical demyelination or oligodendrocyte loss in the gray matter, in contrast to MS cases, which showed cortical demyelination (see Supplementary Fig 1).

Unique Dynamics of AQP4-Negative Astrocytes in Cortical Gray Matter of NMOsd Brains

In inflammatory demyelinated lesions of myelitis in NMOsd, we observed astrocyte necrosis and loss of AQP4 and glial fibrillary acidic protein (GFAP) immunoreactivity secondary to the complement-mediated lysis that follows AQP4-IgG binding (Fig 3C) as previously described.[5,16,17] In all cortical layers of NMOsd brains, reactive astrocytes with swollen cell bodies, clear cytoplasm, prominent nuclei, and multiple, elongated, and thin processes were abundant, with no perivascular

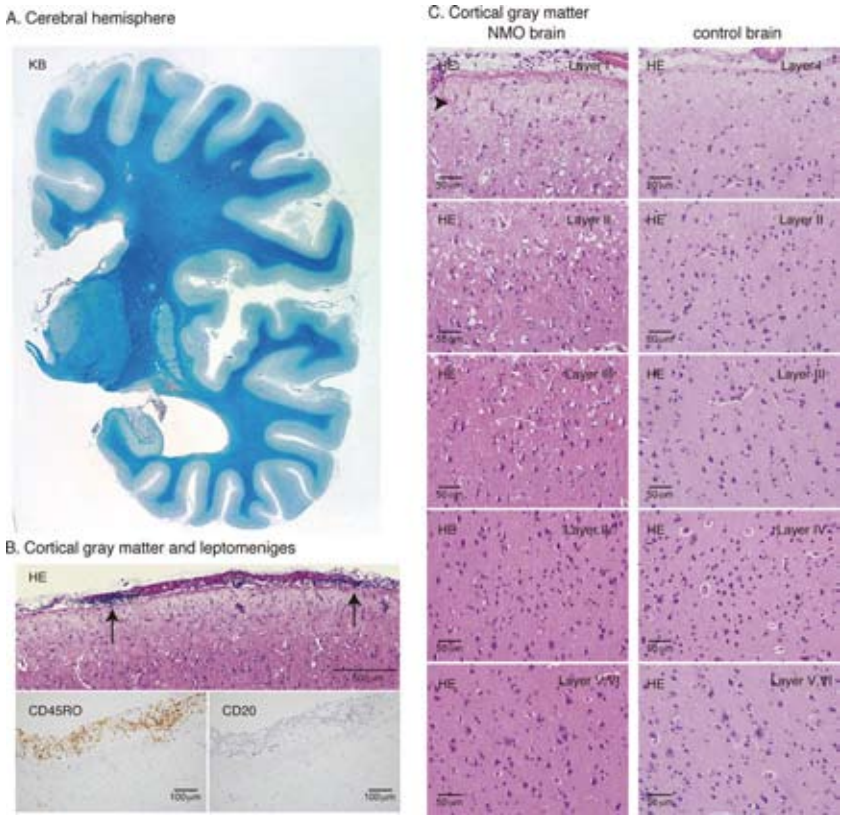

FIGURE 2: Increased meningeal inflammation and cortical neuronal loss, but absence of cortical demyelination in the cerebral cortex of patients with neuromyelitis optica (NMO) spectrum disorder (NMOsd). (A) Cortical demyelination was absent in the entire neocortex from all forebrain lobes, archicortex (hippocampus), mesocortex (parahippocampus, cingulate, and insula), and cerebellar cortex of patients with NMOsd. Myelin was preserved in all cortical layers. (B) Some degree of meningeal cell infiltration including considerable amounts of CD45RO-positive cells and few CD20-positive cells were evident in the brain of patients with NMOsd (*arrows*). (C) Rarefaction and gliosis were prominent in cortical layers II to IV from all forebrain lobes in patients with NMOsd. The cortex in layers II to IV showed dystrophic neurons such as pyknotic neurons on a background of profound astrogliosis in all layers of NMOsd cortices. Intriguingly, reactive astrocytes were abundant in layers I (*arrowhead*) and II of NMOsd cortices. HE = hematoxylin and eosin; KB = Klüver–Barrera.

