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Glutamate Stimulates Local Protein Synthesis in the Axons of Rat Cortical Neurons by Activating α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic Acid (AMPA) Receptors and Metabotropic Glutamate Receptors*

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Wei-Lun Hsu (許瑋倫)[‡], Hui-Wen Chung (鍾惠雯)[‡], Chih-Yueh Wu (巫芝岳)[§], Huei-Ing Wu (吳慧音)[‡], Yu-Tao Lee (李侑道)[¶], En-Chan Chen (陳恩湛)[¶], Weilun Fang (方維倫)[¶], and Yen-Chung Chang (張兗君)[‡§‖1]

From the [‡]Institute of Molecular Medicine, [¶]Department of Power Mechanical Engineering, [§]Institute of Systems Neuroscience, and [‖]Institute of Nanoengineering and Microsystems, National Tsing Hua University, Hsinchu 300, Taiwan

Background: Transient rise of extracellular glutamate occurs in the developing brain.
Results: Glutamate stimulates axonal translation by binding to AMPA receptors and metabotropic glutamate receptors and activating Ca^{2+} and mTOR signaling.
Conclusion: Exposure to glutamate rapidly up-regulates local translation in migrating axons.
Significance: Glutamate-induced stimulation of local translation partakes in regulating axonal functions during development.

Glutamate is the principal excitatory neurotransmitter in the mammalian CNS. By analyzing the metabolic incorporation of azidohomoalanine, a methionine analogue, in newly synthesized proteins, we find that glutamate treatments up-regulate protein translation not only in intact rat cortical neurons in culture but also in the axons emitting from cortical neurons before making synapses with target cells. The process by which glutamate stimulates local translation in axons begins with the binding of glutamate to the ionotropic AMPA receptors and metabotropic glutamate receptor 1 and members of group 2 metabotropic glutamate receptors on the plasma membrane. Subsequently, the activated mammalian target of rapamycin (mTOR) signaling pathway and the rise in Ca^{2+}, resulting from Ca^{2+} influxes through calcium-permeable AMPA receptors, voltage-gated Ca^{2+} channels, and transient receptor potential canonical channels, in axons stimulate the local translation machinery. For comparison, the enhancement effects of brain-derived neurotrophic factor (BDNF) on the local protein synthesis in cortical axons were also studied. The results indicate that Ca^{2+} influxes via transient receptor potential canonical channels and activated the mTOR pathway in axons also mediate BDNF stimulation to local protein synthesis. However, glutamate- and BDNF-induced enhancements of translation in axons exhibit different kinetics. Moreover, Ca^{2+} and mTOR signaling appear to play roles carrying different weights, respectively, in transducing glutamate- and BDNF-induced enhancements of axonal translation. Thus, our results indicate that exposure to transient increases of glutamate and more lasting increases of BDNF would stimulate local protein synthesis in migrating axons en route to their targets in the developing brain.

Axons are long processes that connect neurons to their targets. During early development, highly dynamic growth cones lead the lengthening axons to their targets (1). Short and long range guidance cues that follow spatiotemporal dynamics in a developing brain serve to guide growth cones to navigate through the maze of cells separating neurons and their targets (2). Upon arriving at the target areas, axons form the presynaptic terminals of synapses that transmit signals between neurons and targets.

Glutamate is the principal excitatory neurotransmitter in the mammalian CNS. During development, glutamate also affects the growth, branching, chemotropic turning, and filopodial motility of migrating axons as well as synaptogenesis once axons have arrived at their target areas (3–12). These glutamate-induced effects on axons have been attributed to the activation of various glutamate receptors on the plasma membrane of axons and to the subsequent increases in Ca^{2+} concentration and resultant changes of the cytoskeleton in axons (2, 13–15).

Axons contain components of translation machinery, endoplasmic reticulum, Golgi apparatus, and numerous mRNA species (16–23). The composition of mRNAs in axons is dynamic and undergoes changes as development progresses and as the growing conditions and physiological states of neurons are altered, such as during injury and regeneration (20, 24). Local protein synthesis in axons can be stimulated by treatments with various reagents, such as Netrin-1, semaphorin 3A, Slit2, nerve growth factor, brain-derived neurotrophic factors (BDNF),[2] neurotrophin-3, and sero-

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[1] To whom correspondence may be addressed: College of Life Science, National Tsing Hua University, 101 Section 2 Kuang-Fu Rd., Hsinchu 300, Taiwan. Tel.: 886-3574-2754; Fax: 886-3571-7237; E-mail: ycchang@life.nthu.edu.tw.

[2] The abbreviations and used are: BDNF, brain-derived neurotrophic factor; mGluR, metabotropic glutamate receptor; mTOR, mammalian target of rapamycin; TRPC, transient receptor potential canonical; AHA, azidohomoalanine; FUNCAT, fluorescent noncanonical amino acid tagging; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; DL-APV, DL-2-amino-5-phosphonopentanoic acid; NASPM, 1-naphthyl acetyl spermine trihydrochloride; LY341495, 2*S*-2-amino-2-(1*S*,2*S*-2-carboxycyclopropyl-1-yl)-3-(xanth-9-yl)propanoic acid; 3-MATIDA, α-amino-5-carboxy-3-methyl-2-thiopheneacetic acid; W7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide; SKF96365, 1-{2-(4-methoxyphenyl)-2-[3-(4-methoxyphenyl)propoxy]ethyl}-1*H*-imidazole hydrochloride; MTEP, 3-[(2-methyl-1,3-thiazol-4-yl) ethynyl]pyridine hydrochloride; PLL, poly-L-lysine; DIV, day *in vitro*; ANOVA, analysis of variance; AMPA, α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; DHPG, (*RS*)-3,5-dihydroxyphenylglycine; CPPG, (*RS*)-α-cyclopropyl-4-phosphophenylglycine.

tonin (23). The resultant changes in local protein synthesis have been reported to be involved in the growth, branching, and regeneration of axons and the turning and collapse of axonal growth cones (23, 25–32).

During neural development, transient increases of glutamate in the extracellular space occur as resulting from spillover from immature glutamatergic synapses (33, 34) and being released from astrocytes (35) and axons (36). Conceivably, migrating axons would likely encounter such glutamate rises during development. We are interested to learn whether such rises of extracellular glutamate influence the local protein synthesis in migrating axons. To answer this question, a chip developed by Wu *et al.* (37) was used here. On the chip surface (Fig. 1*A*), neurons dissociated from the cortices of rat embryos are grown in the region 1 area, and axons are guided to grow into a separate and distant area, region 2. Because of the lack of synaptic targets in region 2, axons that have reached this area continue to grow without making synapses for 5–6 more days until region 2 becomes fully occupied by axons (38). The axons in region 2 are used here to model those axons in migrating toward their targets in a developing brain. For assaying local protein synthesis, the axons in region 2 are physically severed from their cell bodies and then subjected to treatments with glutamate, whereas the newly synthesized proteins in axons are metabolically labeled with the methionine analogue azidohomoalanine (AHA) (Fig. 1*B*). Afterward, the AHA moieties incorporated into axons are detected by the fluorescent noncanonical amino acid tagging (FUNCAT) method (Fig. 1*C*) (39). We have first validated the applicability of this procedure by using it to assay the known enhancement effects of BDNF on protein synthesis in cultured cortical neurons and their axons (16, 40). By using the same procedure, we find that glutamate enhances protein synthesis in cortical axons by activating AMPA receptors and metabotropic glutamate receptors (mGluRs). Our results further indicate that both Ca^{2+} and mammalian target of rapamycin (mTOR) signaling cascades mediate glutamate- and BDNF-induced enhancements of protein synthesis in cortical axons. Yet, these cascades may play roles of different weights, respectively, while signaling glutamate and BDNF stimulations to the translation machinery in axons.

Experimental Procedures

Reagents and Antibodies—Pregnant Sprague-Dawley rats were purchased from BioLASCO Taiwan Co., Ltd. (Taipei, Taiwan). The culture medium, including minimum Eagle's medium, neurobasal (NB), B27, DMEM, and methionine-free DMEM, were obtained from Gibco. Azidohomoalanine (AHA) was purchased from AnaSpec; alkyne-Alexa Fluor 647 (A10278), Click-iT cell reaction buffer kit (C10269), alkyne-biotin (C33372), and HRP (horseradish peroxidase)-streptavidin (43–4323) were obtained from Invitrogen. Glutamic acid, BDNF, cycloheximide, $GdCl_3$, and EGTA were purchased from Sigma. The following were obtained from Tocris: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), a selective agonist of AMPA receptors; *N*-methyl-D-aspartic acid (NMDA), a selective agonist of NMDA receptors; 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), an antagonist for both AMPA and kainate receptors; DL-APV (DL-2-amino-5-phosphonopentanoic acid), a selective NMDA receptor antagonist; 1-naphthyl acetyl spermine trihydrochloride (NASPM), a selective antagonist for Ca^{2+}-permeable AMPA receptors; DL-AP3 (DL-2-amino-3-phosphonopropionic acid, a group 1 mGluRs antagonist; LY341495 (2*S*-2-amino-2-(1*S*,2*S*-2-carboxycyclopropyl-1-yl)-3-(xanth-9-yl)propanoic acid), a group 2 mGluRs antagonist; (*RS*)-α-cyclopropyl-4-phosphonophenylglycine, a group 3 mGluRs antagonist; 3-MATIDA (α-amino-5-carboxy-3-methyl-2-thiopheneacetic acid), a selective mGluR1 antagonist; W7 (*N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide), a calmodulin inhibitor; SKF96365 (1-{2-(4-methoxyphenyl)-2-[3-(4-methoxyphenyl)propoxy]-ethyl}-1*H*-imidazole hydrochloride), a TRPC channels blocker; and MTEP (3-[(2-methyl-1,3-thiazol-4-yl) ethynyl]pyridine hydrochloride), a selective mGluR5 antagonist. Rapamycin, an mTORC1 inhibitor, was purchased from Enzo Life Sciences. Mouse anti-βIII-tubulin antibody (ab78078) was obtained from Abcam; rabbit anti-p-4E-BP1 antibody (sc-12884-R) was purchased from Santa Cruz Biotechnology; mouse anti-neurofilament (SMI-312R) antibody was obtained from Covance; mouse anti-GluN1 antibody (MAB363), rabbit anti-GluR1 antibody (05-855R) and rabbit anit-GluN2A/B antibody (AB1548) were obtained from Millipore. Alexa Fluor 488-conjugated goat anti-mouse IgG (115-545-003) and Cy3-conjugated goat anti-rabbit IgG (111-165-144) were purchased from Jackson ImmunoResearch.

