Journal of Neurochemistry

JOURNAL OF NEUROCHEMISTRY | 2013 | 127 | 66–77 doi: 10.1111/jnc.12362

ORIGINAL ARTICLE

AMP-activated protein kinase counteracts brain-derived neurotrophic factor-induced mammalian target of rapamycin complex 1 signaling in neurons

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Abstract

Growth factors and nutrients, such as amino acids and glucose, regulate mammalian target of rapamycin complex 1 (mTORC1) signaling and subsequent translational control in a coordinated manner. Brain-derived neurotrophic factor (BDNF), the most prominent neurotrophic factor in the brain, activates mTORC1 and induces phosphorylation of its target, p70S6 kinase (p70S6K), at Thr389 in neurons. BDNF also increases mammalian target of rapamycin-dependent novel protein synthesis in neurons. Here, we report that BDNF-induced p70S6K activation is dependent on glucose, but not amino acids, sufficiency in cultured cortical neurons. AMP-activated protein kinase (AMPK) is the molecular background to this specific nutrient dependency. Activation of AMPK, which is induced by glucose deprivation, treatment with pharmacological agents such as 2-Deoxy-D-glucose, metformin, and 5-aminoimidazole-4-carboxamide ribonucleoside or forced expression of a constitutively active AMPKα subunit, counteracts BDNF-induced phosphorylation of p70S6K and enhanced protein synthesis in cortical neurons. These results indicate that AMPK inhibits the effects of BDNF on mTORC1-mediated translation in neurons.

Keywords: AMPK, BDNF, mTOR, neuron, protein synthesis, translation.

J. Neurochem. (2013) **127**, 66–77.

Brain-derived neurotrophic factor (BDNF) is the most prominent neurotrophic factor in the brain that promotes differentiation, maturation, and survival of neurons (Lewin and Barde 1996). In addition, BDNF and its cognate receptor tyrosine kinase TrkB play important roles in synaptic plasticity and higher brain functions, such as learning and memory (Nawa and Takei 2001; Poo 2001). Many of the events that underlie plasticity depend on translation (Costa-Mattioli *et al.* 2009; Richter and Klann 2009). We have previously shown that BDNF up-regulates translation by activating mammalian target of rapamycin (mTOR) signaling in neurons (Takei *et al.* 2001, 2004; Inamura *et al.* 2005). mTOR is a serine/threonine protein kinase that integrates signaling mediated by growth factors/receptor tyrosine kinases, and various nutrients, such as amino acids and glucose. mTOR nucleates two distinct complexes, mTOR complex 1 (mTORC1) and mTOR complex 2. mTORC1 plays a central role in mRNA translation and autophagy, suggesting that mTORC1 regulates total protein levels. Interestingly, recent reports have revealed that

Received June 7, 2013; revised manuscript received July 4, 2013; accepted July 5, 2013.

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Abbreviation: 2DG, 2-Deoxy-D-glucose; ACC, acetyl-CoA carboxylase; AICAR, 5-aminoimidazole-4- carboxamide ribonucleoside; AMPK, AMP-activated protein kinase; BDNF, brain-derived neurotrophic factor; GST, glutathione S-transferase; mTORC1, mTOR complex 1; mTOR, mammalian target of rapamycin; p70S6K, p70 S6 kinase; TSC, tuberous sclerosis complex; raptor, the regulatory associated protein of mTOR.

mTOR is a key molecule for translation-dependent learning and memory (Belelovsky *et al.* 2009; Hoeffer and Klann 2010; Qi *et al.* 2010).

Various nutrients, such as amino acids and glucose, serve as not only raw materials and energy sources for protein synthesis, but also signaling molecules that regulate the translation machinery through mTORC1 (Sengupta *et al.* 2010). For instance, branched chain amino acids, in particular leucine, activate mTORC1-mediated signaling (Tokunaga *et al.* 2004; Hara *et al.* 1998; Ishizuka *et al.* 2008). Cellular glucose levels, which are closely related to the AMP/ATP ratio, affect mTORC signaling pathway via AMP-activated protein kinase (AMPK) (Inoki *et al.* 2003; Tokunaga *et al.* 2004; Kimura *et al.* 2003). Ligands of receptor tyrosine kinases are known to control translation levels. It has been well studied that insulin and insulin receptor signaling activates the translation machinery through multiple factors, including mTORC1 (Proud and Denton 1997; Proud 2006). These multiple cascades, amino acids, glucose, and receptor tyrosine kinase signals are merged on mTORC1 and interplay, leading to the downstream cellular responses such as regulation of translation (Hay and Sonenberg 2004; Wullschleger *et al.* 2006; Sengupta *et al.* 2010; Proud 2007).

Our previous studies found that BDNF plays a key role in neurons to control translation via mTORC1 (Inamura *et al.* 2005; Takei *et al.* 2001, 2004). Moreover, leucine activates p70S6 kinase (p70S6K) in neurons in a rapamycin-dependent manner (Ishizuka *et al.* 2008). The aim of this study was to elucidate the interplay between BDNF and certain nutrients in the central nervous system. Since amino acid(s) sufficiency is reported to be essential for the insulin-induce activation of mTORC1 in PC12 and chinese hamster ovary cells (Kleijn and Proud 2000; Campbell *et al.* 1999), we examine the inter-related effects between amino acids or glucose and BDNF in primary cultured cortical neurons. Unlike these cell lines, the effects of BDNF on mTORC1 signaling in neurons are dependent on glucose, but not amino acids.

We found that AMPK suppressed the effects of BDNF on mTORC1-dependent translation in neurons. To date, how neurons integrate information about cellular energy status and BDNF–TrkB signaling has remained unclear. Therefore, we analyzed the role of AMPK in BDNF-induced p70S6K a substrate of mTORC1, activation and consequent protein synthesis in neurons.

AMPK is a heterotrimeric serine/threonine protein kinase, consisting of a catalytic α subunit and regulatory β and γ subunits (Hardie 2004). AMPK serves as a cellular energy sensor by detecting the AMP/ATP ratio. When the ratio is elevated – for instance, during periods of metabolic stress from nutrient insufficiency or hypoxia/ischemia – AMPK activates energy-generating catabolic pathways and suppresses such anabolic pathways as fatty acid synthesis, gluconeogenesis, and protein synthesis (Inoki *et al.* 2003). Of note, AMPK activity is coupled with phosphorylation of Thr172 in the AMPKα subunit (Kemp *et al.* 2003; Witters *et al.* 2006; Hardie 2004). Here, we report that AMPK activation abolishes the effects of BDNF on protein synthesis by specifically suppressing activation of mTOR signaling in cortical neurons.