deposition of complement, C9neo. Immunoreactivities for AQP4 and GFAP on astrocytes were strikingly different between layer I and layers II to VI in NMO brains. Loss of AQP4 immunoreactivity on most astrocytes was evident in cortical layer I in NMO brains, whereas expression of AQP4 and GFAP on all reactive distended astrocytes was preserved in layers II to VI (Figs 3A, B and 4A, B). Some astrocytes in layer I in NMO brains lost GFAP expression, but others showed preserved GFAP. These phenotypic features of astrocytes were consistent with the quantitative analysis of GFAP-positive astrocytes in gray matter, indicating that the number of GFAP-positive astrocytes was decreased in layer I of NMOsd brains, with an increased number of GFAP-positive astrocytes in layers II to VI of NMOsd brains (Fig 5B). Immunoreactivity for excitatory amino acid transporter 2 on astrocytes was relatively preserved within the cerebral cortex of NMOsd brains. Therefore, the gray matter of NMOsd brains was characterized by unique nonlytic astrocytes with a loss of AQP4 and/or GFAP expression, which was limited to cortical layer I. The dynamics of AQP4 in NMOsd brains were completely distinct from those of MS and control brains (see Figs 3 and 4; Supplementary Fig 3).

Substantial Neuronal Loss Was Prominent in Cortical Layer II in NMOsd Brains

To evaluate the cortical pathology in NMOsd, a detailed quantitative analysis of neuronal cell numbers was carried out in NMOsd cases and controls. We used the pan-neuronal marker to quantify neuronal cells in each layer and observed that a striking and significant decrease in the density of cortical neurons in layers II, III, and IV was seen in NMOsd brains compared to control brains (see Fig 5A). Diffuse cortical neuronal loss was spreading throughout the brain, but not localized in neurons and projection fibers related to optic radiations or corticospinal tracts.