Rat Cortical Neuronal Culture—All animal experiments were approved by the Animal Care and Use Committee of National Tsing Hua University at Hsinchu, Taiwan. Primary cortical cultures were prepared from rat fetuses at embryonic day 18 and maintained on conventional glass coverslips and in serum-free medium as described previously (41, 42). Dissociated embryonic day 18 cortical cells were also grown on the chip (Fig. 1*A*) according to the procedure described by Wu *et al.* (37). Briefly, a poly-L-lysine (PLL)-coated pattern was made on the surface of a square glass chip by microcontact printing (see the areas in *deep blue* in Fig. 1*A*). A polydimethylsiloxane stencil (data not shown) with a rectangular opening in register with the region 1 area was placed on the chip surface. Dissociated cortical cells in minimum Eagle's medium supplemented with horse serum (5%, v/v) and fetal calf serum (5%, v/v) at a density of 5×10^5 cells/ml were added to the hole made by the stencil opening and region 1 area (Fig. 1*A*). On day *in vitro* (DIV) 1, the stencil was lifted off, and the medium was replaced by neurobasal medium supplemented with 2% B27, 0.5 mM glutamine, and 25 μM glutamate. On DIV 3, neurons were treated with 5 μM cytosine-β-D-arabinofuranoside for 24 h to curtail the growth of glial cells. Afterward, ⅔ of the medium over the chip was replaced by fresh NB-B27 supplemented with 0.5 mM glutamine every 3–4 days. On DIV 8–9, axons extending from neurons in region 1 and migrating on PLL-coated lines started entering region 2; region 2 was fully occupied by axons at DIV ~15–16 (indicated by areas in *yellow*, Fig. 1*A*) (38).

Nascent Protein Detection Assays—The production of nascent proteins in cultured cortical neurons maintained on conventional coverslips at DIV 16 and that in the axons of cortical neurons in region 2 of chips between DIVs 15 and 17 were

assayed by the FUNCAT method (39) with modifications (Fig. 1*B*). The medium over the coverslips and chips was first replaced by methionine-free DMEM. Forty five min later, AHA was added to the medium to a final concentration of 2 mM. For neurons maintained on chips, the axons on PLL-coated lines between regions 1 and 2 were severed by using a fine stainless steel needle (0.15 mm diameter) at the position indicated by the broken *red line* in Fig. 1*A* right before the addition of AHA. Cells were then incubated at 37 °C and in 5% CO_2 for another 2 h. During this period, drugs were added to the medium at different time points (Fig. 1*B*). In some experiments, cycloheximide (final concentration 40 μM) or rapamycin (final concentration 100 nM) was added to methionine-free DMEM 15 min before the addition of AHA. At the end of the incubation, cells were washed with PBS to remove AHA, followed sequentially by fixation with PBS containing 4% paraformaldehyde and 4% sucrose for 20 min and permeabilization with PBS containing 1% Triton X-100 for 15 min at room temperature. For fluorescence tagging studies, neurons on coverslips and chips were washed with PBS containing 3% BSA three times and then reacted with 5 μM alkyne-Alexa Fluor 647 in Click-iT cell reaction buffer kit for 30 min at room temperature. After washing three times with PBS containing 3% BSA, neurons were rinsed three times in PBS containing 1% (v/v) Tween 20 and 0.5 mM EDTA each for 10 min at room temperature and then subjected to fluorescence immunostaining as described later (Fig. 1*C*). For bioorthogonal noncanonical amino acid tagging analysis (43), cells on coverslips were incubated in lysis buffer (20 mM HEPES at pH 7.0 containing 0.1% SDS, 1 mM PMSF, 1 mM Na_3VO_4, 1 mM NaF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin) on ice for 5 min. Cells were collected by using a rubber scraper, and the resultant lysates were centrifuged at 15,700 × *g* for 20 min at 4 °C to remove cell debris and nuclei. The supernatant was collected and reacted with alkyne-biotin according to the manufacturer's instructions. Proteins were then heated at 95 °C for 10 min in SDS-PAGE sample buffer (62.5 mM Tris-HCl at pH 6.8 containing 2.5% SDS, 5% β-mercaptoethanol, and 10% glycerol) and subjected to SDS-PAGE analysis with 12% polyacrylamide gels. After electrophoresis, proteins on the gels were electrotransferred to a PVDF membrane (Millipore). The resultant blots were incubated in the Tris-buffered saline (20 mM Tris-HCl at pH 7.4 and 50 mM NaCl) containing 0.1% Tween 20, 5% nondairy creamer, and 3% BSA overnight and then probed with HRP-streptavidin for 2 h at room temperature. After reacting with ECL Western blot detection reagent (Amersham Biosciences), HRP-labeled proteins on blots were detected by using ImageQuant^{TM} LAS 4000 mini system (GE Healthcare) and quantified by using ImageJ software (National Institutes of Health).

Fluorescence Immunocytochemistry—After conjugating the metabolically incorporated AHA moieties in nascent proteins with alkyne-Alexa Fluor 647, cells on coverslips or chips were washed with PBS three times and then incubated with mouse anti-βIII-tubulin antibody and, in some experiments, with rabbit anti-p-4E-BP1 antibody at 37 °C for 2 h. Cells were washed with PBS three times and incubated with Alexa Fluor 488-conjugated goat anti-mouse IgG (Cy3-conjugated goat anti-rabbit IgG in experiments wherein p-4EBP1 was probed) at 37 °C for 1.5 h. After washing with PBS three times, cells were sealed in ProLong Gold antifade reagent (P36930, Invitrogen) and then examined by using a confocal microscope (LSM 510, Carl Zeiss).

Image Analysis and Statistics—For neurons grown on conventional coverslips, images of at least five areas of 44,000 μm^2 in size were randomly selected from each coverslip for analysis. For axons in region 2 on chips, images of at least five areas of 17,700 μm^2 in size were randomly selected from each region for analysis (Fig. 1*C*). The regions occupied by cellular structures in images were first defined as regions exhibiting βIII-tubulin immunoreactivity with fluorescence intensities at least 2.5-fold higher than the average fluorescence intensity of the whole image. The fluorescence intensities of Alexa Fluor 647 were then calculated by dividing the summed Alexa Fluor 647 fluorescence intensities by the area of cellular structure-occupied regions. The above analyses were done by using ImageJ software. The Alexa Fluor 647 intensities thus calculated from the samples were then normalized by that calculated from the control sample performed in the same experiment. Results were presented as means ± S.E. Statistical analyses were performed by using two-way ANOVA followed by Fisher's least significant difference test. Two-way ANOVA was also used to analyze the interaction effects, which represent the combined effects of factors on the dependent measure, among stimuli (glutamate and BDNF) and inhibitors (EGTA, SKF96365, rapamycin, and W7) on the AHA signals measured from axons here.