Materials and methods

Materials

2-Deoxy-D-glucose (2DG) was purchased from Wako (Osaka, Japan). Glucose-free Dulbecco's modified Eagle's medium (DMEM) and high-glucose DMEM were purchased from Gibco (Auckland, New Zealand). [^{35}S]Methionine, [γ-^{32}P]ATP, and protein G Sepharose were obtained from GE Healthcare (Tokyo, Japan). Anti-phospho-AMPKα (Thr172), anti-AMPKα, anti-phospho-acetyl-CoA carboxylase (ACC) (Ser79), anti-phospho-p70S6K (Thr389), anti-phospho-p70S6K (Thr421/Ser424), anti-phospho-TSC2 (Thr1462), anti-TSC2, anti-phospho-raptor (Ser792), anti-raptor, and anti-Akt antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-phospho-Akt (Thr308) was purchased from Enogene Biotech (New York, NY, USA). Anti-p70S6K antibodies used for western blotting (H-9) and immunoprecipitation (C-18) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-microtubule-associated protein 2 monoclonal antibody was purchased from Chemicon (Temecula, CA, USA). Anti-β-actin monoclonal antibody was purchased from Calbiochem (San Diego, CA, USA). p70S6K substrate peptide (KEAKEKRQEQIA KRRRLSSLRASTSKSGGSQK, corresponding to amino acids 218-249 of human S6) was obtained from Upstate Biotechnology (New York, NY, USA). The AMPK activator 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR) and metformin were purchased from Cell Signaling Technology and WAKO (Osaka, Japan), respectively. The AMPK inhibitor compound C was purchased from Calbiochem. A rat neuron Nucleofector kit was purchased from Lonza (Koln, Germany).

Animals

Sprague–Dawley rats were purchased from SLC Ltd (Shizuoka, Japan), and maintained under a 12-h light/dark cycle with free access to food and water. All animal experiments were approved by the Animal Use and Care Committee of Niigata University and were carried out in accordance with ARRIVE guidelines. All efforts were made to minimize the number of animals used and their suffering.

Cell culture

Primary cultures of cortical neurons were prepared essentially as previously described (Ishizuka *et al.* 2008; Takei *et al.* 2009). Briefly, cerebral cortices were removed from 16 to 18-day-old embryonic rat fetuses and dissociated with papain and DNase I. Dissociated neurons were seeded in 60-mm^2 culture dishes (5×10^6 cells/dish) for p70S6K activity assays, 6-well plates (2×10^6 cells/well) for western blotting, 24-well plates (5×10^5 cells/well) for the measurements of [^{35}S]methionine incorporation. Neurons for immunocytochemistry were seeded onto cover slips (13 ϕ; 2×10^5 cells/well). Cultures were maintained for 7 days in DMEM containing 10% fetal bovine serum.

Rat fibroblasts were prepared from embryonic meninges, which were digested with trypsin and DNase I and cultivated in DMEM containing 10% fetal bovine serum. To increase the purity of the cell populations, cultures were passed several times before they were used. Cells were starved of serum for 12–16 h before drugs and/or growth factors were applied.

Electrophoresis and western blotting

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and western blotting were performed as described previously (Ishizuka *et al.* 2008). Cells were lysed and sonicated in sample buffer, containing 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2% SDS, 20 mM NaF, 1 mM Na_3VO_4, and Complete Mini Protease Inhibitor Cocktail (Roche, Indianapolis, IN, USA). After centrifugation, supernatant was collected and protein concentrations were determined. Equal amounts of protein (40–50 μg/lane) were subjected to SDS–PAGE and transferred to polyvinylidene fluoride membranes. The membranes were incubated with the indicated primary antibodies and then horseradish peroxidase – conjugated anti-mouse IgG or horseradish peroxidase – conjugated anti-rabbit IgG secondary antibodies (1 : 10000 dilution; Cappel). Peroxidase activity was detected using chemiluminescence (Western Lightning, PerkinElmer, Waltham, MA, USA) and visualized on autoradiographic film.

Immunoprecipitation and p70S6K assays

p70S6K activity was assessed essentially as reported previously (Ishizuka *et al.* 2008). Cells were lysed and supernatants were subjected to immunoprecipitation with anti-p70S6K antibody for 3 h at 4°C. Complexes were isolated with protein G Sepharose. The kinase assay was initiated by adding reaction mixture containing 50 mM 3-morpholino propanesulfonic acid (pH 7.2), 12 mM $MgCl_2$, 2 mM EGTA, 1 mM dithiothreitol, 60 mM ATP, 10 mM β-glycerophosphate, 3 nmol substrate peptide, 10 μCi of [γ-^{32}P] ATP, 5 mM protein kinase C inhibitor peptide, 0.5 mM protein kinase A inhibitor peptide, and 5 mM Compound R24571. Samples were then incubated for 20 min at 30°C. The supernatants were spotted onto P81 phosphocellulose membranes, which were washed, air-dried, and immersed in the scintillation cocktail before radioactivity levels were determined. To calculate net p70S6K activity, background activity was obtained using samples without substrate.

Metabolic labeling with [^{35}S]methionine

Cortical neurons were incubated with 10 μCi of [^{35}S]methionine with or without BDNF for 30 min after treatment with the drugs for the indicated periods of time. Protein synthesis was measured based on [^{35}S]methionine incorporation, as previously reported (Takei *et al.* 2001). Levels of free methionine and methionine that was incorporated into proteins were measured based on radioactivity in the supernatant and pellet, respectively. We then calculated the percentage of total [^{35}S]methionine taken up into the neurons that precipitated in the pellet.

Immunocytochemistry

Immunocytochemistry was performed as previously described (Takei *et al.* 2004). Neurons were incubated with the indicated antibodies for 16–24 h, depending on which primary antibodies were used. Alexa Fluor 488–conjugated anti-mouse IgG or Alexa Fluor 546–conjugated anti-rabbit IgG antibodies (Molecular Probes, Eugene, OR, USA) were used as secondary antibodies. Fluorescence images were obtained using a BZ-9000 digital fluorescence microscope with a 40 × objective lens (Keyence, Osaka, Japan). If primary antibodies were omitted, background fluorescence signals were negligible in all experiments. Captured images were analyzed using a BZ-II Analyzer (Keyence). We identified transfected neurons based on glutathione S-transferase (GST) immunoreactivity and measured the intensity of P-p70S6K-specific signals. The luminance range for the 8-bit digital images was set to 50 to 256 for all pictures to measure fluorescence intensity in transfected neurons. We examined 80 neurons chosen randomly from three or four culture dishes representing each group in a blinded manner.

Electroporation

Sequence encoding constitutively active AMPKα (amino acid residues 1-312) was cloned into pEGB vector as described previously (Crute *et al.* 1998). Empty vector (GST alone) was used as a control. Electroporation was performed using Nucleofector according to the manufacturer's manual. Dissociated cortical neurons (5×10^6 cells) from rat embryos (E16) were resuspended in Nucleofector solution containing 3 μg of plasmid DNA. Neurons were seeded onto culture plates and incubated for 72 h.

Statistical analysis

All values are presented as mean ± SE. Data were subjected to Student's *t*-test or ANOVA to evaluate the differences in western blots, immunocytochemistry, kinase activity and [^{35}S]methionine incorporation. $p < 0.05$ were considered statistically significant.

