Brain-derived neurotrophic factor enhances the basal rate of protein synthesis by increasing active eukaryotic elongation factor 2 levels and promoting translation elongation in cortical neurons.

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Running title: BDNF increases eEF2 and enhances basal translation

The constitutive and activity-dependent components of protein synthesis are both critical for neural function. Although the mechanisms controlling extracellularly induced protein synthesis are becoming clear, less is understood about the molecular networks that regulate the basal translation rate. Here we describe the effects of chronic treatment with various neurotrophic factors and cytokines on the basal rate of protein synthesis in primary cortical neurons. Among the examined factors, brain-derived neurotrophic factor (BDNF) showed the strongest effect. The rate of protein synthesis increased in the cortical tissues of BDNF transgenic mice, whereas it decreased in BDNF knockout mice. BDNF specifically increased the level of the active, unphosphorylated form of eukaryotic elongation factor 2 (eEF2). The levels of active eEF2 increased and decreased in BDNF transgenic and BDNF knockout mice, respectively. BDNF decreased kinase activity and increased phosphatase activity against eEF2 in vitro. Additionally, BDNF shortened the ribosomal transit time, an index of translation elongation. In agreement with these results, overexpression of eEF2 enhanced protein synthesis. Taken together, our results demonstrate that the increased level of active eEF2 induced by chronic BDNF stimulation enhances translational elongation processes and increases the total rate of protein synthesis in neurons.

The synthesis and posttranslational modification of proteins play key roles in neural development, synaptic plasticity, and cognitive brain functions such as learning and memory (1,2). Recent studies have revealed that activity-dependent regulation of translation affects neural plasticity (3,4). Previously, we reported that BDNF—a critical molecule for neural plasticity (5-7)—enhances protein synthesis and activates the translational machinery in CNS neurons (8). In addition, neurotransmitters such as glutamate (9,10), dopamine (11), and serotonin (12) are also reported to facilitate translation in neurons. These observations indicate that endogenous molecules can acutely modulate neuronal translation in response to neural activity. Translation of an mRNA molecule comprises three steps: initiation, elongation, and release (or termination) (13). In the first step, mRNA and methionyl-tRNA^{Met} are recruited to a ribosome. During elongation, aminoacyl-tRNAs are sequentially recruited and the nascent peptide chain lengthens incrementally as amino acids are covalently attached via peptide bonds. Finally, the polypeptide chain is released from the ribosome. Each step is regulated by a variety of factors. The activities of these regulatory proteins are predominantly controlled by phosphorylation and GTP binding. BDNF activates both initiation and elongation by modulating these processes (8,14,15).

In addition to these acute, stimulation-induced changes in the translation rate, the long-term regulation of translation...
plays important roles in developing and mature brains. In fact, recent studies have shown that genetic disruption or overexpression of translation factors or modulator genes alters synaptic plasticity and behavior as well as the basal rate of protein synthesis. Mice lacking the gene encoding GCN2—a kinase that phosphorylates eIF2α—exhibited enhanced translation as well as aberrant long-term potentiation (LTP) and spatial learning (16). Similar phenotypes have been observed in mice carrying a constitutively active mutant variant (Ser52 to Ala) of eIF2α (17). Mice lacking eIF4E-binding protein 2 (4EBP2) exhibited increased cap-dependent translation and altered LTP, long-term depression (LTD), and learning (18,19). Mice expressing a transgene encoding a dominant-negative version of MEK, which inhibits the phosphorylation of eIF4E and protein synthesis, were found to have learning deficits (20). Thus, modifying the rate of protein synthesis can produce deleterious effects on synaptic plasticity and brain function.

Although genetic modifications can affect translation, the mechanisms by which the basal translation rate is controlled in normal neurons are unknown. Here, we demonstrate that the chronic treatment of primary cortical neurons with BDNF increases the level of active, unphosphorylated eukaryotic elongation factor 2 (eEF2) and enhances the rates of elongation and protein synthesis. Analysis of BDNF mutant mice supports a role for this neurotrophin in regulating the basal rate of protein synthesis.