Results

Detection of BDNF-induced Enhancements of Protein Synthesis in Cultured Rat Cortical Neurons and Their Axons—The FUNCAT method has been used widely to detect the production of newly synthesized proteins in various cells (39). This method was used here to analyze protein synthesis in the axons of cultured cortical neurons under various conditions (Fig. 1*B*). The applicability of the procedure shown in Fig. 1*B* was first verified by assessing the effects of BDNF treatments upon protein synthesis in intact cultured cortical neurons and in their axons after the axons were disconnected from their cell bodies. Treating cortical neurons with BDNF for 2 h increased the amount of AHA in neurons, as indicated by the increase in the fluorescence of Alexa Fluor 647 tagged to the incorporated AHA moieties in neurons (Fig. 2, *A* and *B*). This increase was inhibited by cycloheximide, a translation inhibitor, indicating that BDNF treatment stimulated the translation activity in these neurons and thus resulted in the production of more AHA-containing nascent proteins. To test this possibility, proteins were extracted from neurons treated with BDNF while in the presence of AHA. The extracted proteins were reacted with alkyne-biotin to label the incorporated AHA moieties and then subjected to Western blotting by using HRP-conjugated streptavidin (Fig. 2*C*). The results indicated that the amounts of AHA incorporated in proteins extracted from BDNF-treated neurons, as indicated by the sums of the intensities of the stained bands of these samples on blots, were ~1.5-fold higher than that of the control sample (Fig. 2*D*). Including cycloheximide in treatments reduced the amounts of AHA moieties incorporated in proteins extracted from control and BDNF-

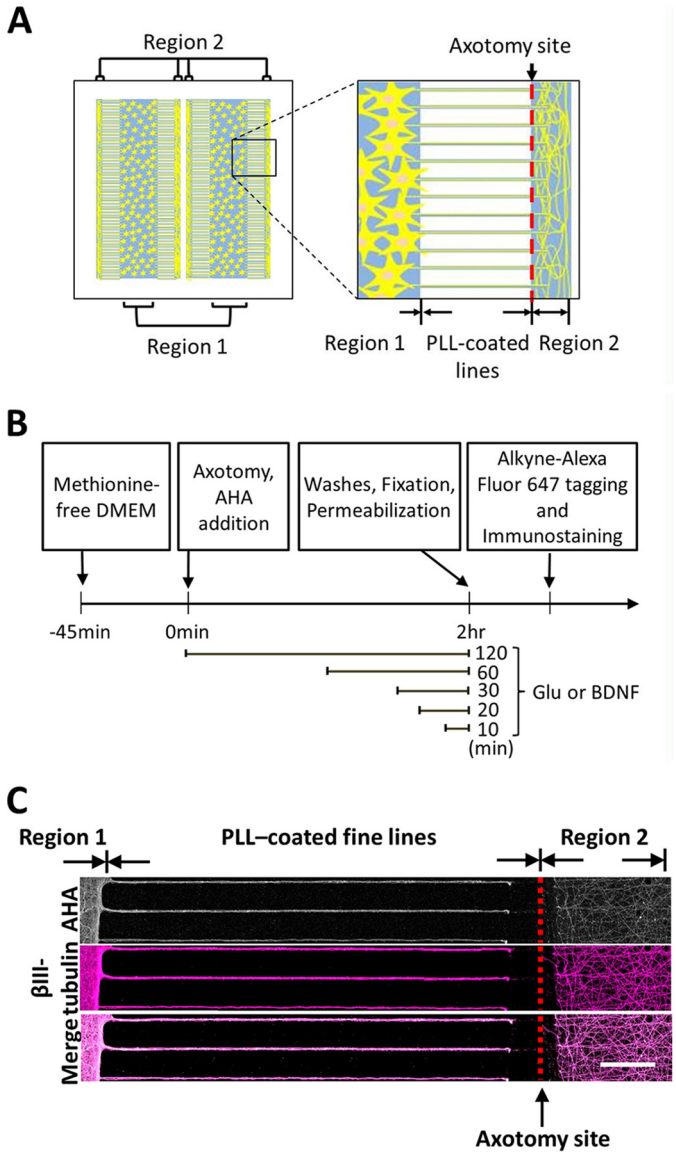

FIGURE 1. **Chip design and experimental procedures.** *A,* schematic presentation of the chip used here. *Left panel,* chip (1.4 × 1.4 cm) contains a PLL-coated micropattern (*blue*), consisting of regions 1 and 2 and the lines connecting these two regions, on the surface. *Right panel,* region enclosed by the *square* in the *left panel* at higher magnification. Fifteen to sixteen days after plating neurons (*yellow*) on the chip, region 2 is almost fully occupied by those axons extending from the neurons in region 1 and migrating along PLL-coated lines. *B,* experimental procedures for metabolically labeling cultured cortical neurons with AHA and for assaying incorporated AHA moieties. Cells on chips are incubated with methionine-free DMEM for 45 min and then with methionine-free DMEM supplemented with AHA for 2 h. The axons connecting regions 1 and 2 are severed at the position as indicated by the *broken red line* in *A* just before the addition of AHA. *Lines* in the *lower part* indicate the periods when glutamate or BDNF is present in different experiments. Cells on chips are then subjected to washes and fixation, followed by alkyne-Alexa Fluor 647 tagging and fluorescence immunostaining. *C,* images obtained from an experiment wherein neurons on the chip surface are assayed by the procedures shown in *B*. *Top* and *middle images*, respectively, show the distributions of Alexa Fluor 647 that tag incorporated AHA moieties and βIII-tubulin immunolabeling in neuronal structures on the chip surface. *Bottom image* is the merge of the *top* and *middle images*. *Scale bar,* 100 μm.

treated cells. The results thus support that the procedures as shown in Fig. 1*B* can detect the BDNF-induced enhancements of protein synthesis in cultured cortical neurons.

BDNF treatment was also found to increase the amount of AHA, as indicated by the increased fluorescence intensity of Alexa Fluor 647, in cortical axons that had been disconnected from their cell bodies (Fig. 3, *A, top row,* and *B*). The BDNF-induced enhancement in severed axons was inhibited by including cycloheximide in the incubation solution. In addition, BDNF treatment increased the content of phosphorylated eukaryotic initiation factor 4E-binding protein (p-4EBP1), which is one of the key signaling molecules downstream of mTOR complex 1 (44), as indicated by the increase of the p-4EBP1 immunoreactivity in axons (Fig. 3, *A, 2nd row,* and *C*). However, BDNF-induced increase of p-4EBP1 immunoreactivity was not affected by cycloheximide (Fig. 3*C*).

These results are consistent with the known enhancement effects of BDNF on the protein synthesis in intact cortical neurons as well as in the axons of cultured cortical neurons (16, 40), and they support the applicability of this process in assaying the changes of protein synthesis in neurons and their axons. The results further indicate that the mTOR signaling path may mediate BDNF-induced stimulations of local translation in axons as it does in dendrites (45).

Glutamate Treatment Enhanced Protein Synthesis in Cultured Rat Cortical Neurons and Their Axons—By using the same fluorescence tagging method as described above, it was found that treatment with glutamate (500 μM, 10 min) resulted in an ~2-fold increase in the amount of AHA, as indicated by the increase in Alexa Fluor 647 fluorescence, in cultured cortical neurons and that this glutamate-induced increase was inhibited by cycloheximide (Fig. 2, *A* and *B*). Western blotting analysis further showed increased incorporation of AHA in the proteins extracted from glutamate-treated cortical neurons when compared with that of control neurons and that this glutamate-induced increase was inhibited by cycloheximide (Fig. 2, *C* and *D*). Together, the results indicate that glutamate treatment enhances protein synthesis in cultured rat cortical neurons. We also investigated the subtypes of glutamate receptors that mediated these glutamate-induced enhancements. The glutamate-induced increases in the incorporation of AHA in neurons was inhibited nearly completely by DL-APV, a selective NMDA receptor antagonist, but not by CNQX, an antagonist for both AMPA and kainate receptors (Fig. 2*E*). The incorporation of AHA in neurons was not affected when they were treated with CNQX or DL-APV alone (Fig. 2*E*). These results are consistent with previous reports showing that glutamate treatments enhance protein synthesis in rat hippocampal neurons by activating NMDA receptors (46, 47). The discrepancy between our results and other earlier reports showing inhibition of protein synthesis in neurons by glutamate treatment (48–51) may be due to differences between the experimental conditions, such as culture medium, temperature, and treatment duration used by us and in these earlier studies, because these experimental conditions have been reported to significantly influence the cellular responses of neurons to glutamate treatment (44, 52, 53).

By using the same fluorescence tagging method, it was found that glutamate treatment (500 μM, 10 min) resulted in an ~2-fold increase in the incorporation of AHA and p-4EBP1 immunoreactivity in cortical axons that had been disconnected from their cell bodies (Fig. 3, *A* and *B*). The increase in the incorporation of AHA, but not that of p-4EBP1 immunoreac-