Results

Effects of various nutrients on growth factor-induced p70S6K phosphorylation in neurons and fibroblasts

To analyze crosstalk between nutrients and growth factors on mTORC1 signaling, we examined the effects of depriving rat cortical neurons and meningeal fibroblasts of amino acids or glucose on growth factor-induced p70S6K phosphorylation at Thr389, a substrate residue of mTORC1. Cells were starved of serum for 16 h. The medium was then changed to HANKS' balanced salt solution or glucose-free DMEM and the cultures were incubated for 1 h before growth factors were added. Neurons and fibroblasts stimulated for 30 min with BDNF (50 ng/mL) and fibroblast growth factor 2 (FGF2) (50 ng/mL), respectively, under nutrient-free conditions were harvested and cell lysates were subjected to SDS–PAGE. Levels of phosphorylated p70S6K were analyzed using western blotting with anti-phosphospecific p70S6K (Thr389) antibody. Similar to previous reports using other cell types, growth factor-induced p70S6K phosphorylation was amino acid-dependent in primary fibroblasts. On the other hand, treatment with fibroblast growth factor 2 (50 ng/mL, Fig. 1) or insulin (10 μg/mL, Figure S1) for 30 min induced p70S6K phosphorylation even in the absence of glucose. Growth factor-induced p70S6K phosphorylation was markedly suppressed when amino acids were removed from the medium. In contrast, in primary cultured cortical neurons, p70S6K phosphorylation

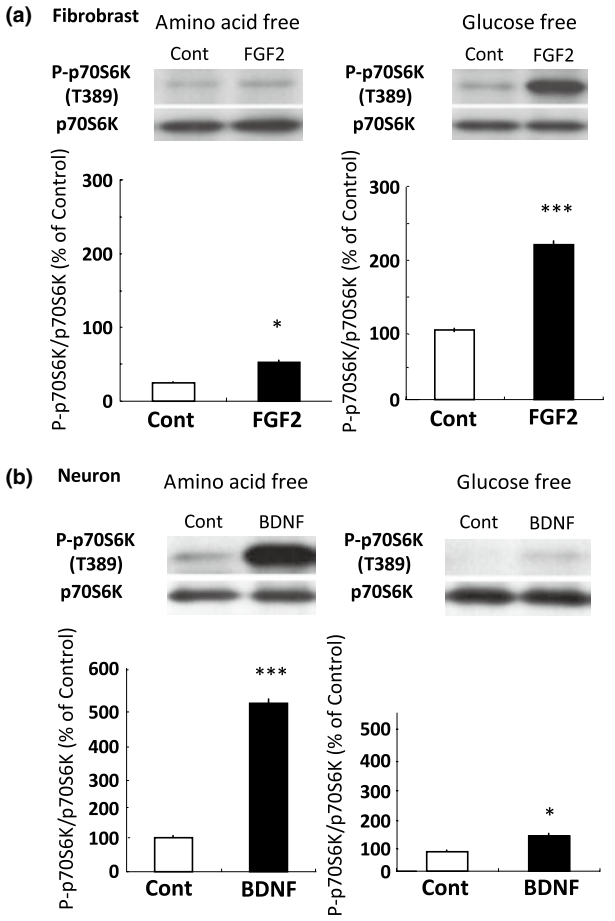

Fig. 1 Effects of nutrients sufficiency on growth factor-induced p70S6 kinase (p70S6K) phosphorylation at Thr389 in fibroblasts and neurons. Upper panels show typical western blots of total p70S6K and p70S6K phosphorylated at Thr389 [P-p70S6K(Thr389)] after stimulation with growth factors under nutrient-free conditions. Fibroblasts (a) and cortical neurons (b) in amino acid-free HANKS' balanced salt solution or glucose-free Dulbecco's modified Eagle's medium were treated with growth factors (50 ng/mL FGF2 or 50 ng/mL brain-derived neurotrophic factor) for 30 min. Lower panels show quantification of the P-p70S6K/p70S6K ratio. Data are presented as means ± SE ($n = 3$). *$p < 0.05$; ***$p < 0.001$ (Student's *t*-test).

induced by BDNF (50 ng/mL, Fig. 1) or insulin (10 μg/mL, Figure S1) was dependent on glucose but not amino acids. Actin levels did not change under any of the conditions (data not shown). These results, together with previous reports, suggest that neurons specifically depend on glucose to exert the action of BDNF on mTORC1 signaling.

Glucose deprivation does not affect BDNF-induced p70S6K phosphorylation at Thr421/Ser424 and Akt phosphorylation at Thr308

Because glucose is the major energy source for neurons and sole source in this culture condition, the most straightforward interpretation of the observed glucose requirement is the need for ample ATP as a phosphate donor. We therefore examined p70S6K phosphorylation at residues that are not substrates for mTORC1. Thr421 and Ser424 of p70S6K are substrates for MAPK and phosphorylation of these residues is induced by BDNF. In addition, we also examined Akt phosphorylation at Thr308. Akt plays a critical role in regulating cell survival and metabolism in many different signaling pathways. Thr308 is phosphorylated by PI3 Kinase pathway(Alessi *et al.* 1996; Burgering and Coffer 1995). Neurons were pre-treated with 25 mM 2DG – a non-metabolizable glucose analog – for 1 h and then stimulated with BDNF. Cells were harvested for western blotting using anti-phosphospecific p70S6K (Thr421/Ser424) and anti-phosphospecific Akt at Thr308 antibodies. Treatment with 2DG did not inhibit BDNF-induced phosphorylation of these sites (Fig. 2). These results indicate that the glucose-deprivation conditions used in this study may reduce but not completely deplete ATP level to an extent that broadly inhibited protein phosphorylation.

AMPK activation in cortical neurons in response to glucose-free conditions, 2DG, metformin, or AICAR

Glucose deprivation and various drugs induce AMPK activity, which is known to counteract mTORC1 signaling. Thus, three AMPK activators with different mechanisms of action were examined (Corton *et al.* 1995; Bolster *et al.* 2002; Lee *et al.* 2006). Neurons were subjected to glucose deprivation for 1 h or were treated with 25 mM 2DG for 1 h, 2 mM AICAR for 4 h, or 10 mM metformin for 4 h. Levels of AMPKα phosphorylated at Thr172, that directly correlates with its enzymatic activity, were examined using western blotting.

Glucose deprivation and 2DG treatment induced AMPKα phosphorylation at Thr172 (Fig. 3a and b). In addition, the AMPK activators AICAR and metformin also induced AMPKα phosphorylation in cortical neurons (Fig. 3i and j), although the degrees of phosphorylation were different. Immunocytochemical analysis showed that AMPKα phosphorylation was induced in neurons and non-neuronal cells (Fig. 3c–h).