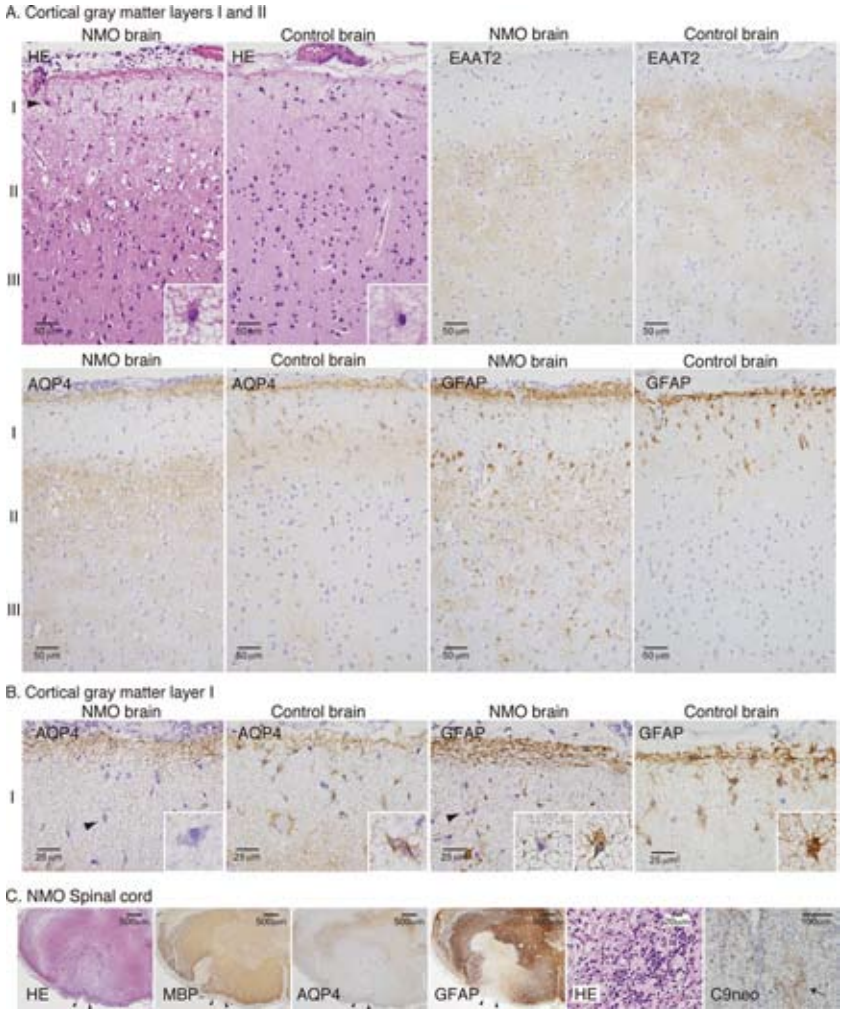

FIGURE 3: Nonlytic reaction of aquaporin-4 (AQP4)-negative astrocytes in cortical layer I of patients with neuromyelitis optica (NMO) spectrum disorder (NMOsd). All cases with NMOsd had typical pathological findings of NMOsd in the spinal cord or optic nerves: pattern-specific loss of AQP4 and glial fibrillary acidic protein (GFAP) immunoreactivities and immunoglobulin deposits colocalizing with a product of complement activation, C9neo, in a vasculocentric pattern around thickened hyalinized vessels in inflammatory demyelinating lesions (C). In contrast to the inflammatory demyelinated lesions of myelitis in NMOsd (C), the cortical gray matter in NMOsd brains showed an abundance of reactive astrocytes with swollen cell bodies, clear cytoplasm, prominent nuclei, and multiple, elongated, and thin processes in all layers (A, B). These changes were not accompanied by perivascular deposition of complement, C9neo, in all cortical layers in NMOsd brains (data not shown). AQP4, GFAP, and excitatory amino acid transporter 2 (EAAT2) immunoreactivities were preserved on astrocytes in cortical layers II to VI in NMOsd brains (A, B). However, intriguingly, loss of AQP4 immunoreactivity on most astrocytes was evident in cortical layer I in NMOsd brains (B; *arrowheads*). Some astrocytes in layer I in NMOsd brains lost GFAP expression, but others showed preserved GFAP (B). These phenotypic features were consistent with the decreased number of GFAP-positive astrocytes in cortical layer I in NMOsd brains (see Fig 4B). Data shown are representative of all NMOsd and control brains. Supplementary Figure 2 shows schematic lesion maps of all NMOsd brains. HE = hematoxylin and eosin; MBP = myelin basic protein.

Increased Microglial Numbers Were Prominent in Cortical Layer II in NMOsd Brains

The density of Iba1-positive microglia was strikingly increased in cortical layer II in NMOsd brains (Fig 6). Iba1-positive microglia in cortical layer II in NMOsd brains mostly displayed a radial ramified morphology, with thick, shortened processes characteristic of activated cells. No accumulation of lymphocytes including CD45RO-positive T cells or CD20-positive B cells was evident in the gray matter of NMOsd brains (data not shown).

Discussion

This study demonstrated cognitive impairment and substantial cortical neuronal loss with unique AQP4 dynamics in astrocytes in NMOsd. We clarified the detailed characteristic features of the cognitive impairment and of immunological, radiological, and pathological abnormalities in NMOsd patients.