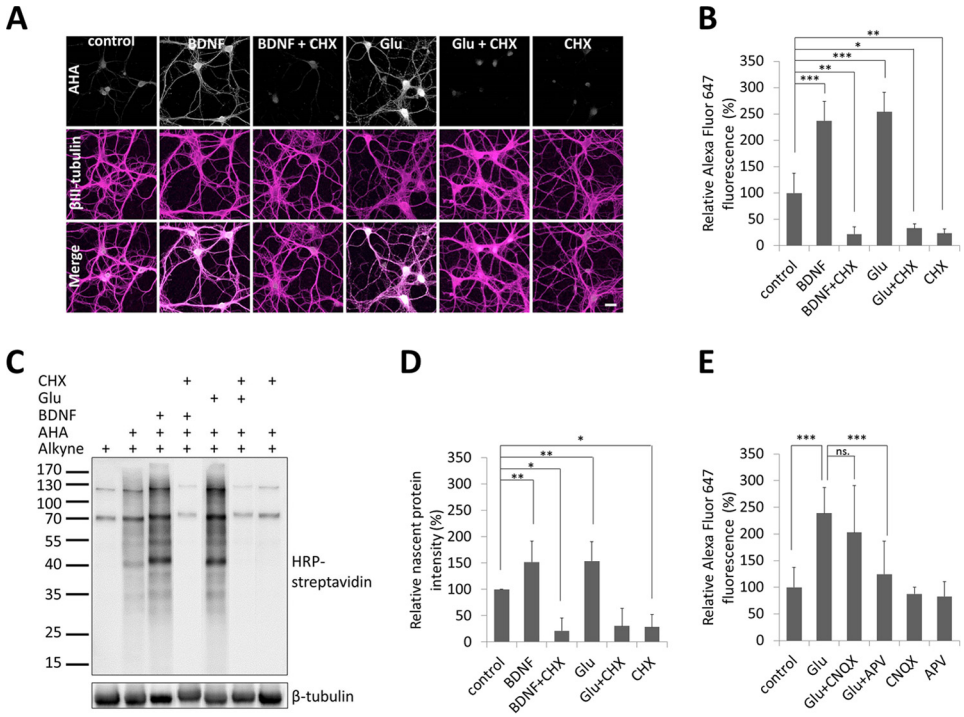

FIGURE 2. **Enhancements of protein synthesis in cultured rat cortical neurons by treatments with BDNF or glutamate.** *A,* cortical neurons were treated with 100 ng/ml BDNF for 2 h or with 500 μM glutamate (*Glu*) for 10 min in the presence or absence of 40 μM cycloheximide (*CHX*) in the medium supplemented with 2 mM AHA. AHA moieties incorporated in proteins were labeled with alkyne-Alexa Fluor 647 (*white*), and cells were subsequently immunostained by using the antibody to βIII-tubulin (*magenta*). *Scale bar,* 20 μm. The results are from a representative experiment of a total of more than five independent experiments. *B,* quantitative analysis of the fluorescence intensities of Alexa Fluor 647 in neurons as shown in *A*. Fluorescence intensities of samples were normalized to that of cells kept in AHA-containing medium for 2 h (*control*). Each datum is the mean ± S.E. of five independent experiments. *, $p < 0.05$; **, $p < 0.01$ and ***, $p < 0.001$ *versus* the control sample. *C,* Western blotting analysis of the proteins extracted from cortical neurons subjected to various combinations of treatments, including 500 μM glutamate, 100 ng/ml BDNF, 40 μM cycloheximide, and 2 mM AHA, as indicated at the top. Extracted proteins were labeled with alkyne-biotin, separated by SDS-PAGE on a 12% polyacrylamide gel, electrotransferred to PVDF membrane, and probed with HRP-streptavidin. The same membrane was also probed with the antibody to β-tubulin as the loading control (*bottom*). Molecular weight markers are indicated to the *left*. *D,* quantitative analysis of the summed intensities of the HRP-streptavidin-stained bands of the samples obtained from cells subjected to treatments as shown in *C*. The summed intensities of samples were normalized to that of the sample obtained from cells kept in AHA-containing medium for 2 h (*control*). Each datum is the mean ± S.E. of four independent experiments. *, $p < 0.05$, and **, $p < 0.01$ *versus* the control sample. *E,* effects of CNQX and DL-APV on glutamate-induced enhancement of protein synthesis in cortical neurons. Cortical neurons were treated with 500 mM glutamate for 10 min in the absence or presence of 200 μM CNQX, a non-NMDA receptor antagonist, or 50 μM DL-APV, an NMDA receptor antagonist, or treated with CNQX or DL-APV alone. After treatments, AHA moieties incorporated in neurons were tagged with Alexa Fluor 647, and the Alexa Fluor 647 fluorescence in neurons was quantified by the same procedure as in *A* and *B*. Each datum is the mean ± S.E. of three independent experiments. ***, $p < 0.001$ *versus* the control or glutamate-treated sample. *ns,* not significant.

tivity in axons, was inhibited by including cycloheximide in the incubation solution. These results indicate that glutamate treatment enhances protein synthesis in axons and further suggest that the mTOR signaling pathway may participate in coupling glutamate stimulation to axonal protein synthesis.

Incubation Time and Concentration Dependences of Glutamate-induced Enhancements of Axonal Protein Synthesis—In severed cortical axons, the extent of glutamate-induced enhancement in protein synthesis varied by changing the duration of treatment. After being treated with 500 μM glutamate for 10 min (Fig. 4, *A,* and *open circles* in *C*), the Alexa Fluor 647 fluorescence in axons became about twice that in the control axons. The fluorescence remained at the similar elevated level when the treatment was lengthened from 10 to 60 min. Further lengthening of the treatment to 120 min resulted in a decrease of fluorescence intensity to a level similar to that of control axons. Because fragmented axons started to appear after incubation for 30 min (indicated by *arrows* in Fig. 4*A*), the leveling off and decrease in the fluorescence intensity found in the axons subjected to glutamate treatments for periods longer than 30 min may be related to the excitotoxic effects of glutamate on axonal structure (52). For comparison, the incubation time dependence of BDNF-induced enhancement of protein synthesis in axons was also studied (Fig. 4*B*). The results indicated that when the incubation time was shorter than 20 min, no enhancement in protein synthesis in axons was detected (*triangles,* Fig. 4*C*). By prolonging the incubation time, the enhancement increased gradually and became statistically significant after 120 min. The axons appeared to remain intact in structure after being treated with BDNF for periods between 10 and 120 min, as judged by the undisrupted microtubule cytoskeleton in axons (Fig. 4*B*).

The increased incorporation of AHA in severed axons as induced by glutamate treatment was also found to be dependent on the concentration of glutamate in a range between 100 and 500 μM when the incubation duration was kept at 10 min (Fig. 4*D*). Treating axons with 1 mM glutamate for 10 min, however, produced widespread fragmented axons in the area of region 2 (data not shown).

Glutamate-enhanced Protein Synthesis in Cortical Axons Was Partly Mediated by Ionotropic AMPA Receptors—Pharmacological tools were then used to identify the receptors that

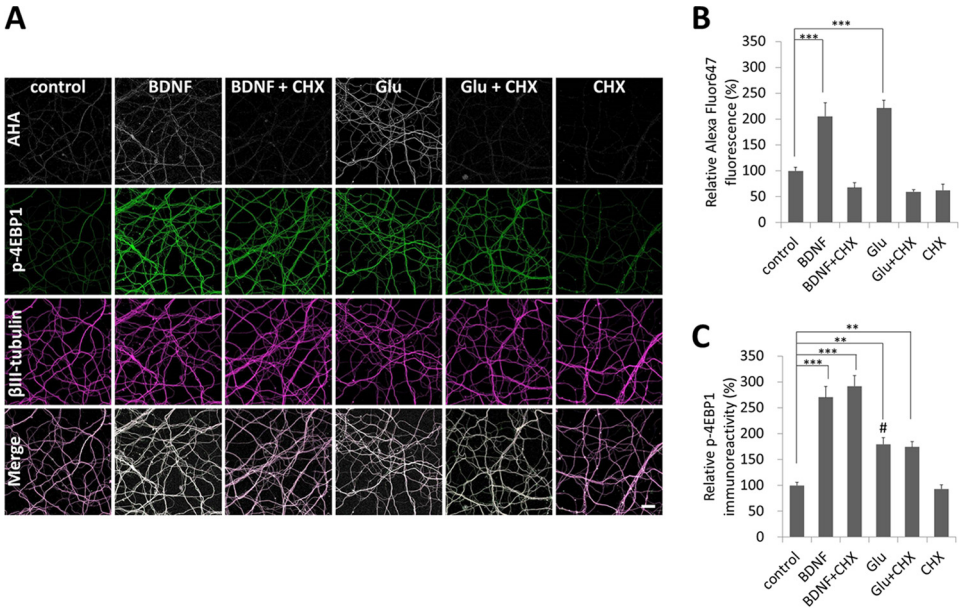

FIGURE 3. **BDNF- and glutamate-induced enhancements of protein synthesis in axons.** *A,* AHA incorporation and p-4EBP1 immunoreactivity in axons in region 2 after treatments with BDNF or glutamate (*Glu*). Axons of cultured cortical neurons at DIV 15 were severed from their cell bodies, maintained in AHA-containing medium, and then treated with 100 ng/ml BDNF for 2 h or with 500 μM glutamate for 10 min in the presence or absence of 40 μM cycloheximide (*CHX*) or treated with cycloheximide alone. Axons were reacted with alkyne-Alexa Fluor 647 (*white*) and then subjected to fluorescence immunostaining with the antibodies to p-4EBP1 (*green*) and βIII-tubulin (*magenta*). Axons maintained in AHA-containing medium for 2 h (control) were also treated by the same procedure. The results were from a representative experiment of a total of eight independent experiments. *Scale bar,* 10 μm. *B,* relative fluorescence intensities of Alexa Fluor 647 in axons subjected to the treatments as in *A*. Intensities of Alexa Fluor 647 measured from these samples were normalized by that measured from the control sample. *C,* relative levels of p-4EBP1 immunoreactivity in axons subjected to treatments as in *A*. Each datum of *B* and *C* is the mean ± S.E. of eight independent experiments. **, $p < 0.01$, and ***, $p < 0.001$ *versus* the control sample; #, $p < 0.05$ *versus* the BDNF-treated sample.