AMPK activators suppress BDNF-induced p70S6K Thr389 phosphorylation and kinase activity via mTORC1 signaling

Next, the effects of AMPK activation on BDNF-induced p70S6K phosphorylation were analyzed using western blotting. As shown in Fig. 4, 2DG and metformin suppressed BDNF-induced p70S6K phosphorylation at Thr389. Furthermore, 2DG treatment inhibited BDNF-induced p70S6K kinase activity, which was measured using p70S6K immunoprecipitated from neurons, a substrate peptide, and [γ-^{32}P] ATP. To analyze the action point of AMPK on BDNF-induced mTORC1 signaling, phosphorylation of tuberous sclerosis complex 2 (TSC2, also known as tuberin) and the regulatory associated protein of mTOR (raptor) was exam-

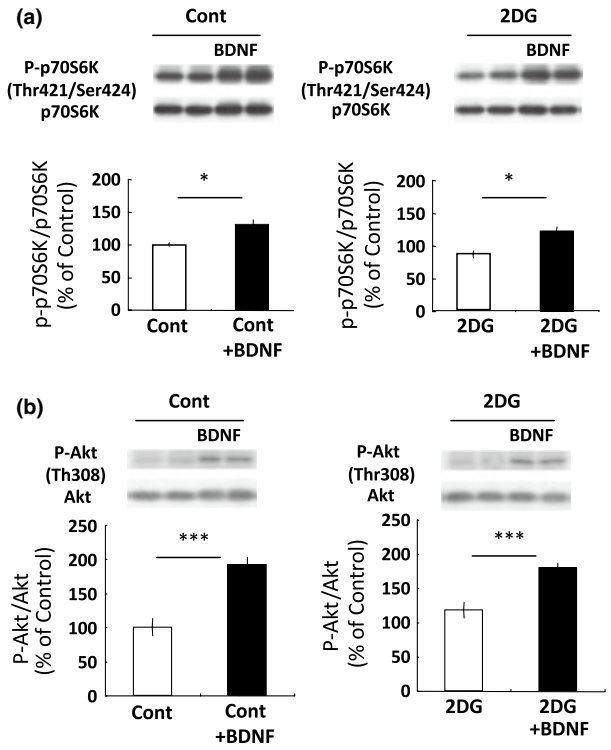

Fig. 2 Glucose-deprivation does not affect p70S6 kinase (p70S6K) phosphorylation at Thr421/Ser424 and Akt phosphorylation at Thr308 in neurons. Upper panels show typical western blots of p70S6K (a) and Akt (b). Blots using phospho-specific antibodies to indicate residues are also presented. Cortical neurons were treated with brain-derived neurotrophic factor (50 ng/mL, 30 min) and pre-treatment for 1 h with or without 25 mM 2DG. Western blots were quantified with NIH image after standardizing the ratio of phospho/total p70S6K (a) and Akt (b). Data are presented as means ± SE ($n = 4$) *$p < 0.05$, ***$p < 0.001$ (t-test).

ined. Tuberous sclerosis complex 1 (TSC1, also known as hamartin) and TSC2 form a tumor suppressor heterodimer that inhibits the mTORC1 signaling. Upon phosphorylation of TSC2 at Thr1462 and Tyr1571, TSC1/2 complex lose its activity of inhibition to mTOR (Manning *et al.* 2002; Aicher *et al.* 2001; Dan *et al.* 2002). Whereas BDNF increased TSC2 phosphorylation at Thr1462, 2DG treatment completely canceled the action of BDNF on TSC2 phosphorylation (Fig. 5a). Raptor is a component of mTORC1 (Hara *et al.* 2002; Kim *et al.* 2002) and acts as a scaffold for recruiting mTORC1 substrates such as p70S6K (Nojima *et al.* 2003). Recently, raptor has been identified as a direct substrate of AMPK. AMPK phosphorylates Raptor on Ser792 and reduces mTORC1 activity (Gwinn *et al.* 2008) Indeed, raptor phosphorylation at Ser792 was enhanced by 2DG, regardless of the presence of BDNF (Fig. 5b) in cortical neurons. These results suggest that AMPK affects both TSC2, an upstream molecule of mTORC1 and raptor, a component of mTORC1 thus counteracting the effect of BDNF.

AMPK activators suppress BDNF-induced protein synthesis

Because AMPK activators inhibited BDNF-induced p70S6K phosphorylation and kinase activity, the effects of these agents on BDNF-induced novel protein synthesis were investigated. As reported previously, BDNF enhanced [^{35}S] methionine incorporation into newly synthesized proteins in cortical neurons in mTORC1-dependent manner (Takei *et al.* 2001). Both 2DG and metformin inhibited protein synthesis induced by BDNF (Fig. 6). These results suggest that AMPK activation interferes with BDNF-induced enhancement of mTORC1-mediated translation.

AMPK inhibitor compound C restores AMPK-induced suppression of p70S6K phosphorylation by BDNF

To further confirm the suppressive role of AMPK on BDNF-induced p70S6K phosphorylation, effect of Compound C, a potent AMPK inhibitor was investigated. Neurons were starved of serum for 16 h. AICAR (2 mM) were added to cortical neurons with or without Compound C (40 μM) for 4 h. Neurons were then stimulated with 50 ng/mL BDNF for 30 min. Compound C inhibited AICAR-induced phosphorylation of AMPKα, ACC, a substrate of AMPK and raptor, respectively, in cortical neurons (Figure S3), indicating that it inhibits AMPK in neurons. As shown Fig. 7, Compound C restored BDNF-induced p70S6K phosphorylation at Thr389 even under the presence of AICAR. The result suggests that AMPK activation itself inhibits BDNF-induced mTORC1 activation.

Expression of CA-AMPKα reduced BDNF-induced phosphorylation of p70S6K and protein synthesis

To ascertain the critical role of AMPK in BDNF-induced p70S6K activation and enhanced protein synthesis, a constitutively active AMPKα subunit (CA-AMPKα) was introduced into neurons using electroporation. C-terminal truncation to a.a.312 of AMPKα (1-312) results in a constitutively active variant, because the enzyme lacks the autoinhibitory domain (Crute *et al.* 1998). Fig. 8a shows the expression of a GST-fused CA-AMPKα. Because the transfection efficacy was low (up to 20%), signals from anti-AMPKα or anti-phospho-AMPKα antibodies showed only a small increase (arrowheads). Note that endogenous AMPKα and GST-CA-AMPKα have similar molecular weights. Signals from anti-GST antibodies, however, revealed that certain amount of CA-AMPKα was expressed in the neurons. ACC phosphorylation increased following transfection (control, 100 ± 5.4% vs. CA-AMPKα 150 ± 4.0%; $n = 4$), demonstrating that AMPK activity was enhanced by CA-AMPKα. Under these conditions, BDNF-induced p70S6K phosphorylation was inhibited partially but significantly (Fig. 8b). Furthermore, CA-AMPKα transfection inhibited BDNF-induced protein synthesis (Fig. 9).