First, we confirmed that as in the French cases,[25] cognitive deficits were prominent in Japanese NMOsd patients, showing that these deficits occur regardless of

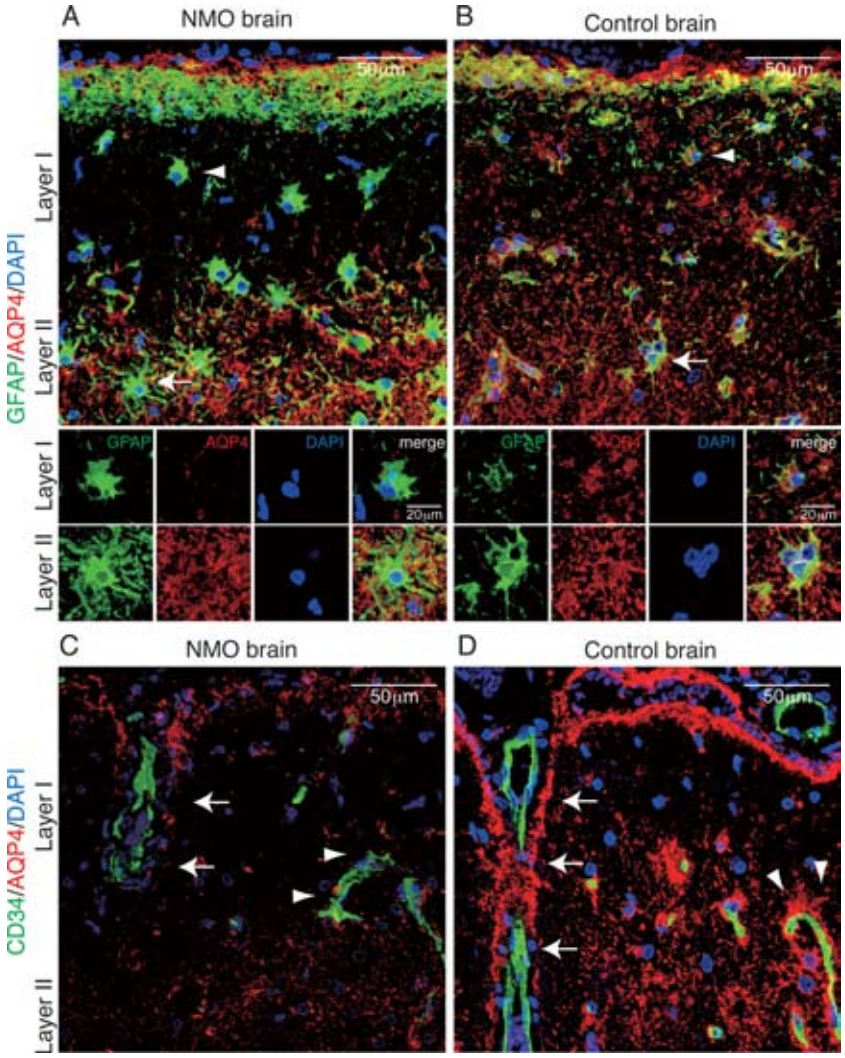

FIGURE 4: Double immunofluorescence demonstrating localization of aquaporin-4 (AQP4) and either glial fibrillary acidic protein (GFAP) or CD34 in neuromyelitis optica (NMO) spectrum disorder (NMOsd) and control brains. In cortical layer I of NMOsd brains, loss of AQP4 (red) in GFAP-labeled (green) astrocytes (eg, an astrocyte indicated by an *arrowhead* in upper panel of A) is evident, whereas AQP4 in GFAP-labeled astrocytes in cortical layer II in NMOsd brains (an example is indicated by an *arrow* in the upper panel of A) and in cortical layers I and II in control brains (examples are indicated by an *arrow* and an *arrowhead* in upper panel of B) is preserved. In cortical layer I of NMOsd brains, loss of AQP4 (red) is evident in astrocyte processes consisting of glia limitans covering the Virchow–Robin spaces (*arrows* in C) and in astrocyte endfeet facing CD34-labeled endothelial cells (green) of cortical blood vessels (an example is indicated by *arrowheads* in C), whereas AQP4 (red) of glia limitans and endfeet (*arrows* and *arrowheads* in D, respectively) in cortical layers I and II of control brains are preserved. These data consistently indicated the loss of AQP4 immunoreactivity in most astrocytes in cortical layer I in NMOsd brains. Nuclei were stained blue with 4,6-diamidino-2-phenylindole (DAPI). Supplementary Figure 3 shows the preservation of AQP4 immunoreactivity in cortical layers I and II in multiple sclerosis brains, in contrast to cortical layer I in NMOsd brains.