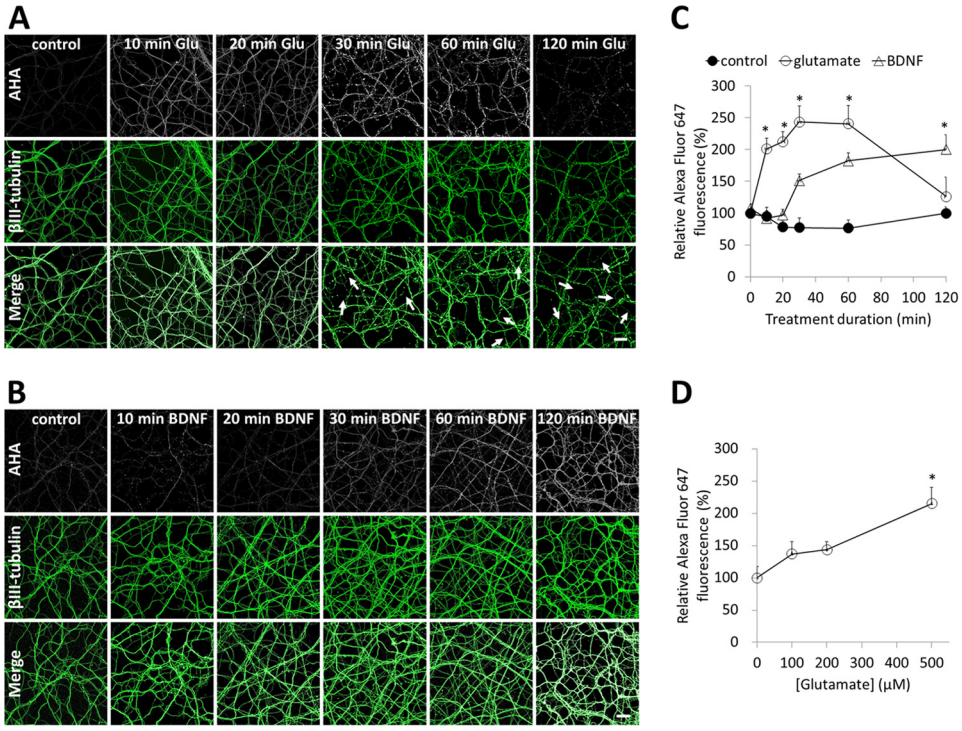

FIGURE 4. **Time courses of glutamate- and BDNF-induced enhancements of protein synthesis in axons and the dependence of protein synthesis in axons upon glutamate concentration.** *A* and *B,* at DIV 15, axons were severed from their cell bodies and treated with 500 μM glutamate (*Glu*) (*A*), 100 ng/ml BDNF (*B*), or PBS vehicle for different lengths of time in the presence of AHA. Afterward, AHA moieties incorporated in axons were tagged with alkyne-Alexa Fluor 647 (*white*), followed by immunostaining with the antibody to βIII-tubulin (*green*). *Arrows* indicate fragmented axons. *Scale bars,* 10 μm. *C,* fluorescence intensities of Alexa Fluor 647 in axons after being treated with glutamate (○), BDNF (Δ), or vehicle (●) for different lengths of time were normalized by that measured from axons kept in AHA for 2 h (control). *, $p < 0.05$ *versus* samples treated with PBS vehicle for the same lengths of incubation time. *D,* axons were treated with 100, 200, or 500 μM glutamate for 10 min in the presence of AHA, and the AHA moieties incorporated in axons were tagged with alkyne-Alexa Fluor 647. The fluorescence intensities of Alexa Fluor 647 measured from these samples were normalized by that measured from axons kept in AHA for 2 h. *, $p < 0.05$ *versus* the control sample. Each datum of *C* and *D* is the mean ± S.E. of three independent experiments.

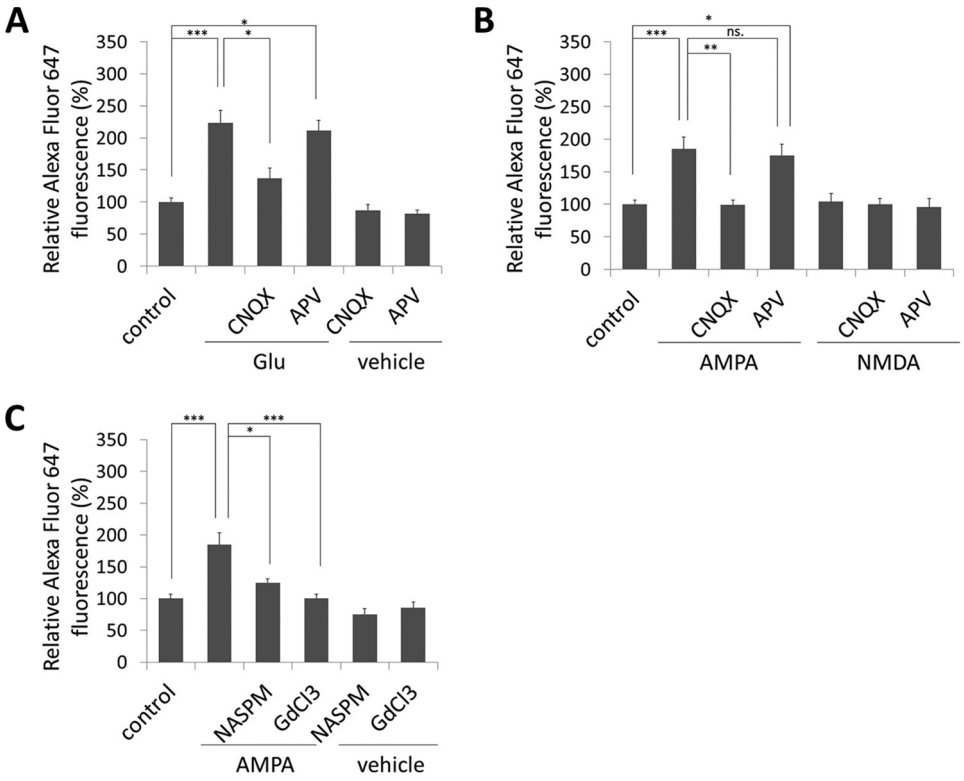

FIGURE 5. **AMPA receptors mediated glutamate-enhanced protein synthesis in axons.** *A,* axons were disconnected from their cell bodies and then treated with 500 μM glutamate (*Glu*) or PBS vehicle for 10 min in the presence or absence of 200 μM CNQX or 50 μM DL-APV or treated with 200 μM CNQX or 50 μM DL-APV alone in the presence of AHA. Afterward, AHA moieties in neurons were tagged with alkyne-Alexa Fluor 647. The fluorescence intensities of Alexa Fluor 647 measured from axons subjected to the above treatments were normalized by that measured from axons maintained in AHA-containing medium for 2 h (*control*). Each datum is the mean ± S.E. of three independent experiments. *B,* relative fluorescence intensities of Alexa Fluor 647 were measured from axons being treated with 200 μM AMPA or 200 μM NMDA in the presence or absence of 200 μM CNQX or 50 μM DL-APV in the presence of AHA by the same procedure as that used in *A*. Each datum is the mean ± S.E. of four independent experiments. *C,* relative fluorescence intensities measured from axons after being treated with 200 μM AMPA or PBS vehicle in presence or absence of 200 μM NASPM or 10 μM $GdCl_3$ for 10 min in the presence of AHA by the same procedure as that used in *A*. Each datum is the mean ± S.E. of three independent experiments. *, $p < 0.05$; **, $p < 0.01$, and ***, $p < 0.001$ *versus* the control or stimulated sample. *ns,* not significant.

mediated the glutamate-induced enhancements of protein synthesis in severed axons. It was found that CNQX inhibited the glutamate-induced enhancement by 70.3 ± 5.5% (mean ± S.D., $n = 3$) (Fig. 5*A*). Axonal protein synthesis could also be enhanced by treatment with AMPA, a selective agonist of AMPA receptors, to a level of 68.3 ± 3.9% (mean ± S.D., $n = 4$) of that induced by glutamate, and AMPA-induced enhancement of axonal protein synthesis was completely inhibited by CNQX (Fig. 5*B*). In contrast, DL-APV did not affect the glutamate- or AMPA-induced increases in axonal protein synthesis (Fig. 5, *A* and *B*), and NMDA, a selective agonist of NMDA receptors, did not enhance protein synthesis in axons (Fig. 5*B*). CNQX and DL-APV by themselves did not affect axonal protein synthesis (Fig. 5*A*). The results thus indicate that glutamate-induced enhancement in axonal protein synthesis is mediated partly by AMPA receptors but not by NMDA receptors. Furthermore, our finding that the AMPA-induced increase of axonal protein synthesis was partly inhibited by NASPM (Fig. 5*C*), a selective antagonist for Ca^{2+}-permeable AMPA receptors, indicated that part of the AMPA receptors mediating AMPA stimulation to axonal protein synthesis were Ca^{2+}-permeable. The AMPA-induced increase in axonal protein synthesis was found to be abolished completely by $GdCl_3$, a general antagonist of voltage-gated Ca^{2+} channels (Fig. 5*C*). NASPM and $GdCl_3$ by themselves did not affect protein synthesis in axons. These latter observations raised the possibility that upon glutamate stimulation Ca^{2+} may flow into axons via Ca^{2+}-permeable AMPA receptors and voltage-gated Ca^{2+} channels and then lead to increases of protein synthesis in axons.