**EXPERIMENTAL PROCEDURES**

**Materials**

BDNF was a generous gift from Sumitomo Pharmaceutical Co. The following antibodies were employed in this study: anti-eIF2α and anti-eEF2K (Cell Signaling Technology); anti-eIF2β, anti-eIF2Bβ, anti-eIF2Be, anti-eIF2α, anti-eIF4G, and anti-eIF5 (Santa Cruz Biotechnology); anti-eIF4E and anti-eIF6 (BD Biosciences); anti-eEF1A and anti-PPP2A C subunit (Upstate); anti-actin (Chemicon); anti-GAPDH (Ambion); and anti-NSE (Polyscience). Anti-eEF2 and anti-phospho-eEF2 antibodies were prepared as described previously (15). Anti-eRF1 and anti-eRF3 antibodies were generous gifts from Dr. Shinichi Hoshino (Nagoya City University, Japan). [35S]methionine and protein G-Sepharose were purchased from GE Healthcare.

**Cell culture**

Primary cultures of cortical neurons were prepared by modifying a previously described method (8,21). Briefly, the cerebral cortices were removed from 18- to 19-day-old embryonic rat fetuses and dissociated with papain and DNase I. Neurons were seeded at 2 x 10^5 cells/cm^2 and cultured in Dulbecco’s modified Eagle medium (DMEM; Nissui) containing 10% fetal bovine serum (Gibco) overnight. The following day, the medium was changed to serum-free medium containing 10mM HEPES, 20nM progesterone, 30nM sodium selenite, 100μM putrescine, transferrin, and 0.01% pyruvate. BDNF (50 ng/ml), other factors, or vehicle (2 mg/ml transferrin in PBS) were added daily for 5 days.

**eEF2 transfection**

Rat eEF2 was cloned by PCR using rat brain cDNA as a template and sequenced. The amplified fragment was subcloned into the pCI vector (Promega). eEF2 cDNA was transfected into neurons by electroporation (Nucleofector™; Amaxa Biosystems) immediately after dissociation. After 72 h, neurons were harvested and used for the assays. Control transfection was performed using the same vector bearing EGFP. The transfection efficacy estimated by EGFP fluorescence was 20-25%.

**BDNF knockout and transgenic mice**

BDNF knockout mice (C57BL/6J-Bdnftm1Jae) were purchased from Jackson Laboratory. BDNF transgenic mice (CBA/C57BL6-BDNFb-actin) were kindly supplied by Regeneron Pharmaceuticals (22). For the biochemical experiments, mice were anesthetized by hypothermia with ice and sacrificed by decapitation at postnatal day 1 or 2. The cerebral cortices were removed, frozen immediately on dry ice, and stored at -80°C until they were assayed. Genotypes were checked by PCR. Because the increase of BDNF protein levels in the cortex of adult transgenic mice is relatively low (22), we determined the levels of BDNF by ELISA (23). All the procedures were performed according to...
the NIH Guideline for the Care and Use of Laboratory Animals and with the permission of the Institutional Committee for Animal Care of Niigata University.

\[^{35}\text{S} \]methionine incorporation

Cortical neurons were incubated with 10 μCi of \[^{35}\text{S} \]methionine and the growth factors for 30 min. Protein synthesis was measured by \[^{35}\text{S} \]methionine incorporation as previously reported (8). Tissue samples were homogenized in 10 volumes of DMEM and centrifuged to remove the nuclei. The resulting supernatants were incubated with \[^{35}\text{S} \]methionine and incorporation into protein was measured as described previously (14). The levels of free methionine and methionine that had been incorporated into proteins were estimated by counting the radioactivity in the supernatant and the pellet, respectively. We then calculated the ratio of \[^{35}\text{S} \]methionine that precipitated to the total \[^{35}\text{S} \]methionine taken up into the neurons.