Immunocytochemistry was further performed to examine the effects of CA-AMPKα in individual neurons. Anti-GST

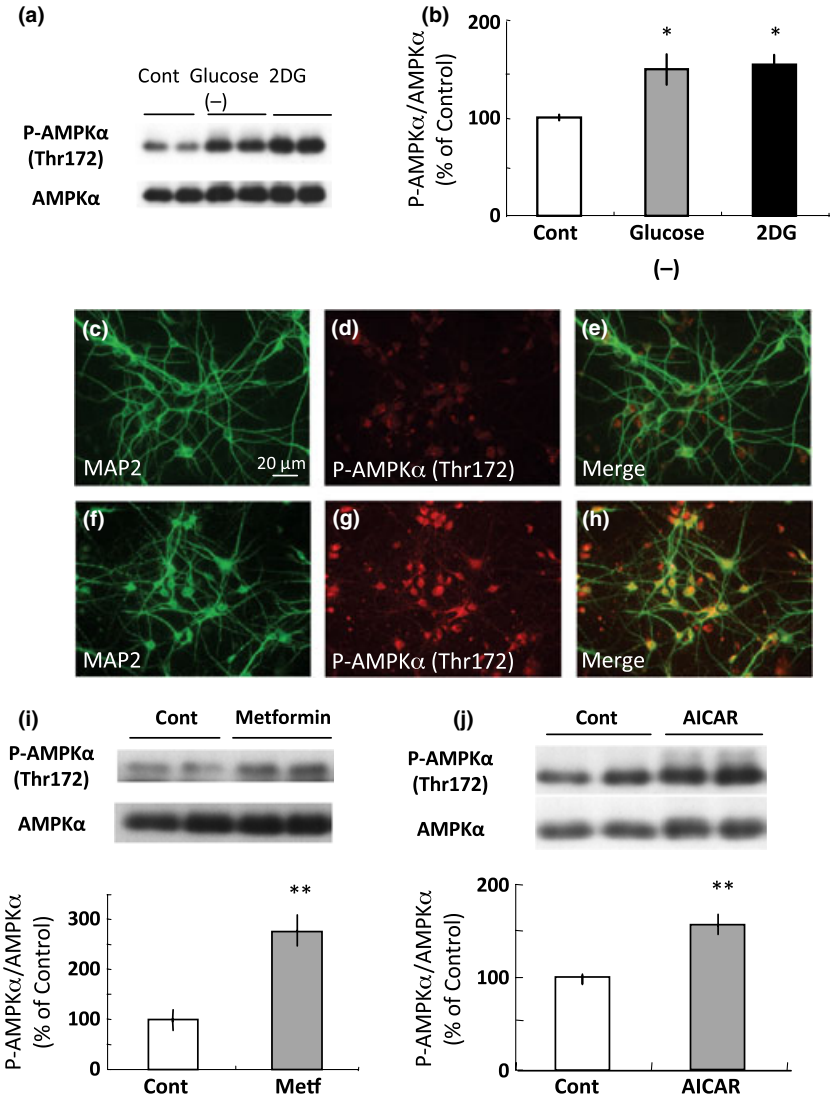

Fig. 3 AMP-activated protein kinase (AMPK) activators induce AMPKα phosphorylation in neurons. Typical western blots of P-AMPKα and total AMPKα in control (Dulbecco's modified Eagle's medium), glucose-free, and 2-Deoxy-D-glucose (2DG)-treated (25 mM) cultures are shown in (a). Ratios of P-AMPKα to AMPKα levels are shown in (b). (c–h) show double-immunocytochemistry with anti-microtubule-associated protein 2 (c, f) and anti-P-AMPKα (d, g) antibodies. Merged images are shown in (e and h). Neurons were treated without (c–e) or with 2DG (f–g) for 60 min. Scale bar, 20 μm. Effects of other AMPK activators on AMPKα phosphorylation, including treatment with metformin (10 mM) (i) and 5-amino-imidazole-4- carboxamide ribonucleoside (2 mM) (J) for 4 h, are also shown. Data are presented as means ± SE ($n = 4$). *$p < 0.05$; **$p < 0.01$ (ANOVA).

and anti-phospho-p70S6K antibodies were used to examine neurons transfected with CA-AMPKα or GST alone (Fig. 10a–l). Arrows show transfected neurons and arrowheads denote neurons that were not transfected. Under control conditions (GST alone), both populations of neurons showed strong signals for phospho-p70S6K in response to BDNF (Figure S5 and Fig. 10d–f). When CA-AMPKα was transfected into the cells, CA-AMPKα-positive neurons show weak signals for phospho-p70S6K, whereas cells that were not transfected showed strong signals (Fig. 10j–l). Signal intensities from the images were quantified (Fig. 10m). Fluorescence intensities representing phospho-p70S6K in GST-positive (transfected) neurons were calculated as described in the Experimental Procedures ($n = 80$ in each group). Because the cultures used for electroporation experiment were relatively young (E16) and the duration of culture was short (3 days total), almost all of the cells (> 98%) were neurons. These data indicate that AMPK activation suppresses BDNF-induced p70S6K phosphorylation. Taken together, the results demonstrate that AMPK activation suppresses BDNF-induced enhancement of mTORC1-mediated translation.

Discussion

This study examined the mutual relationships between BDNF/TrkB signaling and nutrients signals in neurons. Brain function clearly depends on the levels of essential nutrients. A lack of certain nutrients reduces brain activity and leads to a loss of consciousness. Recent studies have shown that amino acids and glucose are not only used as materials for protein synthesis and energy metabolism but also function as signaling molecules. Indeed, in neurons, amino acids – notably, leucine (Ishizuka *et al.* 2008; Cota *et al.* 2006) – and glucose (Dash *et al.* 2006) activate mTORC1 *in vitro* and *in vivo*.