We then investigated whether our observation that AMPA but not NMDA receptors mediated glutamate-induced enhancement of axonal protein synthesis was due to the respective presence and absence of AMPA and NMDA receptors on the axons in the region 2 area. By fluorescence immunostaining, these axons, as positively stained by pan-axonal neurofilament marker (SMI-312R) monoclonal antibody, were found to exhibit both the immunoreactivities of the antibodies to GluR1, an AMPA receptor subunit (Fig. 6*A*, as indicated by region 2), and GluN2A/B, NMDA receptor subunits (Fig. 6*B*, as indicated by region 2). Both of the immunoreactivities to GluR1 and GluN1, another NMDA receptor subunit, were found in the same axons (Fig. 6*C*, *left panels,* as indicated by region 2). The presence of both GluN1 and GluN2A/B subunits in these axons further suggests that they may assemble into functional NMDA receptors (54). The immunoreactivities of GluR1, GluN1, and GluN2A/B were also found in the somatodendrites of cortical neurons in the region 1 area (Fig. 6, as indicated by region 1). When neurons and axons were incubated with the secondary antibodies alone, virtually no immunostaining was observed (Fig. 6*C*, *right panels*). It is thus unlikely that our finding that

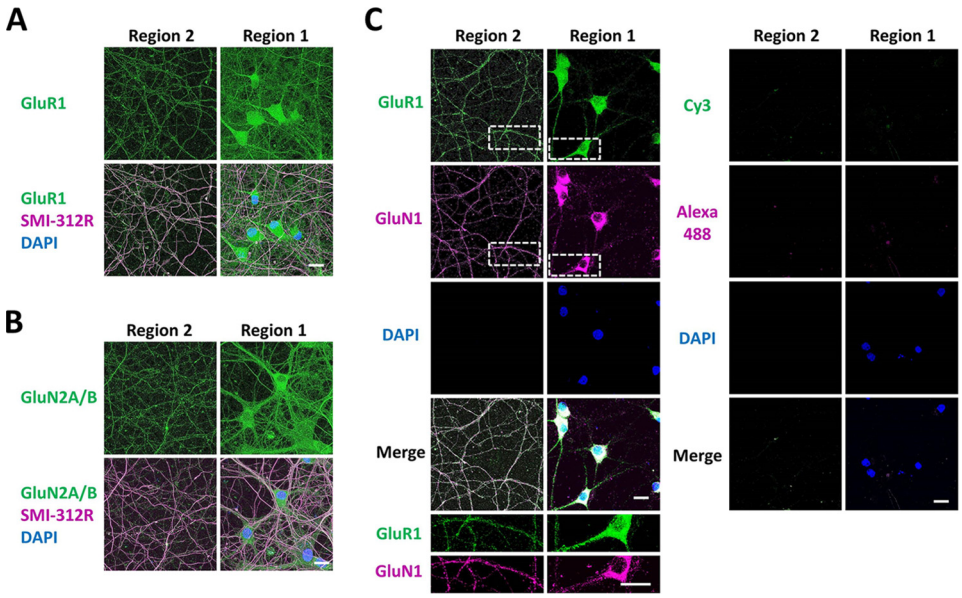

FIGURE 6. **AMPA and NMDA receptors in neuronal structures on chip surface.** *A,* fluorescence immunostaining of the axons in region 2 and neurons in region 1 with anti-GluR1 (*green*) and SMI-312 (*magenta*) antibodies and labeled with DAPI (*blue*). *B,* fluorescence immunostaining of the axons in region 2 and neurons in region 1 with anti-GluN2A/B (*green*) and SMI-312 (*magenta*) antibodies and labeled with DAPI (*blue*). *C, left panels,* fluorescence immunostaining of the axons in region 2 and neurons in region 1 with anti-GluR1 (*green, top row images*) and anti-GluN1 (*magenta, 2nd row images*) antibodies and labeled with DAPI (*blue, 3rd row images*). The two images at *bottom* are the areas enclosed by *white broken lines* in the images above at higher magnification. *Right panels,* axons in region 2 and neurons in region 1 were incubated with Cy3-conjugated goat anti-rabbit IgG (*green, top images*) and Alexa Fluor 488-conjugated goat anti-mouse IgG (*2nd row images*) and labeled with DAPI (*3rd row images*). *Scale bars,* 20 μm.

AMPA but not NMDA receptors mediate glutamate-induced enhancements of protein synthesis in axons is due to the absence of NMDA receptors from these axons. Furthermore, previous studies have indicated that both AMPA and NMDA receptors are present in the axons of young neurons (55–58) and that NMDA receptors are absent from the axons of older neurons (55, 56, 58). Our finding of the presence of AMPA and NMDA receptors in axons emitting from older neurons, at DIV 16, may be related to the fact that these axons do not form synapses in the region 2 area, whereas axons in conventional cultures actively participate in synaptogenesis after the 1st week *in vitro* (59). This possibility needs to be further investigated in the future.

Glutamate-enhanced Protein Synthesis in Cortical Axons Was Also Partly Mediated by Metabotropic Glutamate Receptors—We next asked whether glutamate treatment also activated the metabotropic glutamate receptor and thus enhanced axonal protein synthesis. DL-AP3, a group 1 mGluRs antagonist, and LY341495, a group 2 mGluRs antagonist, respectively, induced an 83.1 ± 1.4% (mean ± S.D., $n = 3$) and 53.3 ± 9.4% (mean ± S.D., $n = 3$) reduction in the glutamate-induced enhancement of axonal protein synthesis (Fig. 7*A*). In contrast, (*RS*)-α-cyclopropyl-4-phosphophenylglycine (CPPG), an antagonist of group 3 mGluRs, did not affect this glutamate-induced enhancement (Fig. 7*A*). The results indicate that among the three groups of mGluRs, group 1 mGluRs play a more prominent role in signaling glutamate stimulation to protein synthesis in axons. We then sought to identify the member of group 1 mGluR, consisting of mGluR1 and mGluR5, that signaled glutamate stimulation to protein synthesis in axons. (*RS*)-3,5-Dihydroxyphenylglycine (DHPG), an agonist for both mGluR1 and mGluR5, resulted in an enhancement of axonal protein synthesis of 56.5 ± 7.2% (mean ± S.D., $n = 3$) of that afforded by glutamate treatment (Fig. 7*B*). (*RS*)-2-Chloro-5-hydroxyphenylglycine, a selective agonist of mGluR5, did not affect axonal protein synthesis significantly. DHPG-induced increases in axonal protein synthesis could be completely abolished by 3-MATIDA, a selective mGluR1 antagonist, but not affected by MTEP, a selective mGluR5 antagonist (Fig. 7*B*). The results are consistent with the interpretation that mGluR1 also mediates glutamate stimulation to protein synthesis in axons.

Activating group 1 mGluRs has been reported to enhance local protein synthesis in dendrites via the mTOR signaling cascade and to evoke postsynaptic currents by activating TRPC channels (60–62). To test the possibilities that the mTOR signaling cascade and TRPC channels also participated in mediating the activation of mGluR1 to protein synthesis in axons, the effects of the inhibitors of mTOR complex 1 and TRPC channels, rapamycin and SKF96365, respectively, on DHPG-induced enhancements of protein synthesis in severed axons were studied. The results indicate that rapamycin induced an 86.1 ± 8.5% (mean ± S.D., $n = 3$) reduction and that SKF96365 almost completely abolished DHPG-induced enhancements of protein synthesis in axons (Fig. 7*C*). The results are consistent with the involvement of the mTOR signaling cascade and TRPC channels in DHPG-induced enhancement of protein synthesis in axons. It was further found that DHPG-induced enhancement was partly inhibited by W7, a calmodulin inhibitor (63), suggesting the involvement of calmodulin in transducing DHPG-induced enhancement of axonal protein synthesis. However, the DHPG-induced enhancement was not affected by APV or CNQX (Fig. 7*D*).