ethnic background. Deficits occurred in sustained attention, concentration, speed of information processing, and verbal memory, whereas the areas of spatial reasoning and verbal fluency on semantic stimulation were relatively spared compared with healthy controls (see Fig 1), suggesting that the major spectrum of cognitive impairment in NMOsd patients was similar to that in MS patients. The profile of cognitive decline found in limited NMO was similar to that in definite NMO (see Fig 1), indicating that these deficits may insidiously present from the very early stages of the disease course, and a secondary progressive clinical course[5,26] may be uncommon in NMOsd. In contrast, patients with a clinically isolated syndrome suggestive of MS had cognitive impairment,[27,28] and $>2/3$ of patients with relapsing–remitting MS experience secondary progression as defined by gradual, unremitting clinical deterioration of neurologic function.[29] Thus, it is likely that the entire course and correlation factors of cognitive impairment in NMOsd are distinct from those in MS.

We then revealed unique pathological findings in the cortical gray matter of NMOsd brains, demonstrating the absence of demyelinating gray matter foci, the

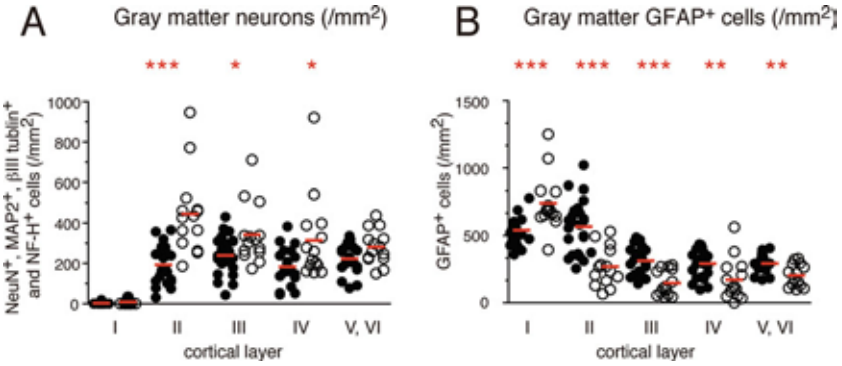

FIGURE 5: Substantial neuronal loss on a background of profound astrogliosis in the gray matter of patients with neuromyelitis optica spectrum disorder (NMOsd). To obtain a detailed analysis of the cellular pathology in the gray matter, we quantified the numerical density of the different cell populations in normal-appearing gray matter (NAGM) in tissue blocks of the frontal lobe from 6 NMOsd cases and 4 controls. Quantitative examination of the numerical density of each cell population was made using pan-neuronal markers including anti-NeuN, microtubule-associated protein 2 (MAP2), βIII tubulin, and neurofilament H (NF-H) antibodies for neuronal cells, and glial fibrillary acidic protein (GFAP) for astrocytes. For verification of the density of intact cells, we used 4,6-diamidino-2-phenylindole (DAPI) for nuclear staining. For each case and each staining, 2 fields (20× objective) were acquired for each cortical layer of NAGM. All values were normalized as cell counts per square millimeter of the respective tissues. All quantifications were performed by 3 independent investigators (K.Y., M.A., and I.K.) blinded to the case identification and status. Bars indicate the mean for each group. (A) The numerical density of pan-neuronal marker-positive and DAPI-positive neurons measured in each cortical layer of the gray matter in NMOsd brains compared to control brains is shown. A striking and significant decrease in the density of cortical neurons was seen in layers II, III, and IV of NMOsd brains compared to control brains, and the greatest loss of neuronal cells was seen in layer II of NMOsd brain. (B) The numerical density of GFAP-positive and DAPI-positive astrocytes measured in each cortical layer of the gray matter in NMOsd brains compared to control brains is shown. A significant increase in the density of cortical GFAP-positive astrocytes was seen in layers II to VI of NMOsd brains compared to control brains. Intriguingly, the number of GFAP-positive astrocytes was inversely decreased in layer I of NMOsd brains compared to control brains. These findings were consistent with the phenotypic features of astrocytes in the gray matter of NMO brains: loss of AQP4 immunoreactivity on most astrocytes in cortical layer I, but preserved expression of AQP4 immunoreactivity on all reactive, distended astrocytes in layers II to VI (see Fig 3). Solid circles = neuromyelitis optica; open circles = control; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