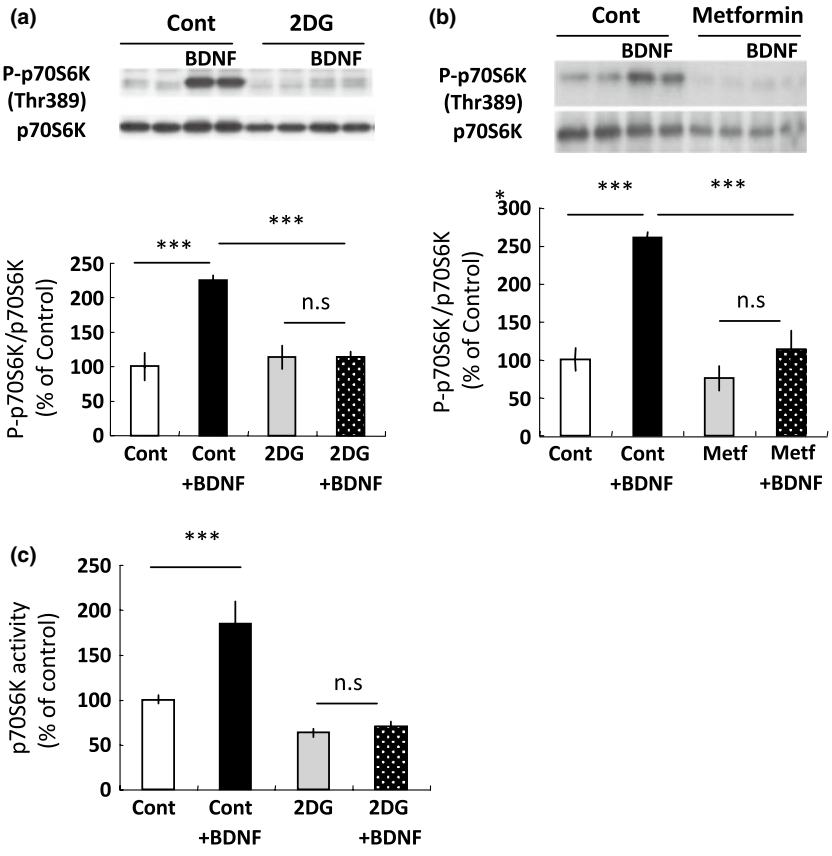

Fig. 4 AMP-activated protein kinase (AMPK) activators suppress brain-derived neurotrophic factor (BDNF)-induced p70S6 kinase (p70S6K) activation and phosphorylation at Thr389. Effects of AMPK activators on BDNF-induced p70S6K activation and phosphorylation in neurons were analyzed using western blotting and kinase assay (a–c). Upper panels show typical western blots of total p70S6K and p70S6K phosphorylated at Thr389 after neurons were stimulated with 50 ng/mL BDNF for 30 min with or without 25 mM 2-Deoxy-D-glucose for 1 h (a), 10 mM metformine for 4 h (b). Lower panels show P-p70S6K/p70S6K ratios. Data are presented as means ± SE ($n = 4$) $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ (ANOVA). (c) shows p70S6K kinase activity. Kinase activity of p70S6K was measured *in vitro* using synthetic peptide as a substrate. Bound radioactivity was counted and expressed as percentage of the control. The experimental design was the same as that used to obtain the data in (a). Data are presented as means ± SE ($n = 12$). $^{***}p < 0.001$ (ANOVA).

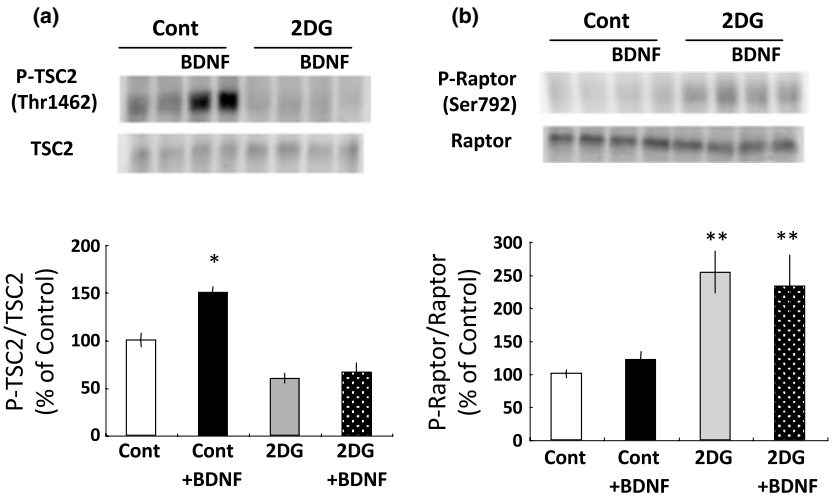

Fig. 5 An AMP-activated protein kinase activator, 2DG affects the phosphorylation states of tuberous sclerosis complex 2 (TSC2) and raptor. Upper panels show typical western blots of phospho- and total-TSC2 (a) and phospho- and total-raptor (b). Cortical neurons were treated with brain-derived neurotrophic factor (50 ng/mL, 30 min) and pre-treatment for 1 h with or without 25 mM 2DG. Western blots were quantified with NIH image after standardizing the ratio of phospho/total TSC (a) and raptor (b). Data are presented as means ± SE ($n = 4$)$^*p < 0.05$; $^{**}p < 0.01$ (ANOVA).

In this study, we found differences in the nutrients required by fibroblasts and neurons for growth factor-induced p70S6K phosphorylation at Thr389, a substrate of mTORC1. In PC12 (Kleijn and Proud 2000) and chinese hamster ovary (Campbell *et al.* 1999) cells, amino acids, but not glucose, are essential for insulin-induced mTORC1 signaling. Although similar results were observed in primary fibroblasts, neurons showed a different profile. BDNF and insulin-induced p70S6K phosphorylation at Thr389 even in the absence of amino acids, whereas the effects of these molecules were largely suppressed under glucose-free conditions. These data raise the question of how glucose deprivation interrupts BDNF/TrkB activation of mTORC1 in neurons.

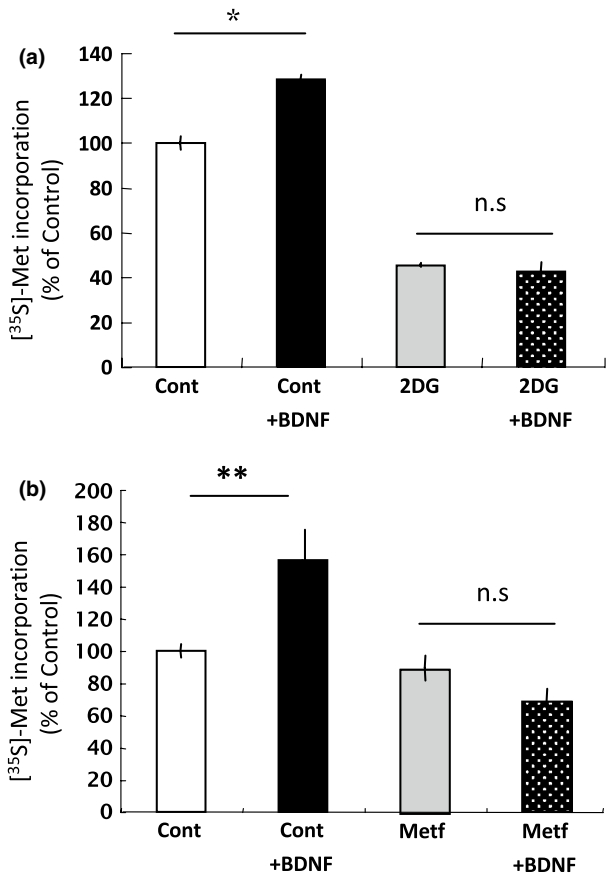

Fig. 6 AMP-activated protein kinase activators suppress brain-derived neurotrophic factor (BDNF)-induced protein synthesis. Neurons were treated with or without BDNF (50 ng/mL, 30 min) after pre-incubation with 2-Deoxy-D-glucose (25 mM, 1 h) (a) or metformin (10 mM, 4 h) (b). [^{35}S]Methionine was present in the cultures for the final 30 min. Radioactivity incorporated into newly synthesized proteins was measured and calculated as a percentage of the results observed in control samples. Data are presented as means ± SE ($n = 6$). *$p < 0.05$; **$p < 0.01$ (ANOVA).