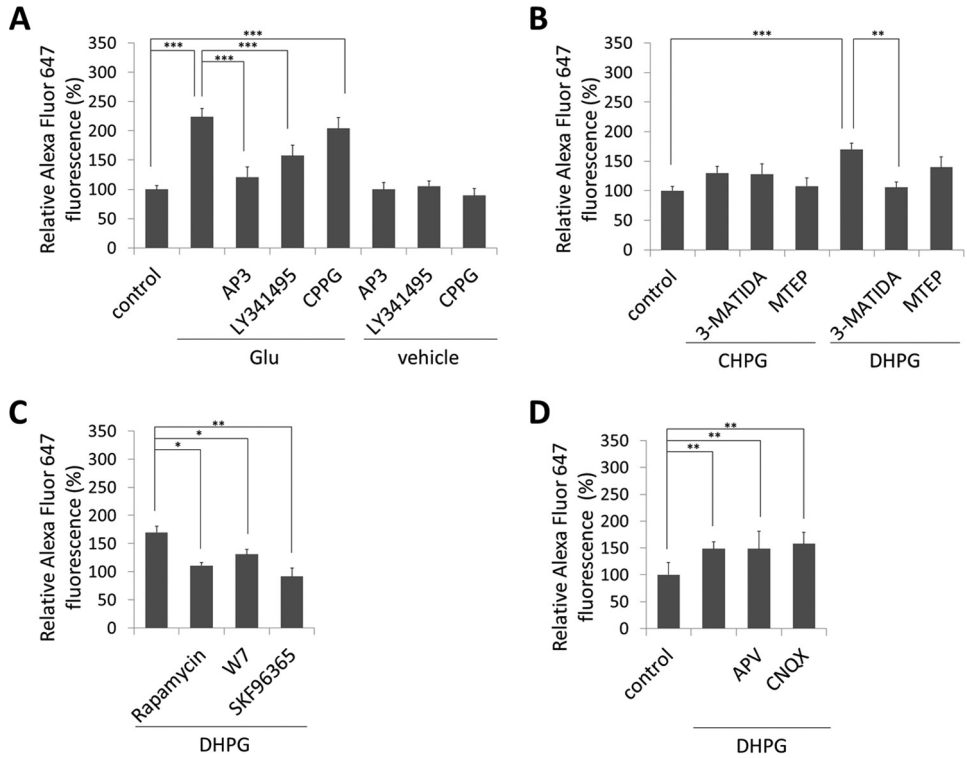

FIGURE 7. **Metabotropic glutamate receptors mediated glutamate-induced enhancements of protein synthesis in axons.** *A,* axons were treated with 500 μM glutamate for 10 min in the absence or presence of 300 μM DL-AP3, a group I mGluR antagonist; 1 μM LY341495, a group II mGluR antagonist; or 1 μM CPPG, a group III mGluR antagonist, or treated with 300 μM DL-AP3, 1 μM LY341495; or 1 μM CPPG alone for 10 min in the presence of AHA. Afterward, the AHA moieties incorporated in neurons were tagged with alkyne-Alexa Fluor 647. The fluorescence intensities of Alexa Fluor 647 measured from the above samples were normalized by that of axons kept in AHA-containing medium for 2 h (control). *B,* relative fluorescence intensities of Alexa Fluor 647 in axons being treated with 100 μM *RS*)-2-chloro-5-hydroxyphenylglycine, a selective mGluR5 agonist, or 100 μM (*S*)-3,5-DHPG, a selective group I mGluRs agonist, in the presence or absence of 10 μM 3-MATIDA, a mGluR1 antagonist, or 10 μM MTEP hydrochloride, a mGlu5 antagonist, in the presence of AHA, as calculated by the same procedure as that used in *A*. *C,* relative fluorescence intensities of Alexa Fluor 647 in axons being treated with 100 μM (*S*)-3,5-DHPG in the absence or presence of 100 nM rapamycin, 50 μM W7, or 30 μM SKF96365, as calculated by the same procedure as that used in *A*. *D,* relative fluorescence intensities of Alexa Fluor 647 in axons being treated with 100 μM (*S*)-3,5-DHPG in the absence or presence of 50 μM DL-APV or 200 μM CNQX in the presence of AHA by the same procedure as that used in *A*. Each datum is the mean ± S.E. of three independent experiments. *, $p < 0.05$; **, $p < 0.01$ and ***, $p < 0.001$ *versus* the control or stimulated sample.

Both Ca^{2+} and the mTOR Signaling Paths Mediated Glutamate and BDNF Stimulations to Protein Synthesis in Axons—We further investigated whether extracellular Ca^{2+} ions were required for triggering the glutamate-induced enhancement of protein synthesis in axons. The results indicated that this enhancement was nearly completely abolished by chelating extracellular Ca^{2+} with EGTA (Fig. 8*A*), suggesting a requirement of extracellular Ca^{2+}. That the enhancement was partially inhibited by W7 treatment (Fig. 8*A*) further indicated that free Ca^{2+} ions flown into axons upon glutamate stimulation might bind to calmodulin to elicit their effects on the translation machinery in axons. That the enhancement was also partially inhibited by rapamycin treatment (Fig. 8*A*) supports the involvement of the mTOR signaling cascade in glutamate-induced enhancement of axonal protein synthesis. These results are all consistent with those studies wherein AMPA and DHPG were used as stimulators (Figs. 5*C* and 7*C*).

Earlier studies have indicated that the mTOR signaling cascade plays important roles in coupling BDNF stimulation to protein synthesis in dendrites (45, 64). BDNF could also activate TRPC channels and result in Ca^{2+} influxes into growth cones and cell bodies (65–67). We then examined whether the mTOR signaling and TRPC channels are involved in the BDNF-induced enhancement of protein synthesis in axons. We found that this enhancement was partially inhibited by rapamycin, SKF96365, and EGTA treatments (Fig. 8*A*), suggesting the involvement of mTOR cascade and TRPC channels. That this enhancement was also partly inhibited by W7 treatment further indicated the involvement of calmodulin in signaling BDNF stimulation to protein synthesis in axons. Treating severed axons with both glutamate and BDNF did not affect the enhancement as induced by treating with glutamate or BDNF alone (Fig. 8*B*). The lack of additive effect further suggests that BDNF and glutamate act within the same signaling cascades in axons.

Our results together indicate that both Ca^{2+} and the mTOR signaling paths are involved in transducing glutamate- and BDNF-induced enhancements of protein synthesis in axons and that these enhancements could be inhibited to different degrees by EGTA, SKF96365, rapamycin, and W7. Two-way ANOVA is used to analyze the interaction effects of these inhibitors on the glutamate- and BDNF-induced enhancements. The results indicate that interaction effects of EGTA on glutamate- and BDNF-induced enhancements of the Alexa Fluor 647 fluorescence intensities in axons are of statistical significance ($p < 0.001$, $n = 3$). However, the interaction effects between BDNF and glutamate with SKF96365, rapamycin, and W7 treatments are not statistically significant. This result

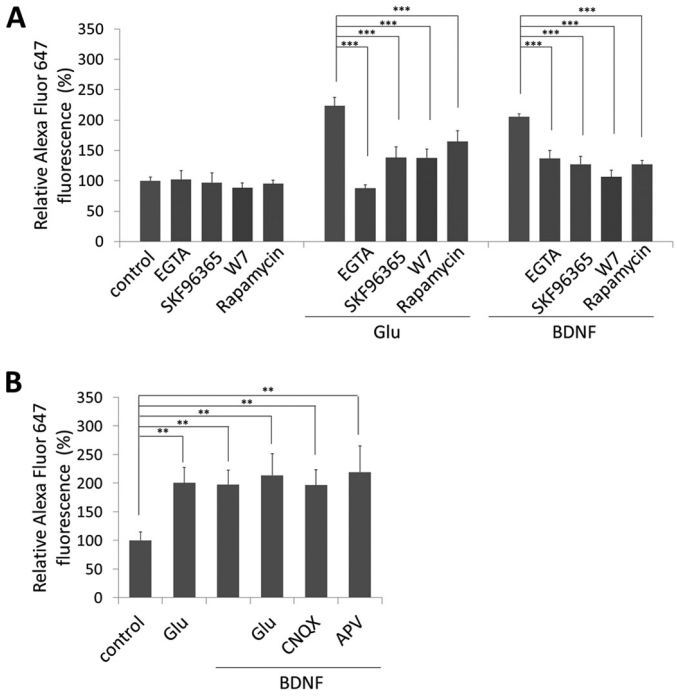

FIGURE 8. *A,* Ca^{2+} and the mTOR signaling mediated glutamate (*Glu*)- and BDNF-induced enhancements of protein synthesis in axons. Axons were severed from their cell bodies and then treated with 500 μM glutamate for 10 min or 100 ng/ml BDNF for 2 h in the absence or presence of 1.8 mM EGTA, 30 μM SKF96365, 50 μM W7, or 100 nM rapamycin in the medium containing AHA. Axons were also treated with 1.8 mM EGTA, 30 μM SKF96365, 50 μM W7, or 100 nM rapamycin in the medium containing AHA for 2 h alone. Afterward, AHA moieties incorporated in axons were tagged with alkyne-Alexa Fluor 647. *B,* glutamate and BDNF do not produce additive effects on the protein synthesis in axons. Severed axons were treated with 500 μM glutamate for 10 min, 100 ng/ml BDNF for 2 h, 100 ng/ml BDNF for 2 h with 500 μM glutamate being added at the last 10 min, 100 ng/ml BDNF for 2 h in the presence of 200 μM CNQX, or 100 ng/ml BDNF for 2 h in the presence of 50 μM DL-APV in the medium containing AHA. Afterward, AHA moieties incorporated in axons were tagged with alkyne-Alexa Fluor 647. The fluorescence intensities measured from the above samples were normalized by those measured from axons being kept in AHA-containing medium for 2 h (*control*). Each datum is the mean ± S.E. of three independent experiments. **, $p < 0.01$; ***, $p < 0.001$ *versus* the drug-treated sample.

seems to suggest that the Ca^{2+} signaling path carries more weight in coupling glutamate stimulation than in coupling BDNF stimulation to protein synthesis in axons.

Discussion

Here, we report that glutamate treatment enhances protein synthesis in the axons of rat cortical neurons. This process involves ionotropic and metabotropic glutamate receptors on the plasma membrane and Ca^{2+} and the mTOR signaling cascades in the axoplasm (Fig. 9).

The axons wherein glutamate-induced enhancements of protein synthesis are examined here originate from rat cortical neurons in culture. These axons are guided to grow into an area (region 2) that lacks any cells that could serve as the synaptic targets for these axons (Fig. 1*A*). These axons thus mimic cortical axons in the middle of migration toward their synaptic target areas and before making synaptic contacts with target cells during development. Before assays, these axons are physically disconnected from their cell bodies by cutting with a fine needle. This ensures that all nascent proteins detected in axons after axotomy are synthesized locally but are not imported from the somatodendritic compartments. All experiments of this study have been performed during the 2-h period after axotomy. As judged by their morphology and microtubule cytoskeleton, the distal parts of severed axons in region 2 remain intact during this 2-h-long window. This observation is consistent with earlier studies showing that the severed distal ends of cultured cortical axons remain intact for periods up to 2–3 h (68) and that Wallerian degeneration of damaged axons begins hours after the injury (69). The axons of neurons in the peripheral nervous system and CNS have been reported to undergo injury-induced protein synthesis in a calcium-dependent fashion (70–74). Vuppalanchi *et al.* (75) have shown that release of calcium stores from the endoplasmic reticulum triggers translation of axonal calreticulin mRNA. The possibility that similar signaling pathways as those mediating local protein synthesis in peripheral nervous system axons may also partly contribute to the responses of cortical axons to glutamate and BDNF treatments cannot be ruled out completely.