Pulse-chase

Neurons were incubated with \[^{35}\text{S} \]methionine for 1h in methionine-free DMEM at 4 days in culture. To analyze degradation rate, cultures were washed three-times by normal DMEM and incubated further 5h in 10% FBS-containing DMEM. Samples from 1h and 6h after labeling were immunoprecipitated with anti-eEF2 antibody. Resultant immunoprecipitates were analyzed by SDS-PAGE and autoradiograms were taken.

Measurement of the ribosomal transit time

Ribosomal transit times were measured as described previously (15). Neurons were cultured without or with BDNF (50 ng/ml) and were incubated with 3 μCi of \[^{35}\text{S} \]methionine. At 1, 5, 15 and 20 min after the addition of the radiolabeled methionine, neurons were washed with PBS and lysed with 1 ml of extraction buffer [20 mM HEPES at pH 7.2, 100 mM KCl, 3 mM MgCl\(_2\), 1 mM dithiothreitol, 100 μg/ml cycloheximide, 0.5% sodium deoxycholate, 0.5% Triton X-100, and complete protease inhibitor cocktail (Complete™, Roche)]. After pelleting the nuclei by centrifugation, the supernatant was aliquoted to measure the “completed” peptide chains and the “total” amount of newly synthesized (completed + ribosome-bound peptide chains) proteins.

Lysate (0.5 ml) was layered onto 1 ml of extraction buffer lacking detergent and containing 0.8 M sucrose. Polysomes were pelleted by centrifugation at 12,000 x g for 2.5 h. Samples of postribosomal supernatant (1.2 ml) were collected to measure the incorporation of \[^{35}\text{S} \]methionine into completed proteins. Newly synthesized proteins representing the completed and ribosome-bound, elongating peptide chains were measured by determining the incorporation of \[^{35}\text{S} \]methionine into the postnuclear supernatant. The transit time was determined as the difference in the positions of the intercepts on the time axis of the lines for the “total” and “completed” polypeptide chains. This difference between the intercepts is equivalent to half the ribosomal transit time (24).

Measurement of eEF2K and protein phosphatase 2A (PP2A) enzymatic activities

The kinase activity of eEF2K was measured as previously described (15) with immunopurified unphosphorylated eEF2 as a substrate. Rat brain homogenate was centrifuged at 100,000 x g for 30 min and resulting supernatant was absorbed with protein G and anti-phospho eEF2 antibody. Then, the supernatant was applied to a column containing anti-eEF2 antibody bound to an Affi-Gel Hz (BioRad). eEF2 was eluted with Immunopure Ag/Ab gentle elution buffer (Pierce), dialyzed, and examined by SDS-PAGE.

The phosphatase activity of PP2A was measured using a PP2A assay kit (Upstate). Lysates from control and BDNF-treated neurons were incubated with anti-PP2A subunit C antibody for 2 h at 4°C. Protein G-Sepharose was added and the samples were incubated for another 1 h. PP2A phosphatase activity in these immunoprecipitates was then assayed according to the manufacturer’s protocol.

Electrophoresis, Western blotting, and immunocytochemistry

SDS-PAGE and Western blotting were performed as described previously (14). Cells or tissues were lysed and sonicated in sample buffer [10 mM Tris-HCl, 150 mM NaCl, 2% SDS, 20 mM NaF, 1 mM Na\(_2\)VO\(_4\), and complete mini protease inhibitors (pH 7.5)]. After centrifugation, supernatant was collected and protein concentrations were determined.
Equal amounts of protein (25-40 μg/lane) were subjected to SDS-PAGE and transferred to PVDF membranes. The membranes were incubated with the primary antibodies and then with horseradish-peroxidase-conjugated anti-mouse IgG or horseradish-peroxidase-conjugated anti-rabbit IgG secondary antibodies (Cappel; dilution, 1:2000). Peroxidase activity was visualized on X-ray film after being treated with chemiluminescence reagents (Western Lightning, Perkin Elmer). Immunocytochemistry using anti-eEF2 and anti-phospho-eEF2 antibodies was performed essentially as described previously (25).