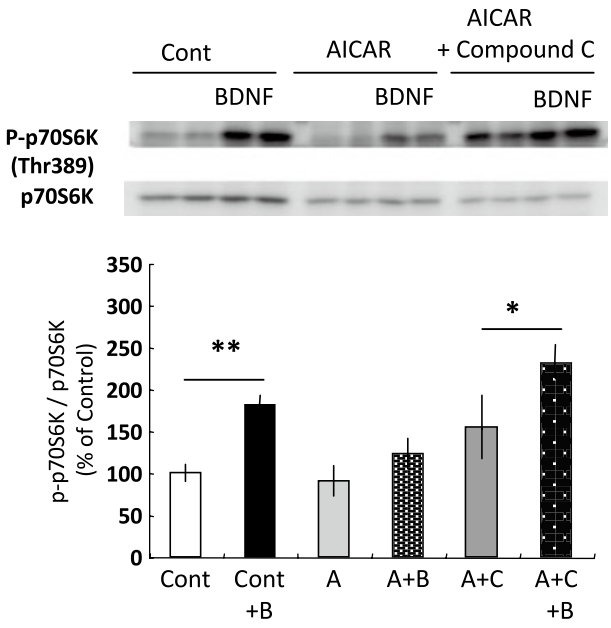

Fig. 7 An AMP-activated protein kinase inhibitor, compound C restores response of p70S6 kinase (p70S6K) to brain-derived neurotrophic factor (BDNF). Upper panels show typical western blots of total p70S6K and p70S6K phosphorylated at Thr389 [P-p70S6K(Thr389)] with or without BDNF under the presence of 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR) and Compound C. Cortical neurons were starved of serum for 16 h. 2 mM AICAR were added to cortical neurons with or without 40 μM Compound C for 4 h. Then, neurons were treated with 50 ng/mL BDNF for 30 min. Data are presented as means ± SE ($n = 6$). *$p < 0.05$; **$p < 0.01$ (ANOVA). A; AICAR, B; BDNF, C; Compound C.

Several lines of evidence showed that the glucose deprivation protocol used in this study decreased but did not completely deplete ATP levels. Phosphorylation was not generally defected (Fig. 2). Thus, we focused on AMPK because it serves as an intracellular energy sensor that detects decreases in ATP concentrations and increases in AMP levels (Kemp *et al.* 2003; Witters *et al.* 2006; Hardie 2004). AMPK activates tuberous sclerosis complex (TSC), GTPase-activating proteins for Rheb, resulting in inhibition of mTORC1 activity (Inoki *et al.* 2003). After confirming that glucose deprivation and 2DG treatment induced AMPKα phosphorylation at Thr172, that represents enzymatic activation, the effects of other AMPK activators were examined in neurons. The efficacy of these compounds differ among cell type, possibly because their permeability or uptake (Kimura *et al.* 2003). AICAR, a structural analog of AMP, and the biguanide antidiabetic drug metformin induced AMPKα phosphorylation and activation in cortical neurons, a process that was not related to reduce ATP levels (Corton *et al.* 1995; Bolster *et al.* 2002). These agents inhibited BDNF-induced activation of p70S6K in cortical neurons (see also Figs 4 and 7). Further confirmation of the effects of AMPK activity on BDNF-induced mTORC1 signaling was achieved by transfecting neurons with CA-AMPKα. AMPK is a tri-heteromeric enzyme that is composed of a catalytic α subunit and regulatory β and γ subunits. The construct composed of AMPKα1 amino acid residues 1-312 lacked the autoinhibitory domain, resulting in a constitutively active enzyme. In addition, the mutant form is more stable than wild-type AMPKα1 (Crute *et al.* 1998). Considering the low efficacy of transfection (about 20%), partial inhibition of p70S6K phosphorylation induced by BDNF is quite reasonable. Our results indicate that AMPK activation interferes with BDNF-induced mTORC1 signaling and protein synthesis in neurons. Interestingly, neither BDNF nor insulin suppresses AMPKα phosphorylation (Figure S4). Whereas *in vivo* experiments showed that insulin suppressed AMPK activity in hypothalamus (Minokoshi *et al.* 2004), no

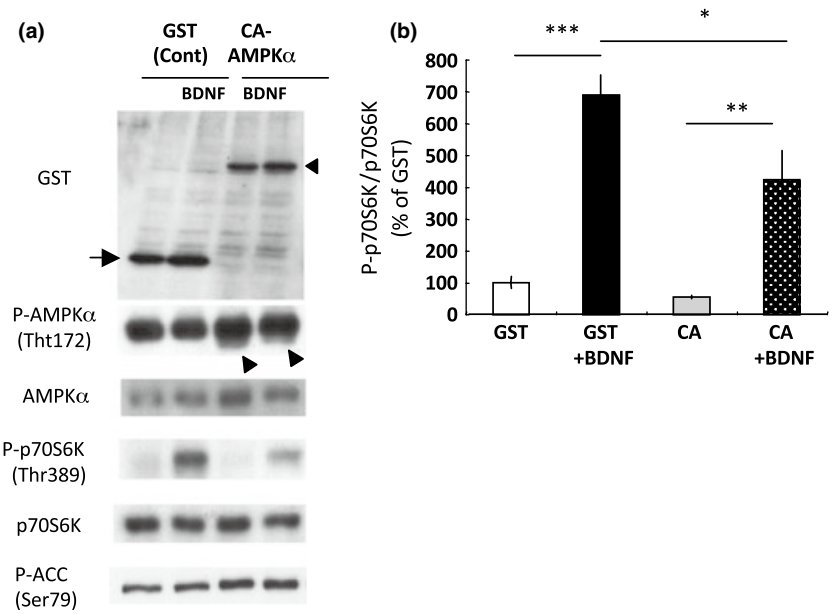

Fig. 8 Over-expression of CA-AMPKα suppresses brain-derived neurotrophic factor (BDNF)-induced p70S6 kinase (p70S6K) phosphorylation. cDNA encoding GST-fused CA-AMPKα or GST alone was transfected into neurons using electroporation. Forty-eight hours after transfection, neurons were treated with or without BDNF (50 ng/mL) for 30 min. (a) shows western blotting for the indicated molecules. Arrowheads indicate CA-AMPKα, whereas the arrow indicates GST. (b) shows the P-p70S6K/p70S6K ratios under each condition. Data represent means ± SE ($n = 6$). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ (ANOVA).

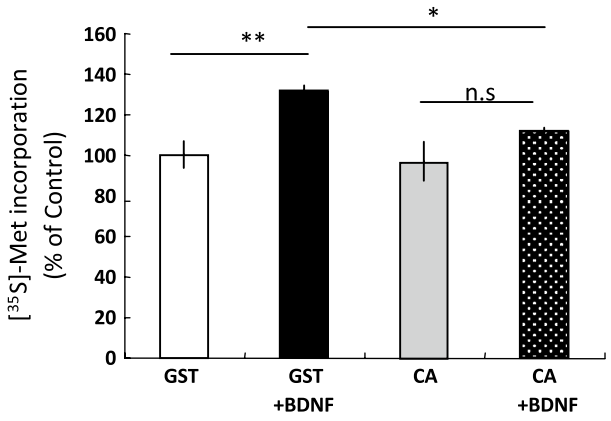

Fig. 9 Over-expression of CA-AMPKα suppresses brain-derived neurotrophic factor-induced protein synthesis. Over-expression experiments were performed as described in Fig. 8. [^{35}S]Methionine was present during the final 30 min of the incubations. Radioactivity incorporated into proteins was measured and calculated as a percentage of the results observed in control samples (GST alone). Data represent means ± SE ($n = 6$). *$p < 0.05$; **$p < 0.01$ (ANOVA).

direct effect was observed in cultured cortical neurons (Figure S4).