presence of cortical neuronal loss in cortical layer II, the presence of nonlytic reaction of AQP4-negative astrocytes in layer I, the abundance of reactive astrocytes with preservation of AQP4 in layers II to VI, massive activated microglia in layer II, and meningeal inflammation including CD45RO-positive T cells, but no B-cell follicle-like structures. These pathological changes in cortical gray matter of NMOsd confirm previous cortical MRI findings[6–9] and a previous pathological study indicating the absence of cortical demyelination and the presence of some pyknotic neurons in NMO brains.[30] Regarding the expression of AQP4 in each cortical layer of NMOsd brains, we found nonlytic reaction of AQP4-negative astrocytes in cortical layer I and the abundance of reactive astrocytes with preservation of AQP4 in cortical layers II to VI in NMOsd brains. The pathological changes in cortical gray matter in NMOsd brains were strikingly distinct from those in MS brains, which were characterized by cortical demyelinating lesions with meningeal inflammation even at the earliest clinical stages.[11,23,24,31,32] Several disease mechanisms of gray matter pathology in MS have been proposed,[12,33,34] including meningeal inflammation as a cause of subpial cortical damage,[11,23] demyelinated hippocampi as a cause of synaptic alterations,[35] selective vulnerability of neuronal subpopulations, growth factor dysregulation,[36] glutamate excitotoxicity,[37] and mitochondrial abnormalities.[38]

As chronic and acute inflammation is a major hallmark of NMOsd pathology as well as MS pathology, we here propose that the pathogenesis of the gray matter damage in NMOsd may be caused by primary selective damage of the gray matter, which is a site of high AQP4 expression, and there may be several neurodegenerative processes in NMOsd brains in conjunction with inflammation, including:

- The binding of NMO-IgG/AQP4 antibodies may cause incomplete internalization of AQP4 to the endo-lysosomal compartment in astrocytes, followed by disruption of water and/or glutamate homeostasis with excitotoxic consequences on neurons in the brain, similar to the hypothesis proposed in a previous report.[39] The 2 major AQP4 isoforms, M1 and M23, have identical extracellular residues. An in vitro study showed that the M1 isoform is completely internalized, but M23 resists internalization and is aggregated into larger-order orthogonal arrays of particles that activate complement more effectively than M1 when bound by NMO-IgG/AQP4 antibodies.[39] Thus,