**RESULTS**

### Chronic effects of various growth factors and cytokines on the rate of protein synthesis

The rate of protein synthesis in primary cortical neurons was examined after chronic treatment with various neurotrophic factors and cytokines. Neurons were treated daily for 5 days and [35S]methionine incorporation into newly synthesized proteins was measured. Among the factors tested, the neurotrophins and insulin enhanced the rate of protein synthesis (Fig. 1). Because the largest effect was observed with BDNF treatment, further analysis was performed to investigate the underlying mechanisms. Immunocytochemistry with anti-MAP-2 antibody revealed that BDNF did not affect cell viability under these culture conditions (Supplemental Fig. 2), thus, protein synthesis rates are calculated from the same cell number.

**The rate of protein synthesis in the cortices of BDNF mutant mice**

Because BDNF increased the basal rate of protein synthesis in culture, we investigated the in vivo effects of BDNF using BDNF transgenic and knockout mice. The rates of protein synthesis in cortical tissues from wild-type littermates, BDNF knockout mice (+/-, -/-), and BDNF transgenic mice were examined by [35S]methionine incorporation. Cortical tissue from homozygous (-/-) BDNF knockout mice exhibited a reduced rate of protein synthesis (Fig. 2A). Lower protein synthesis rates were also observed in tissue from heterozygous (+/-) mice, which express BDNF protein at approximately half of the levels observed in wild-type littermates. BDNF protein in the cortices of both wild-type and transgenic mice was measured by ELISA. In P1-2 mice, the BDNF levels were 5.3 ± 0.8 pg/mg protein (n = 6, mean ± SD) in wild-type cortices, whereas they were 12.8 ± 2.2 pg/mg protein (n = 6, mean ± SD) in the transgenic cortices, which represented a significant increase (p < 0.001, t-test). In the cortical tissue from transgenic mice overexpressing BDNF, the rate of protein synthesis was higher than in wild-type cortex (Fig. 2B).

**BDNF increased the level of eEF2 but not other translation factors**

Translational processes are controlled by various translation factors. We examined...
whether chronic BDNF treatment altered the levels of various translation initiation factors, elongation factors, and release factors. Control cultures and neurons treated with BDNF (50 ng/ml) for 5 days in culture were harvested and their lysates were subjected to SDS-PAGE. The levels of various translation factors were analyzed by Western blotting (Fig. 3) and were quantified using densitometry (Supplemental Fig.1). As shown in Fig. 3 and 4, BDNF specifically and significantly upregulated the level of eEF2.

To examine whether the increase of eEF2 protein level is dependent on transcription or translation, RT-PCR and pulse-chase experiment were performed. BDNF treatment did not alter the eEF2 mRNA level at culture day 3 (data not shown) or day 5 as revealed by quantitative RT-PCR (Fig.4A). In contrast, newly synthesized eEF2 protein (1h after labeling) was significantly higher in BDNF-treated neurons, as revealed by pulse-chase and immunoprecipitation (Fig. 4B). In addition, degradation rates of eEF2 after 6h were the same in both control and BDNF-treated neurons (in both cases, eEF2 levels at 6h were about the half of that of 1h). These results indicate BDNF enhances eEF2 translation but neither its transcription nor degradation.

**BDNF decreased the phosphorylation of eEF2**

The elongation activity of eEF2 is regulated by phosphorylation at one position, serine 56. To determine whether the phosphorylation of this position was influenced by BDNF, we quantified the levels of phosphorylated eEF2 (P-eEF2) and total eEF2 on Western blots using phospho-eEF2 specific antibody and pan-eEF2 antibody. Whereas BDNF increased the total amount of eEF2 protein, it markedly reduced the level of phospho-eEF2, which does not contribute to translation elongation (Fig. 4D-F). Immunocytochemical analysis shows the same result. Untreated neurons exhibited faint immunoreactivity for eEF2 but rather strong signals for phospho-eEF2. Neurons treated with BDNF displayed stronger eEF2 immunoreactivity, whereas the signal for phospho-eEF2 was weak. Both total and phospho-eEF2 immunoreactivity was evident along neurites as well as in the cell body (Supplemental Fig. 2). We concluded that the net increase of the active form of eEF2 (unphosphorylated eEF2) was induced by BDNF treatment, consistent with an increased basal rate of protein synthesis.