It is unclear why neurons depend on glucose but not amino acids in the experiments described here. One possibility is that intracellular levels of free amino acids in neurons may be higher than in other cell types. Alternatively, rates of autophagy in neurons may be fast enough to create a sufficient supply of amino acids for the time window (2 h) of the experiments in this study.

AMPK phosphorylates Thr1271 and Ser1387 of TSC2, thereby activating its GTPase-activating protein activity to Rheb, whereas Akt, a downstream effector of BDNF/TrkB, phosphorylates several other residues and inhibits its activity (Huang and Manning 2008). Although further experiments are required, the results presented here suggest that Thr1271 (and possibly Ser1387) phosphorylation may overcome the effect of Akt-induced phosphorylation to induce the GTPase-activating protein activity of TSC2. Interestingly, AMPK activation by 2DG also suppressed the phosphorylation of Akt at Ser473 induced by BDNF (Figure S2). Because Ser473 is a substrate residue of phosphorylation by mTORC2 (Sarvassov *et al.* 2005), AMPK may affect the activity of mTORC2 as well, possibly through TSC2.

In addition, AMPK phosphorylates Raptor at Ser722/792 and induces binding to 14-3-3, thus inhibiting mTORC1 activity (Gwinn *et al.* 2008). In cortical neurons, AMPK activation also induced raptor phosphorylation at Ser 792. The results suggest that AMPK activation attenuated BDNF-induced activation of mTORC1 signaling both through TSC2 and raptor.

Glucose-deprivation or adding 2DG creates a relatively extreme environment, which may mimic pathological conditions, such as ischemia and hypoglycemia induced by hyperinsulinemia. AMPK, however, can be activated under physiologic conditions. Several endogenous molecules regulate AMPK activity in the brain (Lim *et al.* 2010). For example, ghrelin (Andersson *et al.* 2004) and adiponectin (Kadowaki *et al.* 2008) activate AMPK, whereas leptin (Minokoshi *et al.* 2002) and glucagon-like peptide 1 (Seo *et al.* 2008) inhibit the enzyme in the brain. These hormones centrally regulate appetite. Interestingly, BDNF is reported to be a strong anorexigenic factor (Pelleymounter *et al.* 1995), and there may be crosstalk between feeding-related molecules and BDNF in the brain via AMPK and mTORC1.

In addition to eating behaviors, AMPK may contribute to synaptic plasticity (Potter *et al.* 2010) and other higher brain

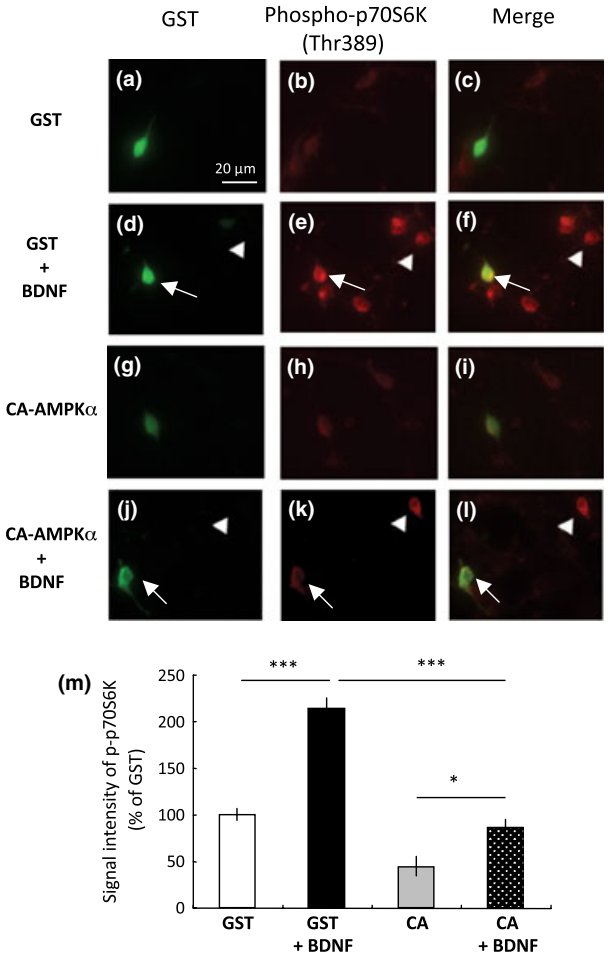

Fig. 10 Immunocytochemical analysis of CA-AMPKα-mediated suppression of brain-derived neurotrophic factor-induced p70S6 kinase (p70S6K) phosphorylation. CA-AMPKα was over-expressed as described in Fig. 8. Neurons were fixed and labeled with anti-GST (a, d, g, j; green) and anti-P-p70S6K (b, e, h, k; red) antibodies. Merged images are shown in (c, f, i, and l). Arrows indicate transfected (GST-positive) neurons, whereas arrowheads identify neurons that were not transfected (GST-negative). Note the weak P-p70S6K-specific signal in the neuron marked with the arrowhead, whereas a strong signal is observed in the neuron denoted with an arrow in (k). Signals related to P-p70S6K in GST-positive neurons were measured and the intensity was calculated (m). Data represent the means ± SE from 80 cells. $*p < 0.05$, $**p < 0.001$ (ANOVA). Scale bar, 20 μm.

functions, such as learning and memory, by interfering with the BDNF–mTORC1 signaling pathway. As noted previously, BDNF is critical for learning and memory (Lewin and Barde 1996; Nawa and Takei 2001; Poo 2001). Moreover, mTORC1 plays a central role in translation-dependent learning and memory (Hoeffer and Klann 2010; Qi *et al.* 2010). Several kinases mediate AMPKα phosphorylation, leading to enzymatic activation (Lim *et al.* 2010). Among them, calcium/calmodulin-dependent protein kinase kinase β (Hawley *et al.* 2005; Woods *et al.* 2005) is primarily expressed in neurons, suggesting an important role in AMPK activation in the brain under physiologic conditions. Regulated by Ca^{2+}, calcium/calmodulin-dependent protein kinase kinase β may transduce signaling from neurotransmitters and neuropeptides to activate AMPK and inhibit the activity of BDNF.

Acknowledgements

We are grateful Sumitomo Pharmaceuticals for supplying the recombinant BDNF. This study was supported by research grants from the Japan Society for the Promotion of Science (a Grant-in-Aid for Scientific Research B, C). All of the authors have no conflict of interest regarding the manuscript.

Supporting information

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

Figure S1. Effects of nutrients sufficiency on insulin-induced p70S6K phosphorylation at Thr389 in fibroblasts and neurons.

Figure S2. Glucose deprivation suppresses BDNF-induced Akt phosphorylation at Thr473 in neurons.

Figure S3. AICAR induces phosphorylation of AMPKa, ACC and Raptor and Compound C reverses the effect.

Figure S4. BDNF and insulin do not affect AMPKα phosphorylation.

Figure S5. BDNF induces p70S6K phosphorylation both naive and GST-transfected neurons.

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