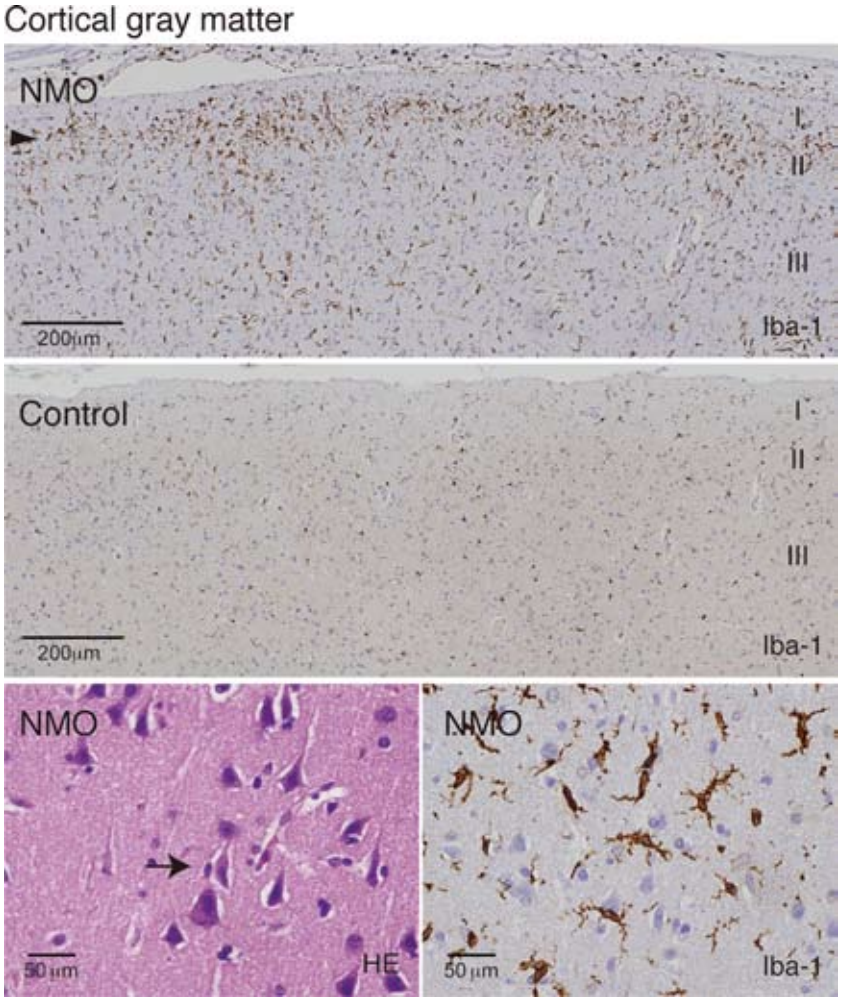

FIGURE 6: Increased numbers of microglial were prominent in cortical layer II, but no accumulation of lymphocytes was seen in neuromyelitis optica (NMO) spectrum disorder (NMOsd) brains. No cortical layers in NMOsd brains showed accumulation of lymphocytes, including CD45RO-positive T cells, CD20-positive T cells, and CD56-positive myelin-laden macrophages. However, all NMOsd cases showed prominent activation of parenchymal microglia with Iba-1 immunoreactivity in all cortical layers, particular in layer II (*arrowhead*). The Iba1-positive microglia in cortical layer II of NMOsd brains mostly displayed a radial, ramified morphology, with thick, shortened processes that are characteristic of activated cells. Interestingly, some microglia were located in the proximity of neurons in cortical layer II in NMOsd brains (*arrow*). HE = hematoxylin and eosin.

differences in the nature and anatomical distribution of NMOsd lesions could be influenced by regional and maturational differences in the ratio of M1 to M23 proteins in astrocytic membranes. In other words, the multiple potential sequelae of NMO–IgG interactions with AQP4 plausibly account for diverse pathological manifestations of NMOsd, including inflammatory demyelination, necrosis, edema, and neuronal degeneration in different anatomical regions of the CNS. These concepts of AQP4 dynamics may be in accordance with our findings that the characteristics of gray matter pathology in NMO brains were distinct from inflammatory demyelinating lesions in NMOsd myelitis.[5,15–17]

- Soluble neurotoxic factors produced by meningeal inflammatory cells may diffuse into gray matter and trigger neurodegeneration in the NMOsd brain, either directly or indirectly through the activation of microglia, in keeping with a theory of nonspecific antibody-independent cytotoxicity. In progressive MS, a clear gradient of neuronal loss was observed in gray matter demyelinating lesions and NAGM in cases with meningeal inflammation and lymphoid tissue formation such as B-cell follicle-like structures, suggesting that cytotoxic factors diffusing from the meningeal compartment contribute to gray matter pathology.[11,23] As some degree of meningeal cell infiltration including CD45RO-positive cells, but not B-cell follicle-like structures, was evident in the brains of NMOsd subjects, these findings raise the intriguing possibility that these inflammatory foci in meninges may influence cortical dysfunction and cognitive impairment in NMO, similar to the pathogenesis in MS.