BDNF is a major neurotrophic factor in the CNS and plays important roles in supporting neuronal survival and in shaping and maintaining the synaptic connectivity in the brain (76–78). The applicability of the experimental procedures used here has been validated first by showing that they could be used to detect the known BDNF-induced enhancements of protein synthesis in cultured cortical neurons and their axons. In addition, our observations that BDNF treatment increases the content of p-4EBP1 in axons and that BDNF-induced enhancement of the protein synthesis in axons is partly inhibited by rapamycin are in agreement with earlier studies that BDNF could activate the mTOR signaling pathway and in turn increases the translation machinery (45, 79). Our observations that BDNF-enhanced protein synthesis in axons is also partly inhibited by SKF96365, W7, and EGTA further indicate that BDNF could also activate TRPC channels that allow Ca^{2+} to flow into axons, as it does at the growth cones of rat cerebellar granule cells (65, 66). Ca^{2+} flowing into axons could bind to calmodulin and then activate the translation machinery in axons.

Ca^{2+} and the mTOR signaling pathways also participate in transducing glutamate-induced enhancements of protein synthesis in severed axons. Our pharmacological studies indicate that this process begins by the binding of glutamate to AMPA receptors, mGluR1 and group 2 mGluRs on the plasma membrane. The requirement of extracellular Ca^{2+} and inhibition by Gd^{3+} indicate that this process involves Ca^{2+} influx via voltage-gated Ca^{2+} channels, which are likely opened by the depolarization as resulting from the activation of AMPA receptors. The inhibition of NASPM suggests that upon glutamate treatment Ca^{2+} may flow into axons via those AMPA receptors that are Ca^{2+}-permeable. The inhibitory effects of SKF96365 on the DHPG- and glutamate-induced enhancements of protein synthesis in axons further indicate Ca^{2+} ions may also flow into axons via TRPC channels, probably by a mechanism similar to that underlying the slow mGluR1-mediated excitatory postsynaptic current found in rat cerebellar Purkinje cells (60). Therefore, upon glutamate stimulation, there are at least three paths allowing Ca^{2+} to flow into axons and that lead to enhancements of protein synthesis, namely voltage-gated Ca^{2+} channels,

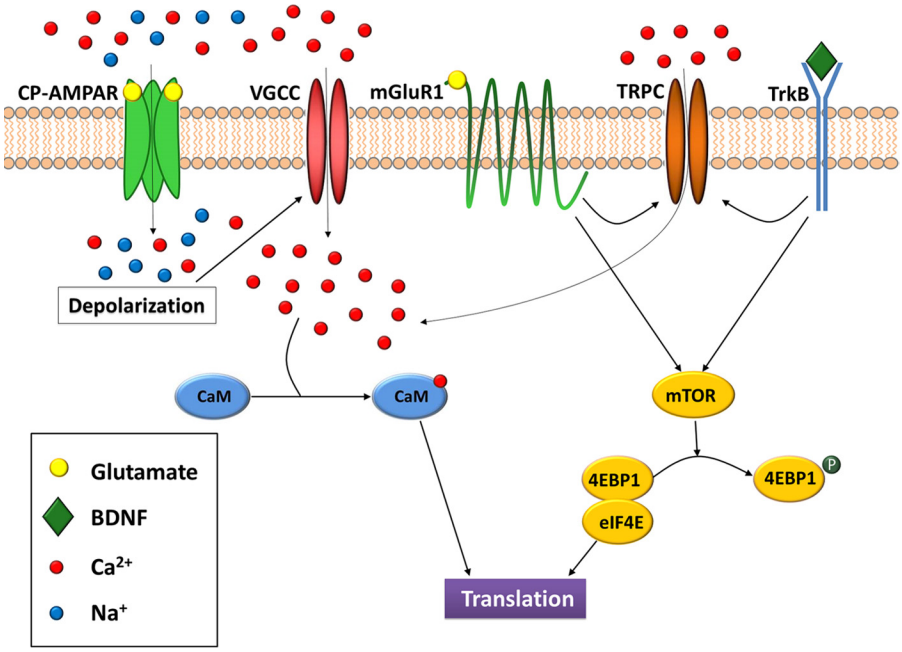

FIGURE 9. **Model of the signaling paths mediating glutamate- and BDNF-induced enhancements of local protein synthesis in the axon.** Glutamate first activates both AMPA receptors (*R*) and mGlu receptors on the plasma membrane of axons. This results in Ca^{2+} influxes into the axon via activated calcium-permeable AMPA receptors (*CP-AMPARs*), voltage-gated calcium channels (*VGCC*), and transient potential receptor canonical (*TRPC*) channels. Ca^{2+} binds calmodulin (*CaM*) in the cytoplasm, and the resultant complex interacts with various components of the translation machinery and stimulates protein synthesis. In addition, activation of mGlu receptors leads to the stimulation of mTOR path and, consequently, the phosphorylation of 4EPB1, thereby releasing eIF4E to participate in translation initiation. BDNF is depicted here to bind the TrkB receptor on the plasma membrane and next leads to the activation of TRPC channels as well as mTOR signaling path. The resultant Ca^{2+} influxes and phosphorylation of 4EBP1 also enhance protein synthesis in the axon.

Ca^{2+}-permeable AMPA receptors, and TRPC channels. The inhibitory effect of rapamycin and increases in the content of p-4EBP1 are consistent with the involvement of mTOR cascade in signaling glutamate stimulation to protein synthesis in axons.

In whole neurons, activating glutamate receptors has been reported to affect protein synthesis differentially depending on the source of glutamate (bath application or activity-induced release from synapses), treatment duration (ranging from seconds to tens of minutes), and ages of neurons (before or after the establishment of extensive synaptic connections) (46, 47, 51). Here, we find that AMPA but not NMDA receptors mediate the glutamate-induced enhancement of protein translation in axons and that, in reverse, NMDA but not AMPA receptors mediate the glutamate-induced enhancement of global translation in neurons. Our immunostaining results, however, indicate that this difference is unlikely to arise from a lack of NMDA receptors in axons. Although the reasons for this difference are presently unclear, several possibilities exist. First, because both synaptic and extrasynaptic glutamate receptors are present in whole neurons, and only extrasynaptic glutamate receptors exist in the severed axons, the different cellular signals initiated by these two types of glutamate receptors may influence the translation machineries in the whole neurons and axons differently (80–82). A possibility also exists that the signaling pathways mediated by glutamate receptors in whole neurons and axons are distinct. Finally, we cannot completely rule out the possibility that this difference may be related to the artificial setting of the chip used in the experiments.

Studies have indicated that glutamate induces diverse effects on migrating axons. Exposure to a glutamate gradient has been reported to induce the turning of growth cones of cultured rat cerebellar granule cells toward the glutamate source (5, 65), and asymmetric distribution of filopodia in growth cones facing the glutamate source has been reported to form before the axons of *Xenopus* spinal neurons begin to turn (5). Bath application of glutamate has been reported to inhibit the movement of the axonal filopodia of cultured rat hippocampal neurons (8), although similar treatment has been reported to accelerate the growth and growth cone splitting of the axons of mouse ventral midbrain dopaminergic neurons (3). Brief exposure of mouse hippocampal neurons to kainate, an agonist of non-NMDA glutamate receptors, stalls the motility of their axonal growth cones (10). During development, various regions of the CNS, including neocortex, hippocampus, brain stem, and spinal cord, produce patterned spontaneous activities (33, 34). Because of the delayed expression of the transporters of neurotransmitters, these activities result in the spillover of neurotransmitters, including glutamate (83), from activated synapses into intercellular space. Glutamate is also released from activated astrocytes (35) and axons (36). When passing through brain regions generating patterned spontaneous activities or encountering astrocytes and axon bundles, migrating axons are expected to be exposed to increases in extracellular glutamate. Our results indicate that protein synthesis will be stimulated rapidly and locally in axons upon exposure to such transient glutamate increases. How the resultant nascent proteins affect an axon with regard to its growth, turning, and branching still awaits further elucidation.

Author Contributions—Y.-C. C. and W.-L. H. conceived and coordinated the study and wrote the paper. W.-L. H. designed, performed, and analyzed the all the experiments. H. W. C. designed and performed the experiments shown in Fig. 2*C*. C.-Y. W. contributed to the preparation of Figs. 7*D* and 8*B*. H.-I. W., Y.-T. L., E.-C. C., and W. F. provided technical assistance of the chip device. All authors reviewed the results and approved the final version of the manuscript.

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Glutamate Stimulates Local Protein Synthesis in the Axons of Rat Cortical Neurons by Activating α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic Acid (AMPA) Receptors and Metabotropic Glutamate Receptors

Wei-Lun Hsu, Hui-Wen Chung, Chih-Yueh Wu, Huei-Ing Wu, Yu-Tao Lee, En-Chan Chen, Weilun Fang and Yen-Chung Chang

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