**eEF2 levels and its phosphorylation status in cortices from BDNF mutant mice.**

Levels of total and phosphorylated eEF2 in the cerebral cortices of BDNF knockout and BDNF transgenic mice were examined by Western blotting. Similar to the results from the neuronal cultures, an increased level of total eEF2 and a decreased level of phosphorylated eEF2 were observed in cortices from BDNF transgenic mice (Fig. 5). On the other hand, the level of eEF2 was lower and the level of phosphorylated eEF2 was higher in cortices from BDNF knockout (-/-) mice (Fig. 5). The ratio of phosphorylated eEF2 to total eEF2 is shown in Fig. 5C.

**BDNF decreased the levels and activity of eEF2 kinase but increased the levels and activity of PP2A**

We next investigated whether the decrease in eEF2 phosphorylation induced by BDNF treatment was a consequence of decreased kinase activity or increased phosphatase activity. Protein levels and activities of the eEF2 kinase eEF2K (also called calcium/calmodulin-dependent protein kinase III), and the eEF2 phosphatase PP2A were examined. Western blot analysis revealed that chronic BDNF treatment decreased the level of eEF2K to about half that observed in control samples (Fig. 6). eEF2K kinase activity present in cell lysates from control and BDNF-treated cultures was measured using immunopurified eEF2 as a substrate. BDNF treatment decreased the level of eEF2K activity, as well as its protein level (Fig. 6C). The level and activity of PP2A were also measured. PP2A was immunoprecipitated from control and BDNF-treated neurons with anti-PP2A C subunit antibody and the phosphatase activity of PP2A in the precipitates was measured. BDNF treatment increased the levels of PP2A protein and activity (Fig. 6). Thus, the increased level of unphosphorylated eEF2 was likely due to a combination of a decrease in kinase activity and an increase in phosphatase activity.

**Chronic BDNF treatment enhanced the elongation rate**
Unphosphorylated (active) eEF2 induces aminoacyl-tRNA translocation from the A site to the P site of a ribosome to promote elongation. Because BDNF increased the level of unphosphorylated eEF2, we examined the rate of translation elongation in cortical neurons with or without BDNF treatment. To accomplish this, we measured the ribosomal transit time—a parameter inversely correlated with the rate of translation elongation. The transit times for control neurons and BDNF-treated neurons were approximately 9 min and 6 min, respectively (Fig. 7A). Thus, BDNF treatment significantly shortened the transit time (Fig. 8B), demonstrating that BDNF enhances the elongation rate in cortical neurons. 

eEF2 overexpression increased the rate of protein synthesis

To directly examine the causal relationship between the increased level of eEF2 and the enhanced protein synthesis rate, we transfected primary cultured neurons with cDNA encoding rat eEF2 cDNA. Electroporation of eEF2 cDNA into neurons resulted in an approximately 1.4-fold increase in the level of eEF2 protein compared to that in EGFP-transfected, control neurons (Fig. 8A). The transfection efficacy estimated by counting EGFP fluorescent cells was 20-25% in five individual experiments. In these experiments, the levels of phosphorylated eEF2 were similar following eEF2 or control transfection. eEF2-overexpressing neurons showed an increased protein synthesis rate (Fig. 8B). These results strongly suggest that the increased level of unphosphorylated eEF2 in neurons enhanced the level of protein synthesis.

DISCUSSION

Here we have demonstrated that BDNF increases the level of eEF2 and decreases its phosphorylation to promote translation elongation and the constitutive protein synthesis rate in central neurons. eEF2 overexpression increased the rate of protein synthesis, suggesting that the BDNF-induced upregulation of eEF2 is directly linked to the enhanced protein synthesis. We also confirmed the results of our in vitro experiments using BDNF knockout and transgenic mice.