- Innate immunity including activated microglia may be a key element of the neurodegeneration in NMOsd (see Fig 6). In MS, it has been proposed that mitochondrial injury, resulting in energy failure, is a key element of neurodegeneration in MS and is apparently driven by radical production by activated microglia.[34,40] We observed that the neuronal loss in layer II was prominent in the proximity of activated microglia in NMOsd brains (see Fig 6), suggesting that the neurodegenerative process may be associated with innate immunity, including microglia reacting in an analogous manner in MS.
- The autoimmune properties of unidentified neural antigens may cause selective neuronal damage in NMOsd brains, as NMOsd has broad-spectrum immunogenic specificities not only for AQP4 but also for other antigens such as myelin[41–43] and GFAP.[44]

Intriguingly, the most dramatic neuronal loss selectively targeting layer II of the gray matter was evident in NMO brains. In the beginning stages of Alzheimer disease, significant loss of neurons in layer II of the entorhinal cortex occurs.[45–47] It remains unclear whether this apparent lesion sensitivity is intrinsic to the neurons or is imposed by external factors such as the pattern of connections or change in AQP4 dynamics in layer I.

This study is not without limitations. The subjects in this study evaluated for cognitive impairment were different from those who were pathologically examined. However, we had a chance to perform both neuropsychological and neuropathological assessments in 2 autopsy cases of NMOsd patients with cognitive impairment (NMOsd cases 3 and 5 in Supplementary Table 2; Supplementary Fig 4). Although cognitive assessment of these patients was not performed using BRBN, both cognitive impairment and substantial cortical neuronal loss with unique AQP4 dynamics in astrocytes were evident in these patients. The exact relationship between cognitive impairment and cortical lesions in NMOsd and MS[32] awaits future research using the prospective study design.

In conclusion, NMOsd and MS patients have similar cognitive profiles, as represented by the latent start of impairment from the very early stage of the disease course. Conversely, compared to MS, NMOsd patients conclusively have distinct characteristic features of neuropsychological, radiological, and pathological modalities in the brain. These significant hallmarks of NMOsd may influence neuronal dysfunction in the brain and cognitive impairment, given the detrimental effects including cytokine diffusion, disruption of water homeostasis associated with AQP4 dynamics, or other unidentified mechanisms. Further research must now investigate the essential mechanisms of cognitive impairment and neurodegeneration.

Acknowledgment

This work was supported in part by a JSPS KAKENHI Grant Number 23591236 (I.K.), a grant for the Promotion of Niigata University Research Projects (I.K.), and a Health and Labor Sciences Research Grant for Intractable Diseases (Neuroimmunological Diseases) from the Ministry of Health, Labor, and Welfare of Japan (M.N.).

We thank Mrs. S. Kawaguchi, Mrs. Y. Watanabe, Mrs. A. Chikuzen, and Mrs. M. Kaneko for technical assistance with assays for AQP4 autoantibody, Dr. M. Kawamura for advice on the cognitive assessments, Dr. K. Tanaka for advice on the neurological and immunological assessments, Dr. M. Tsujita and Dr. K. Sakimura for the provision of an AQP4 expression vector, Dr. B. P. Morgan for the provision of complement antibody, and Dr. Y. Takeuchi for help in the recruitment of healthy controls.

Potential Conflicts of Interest

A.K.: grants/grants pending, Japan Epilepsy Research Foundation; speaking fees, Kyowa Kirin, Eisai, Daiichi-Sankyo, Dainippon-Sumitomo.

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