It has been shown that acute and transient extracellular stimuli, such as hormones, growth factors, and nutrients, can alter the phosphorylation of translation factors and the rate of protein synthesis (28). Previously, we reported that BDNF acutely activates both translation initiation (8,14) and elongation (15) to enhance protein synthesis in central nervous system neurons. Chronic changes of the translation rate induced by extracellular stimuli, however, have not been examined in the nervous system. Interestingly, overexpression of factors such as eIF4E may contribute to the abnormal cell growth and/or proliferation within tumors (29). In fact, overexpression of eIF4E results transformation of fibroblast (30). On the other hand, the cellular and molecular bases of the determinants that regulate the baseline rate of translation in normal proliferating or postmitotic cells have not yet been identified.

A number of lines of evidence have implicated protein synthesis as a critical component of synaptic plasticity, neuronal development, and cognitive functions in the brain. The signaling mechanisms that underlie translational control in neurons and the brain are beginning to be elucidated (3). Recently, studies using genetically modified mice have shown the importance of translation modulation factors on the neural plasticity and learning ability (16-20). In these mice, the rate of protein synthesis is constitutively upregulated or downregulated due to the genetic manipulations. Understanding what conditions and stimuli modify the basal rate of translation will improve our understanding of the molecular basis of neural plasticity and brain function. We have identified BDNF, a neurotrophic factor implicated in synaptic plasticity and learning (5-7), as a key molecule for the sustained regulation of translation. Sustained upregulation of translation often leads to increases in cell volume, as seen for cancer cells. BDNF increases neuronal volume (31) and facilitates dendritic arborization (32). Moreover, reduced brain volume has been reported for BDNF knockout mice (33). BDNF enhancement of translation may explain these biological responses.

To determine the molecular mechanisms contributing to the increase of the protein synthesis rate induced by BDNF, we examined the levels of initiation, elongation, and release
factors following chronic BDNF treatment. BDNF specifically increased the eEF2 protein level. BDNF enhanced eEF2 protein synthesis but did not alter either its stability or its mRNA level, strongly suggesting the results of translational control of eEF2. eEF2 mRNA has 5'-terminal oligopyrimidine tract (5'-TOP) in its 5'-UTR (34). mRNAs which have these sequence is known to be regulated downstream of mTOR. As previously reported (8,14), BDNF activates mTOR cascade. Thus increased eEF2 protein by chronic BDNF may be a result of accumulation of newly synthesized eEF2 in response to daily BDNF stimulation. However, BDNF did not affect the levels of eEF1A and S6 that also have 5'-TOP sequences in their mRNA (Supplemental Fig. 3). Thus, the specific increase of eEF2 translation might be cell-type dependent or stimulation (BDNF) dependent.

It is rather surprising because rate-limiting step for translation is believed to be the initiation process (13). In fact, insulin, which also enhanced protein synthesis (Fig. 1), increased several initiation factors (data not shown). It suggests that BDNF and insulin enhances protein synthesis through the different mechanism.

Protein synthesis is regulated at both the initiation and elongation stage of translation (13,35,36). For example, phosphorylation of elongation factors affects their activity and the overall elongation rate (28,37). In eukaryotes, phosphorylated, GTP-bound eEF1A increases the level of aminoacyl-tRNA recruitment. Subsequently, unphosphorylated, GTP-bound eEF2 activates peptidyl-tRNA translocation from the A site to the P site. It is not yet clear if the increase in the level of eEF2 induces an upregulation of protein synthesis. Thus, we examined effects of eEF2 overexpression. cDNA encoding rat eEF2 was introduced into primary neurons by electroporation, resulting in an approximately 1.4-fold increase in the level of eEF2 and a 1.3-fold increase in the protein synthesis rate compared to EGFP-transfected control samples. This is the first direct evidence that an increase in the level of a certain translation factor enhances the rate of protein synthesis in neurons. Considering the relatively low transfection efficacy (20-25%) and shorter period of overexpression (up to 72 h), these results are parallel to the results observed for BDNF treatment. BDNF might also affect initiation phase by modulating phosphorylation of initiation factors, not by increasing their levels. Indeed, chronic BDNF increased phosphorylation of several initiation factors and modulator (Supplemental Fig. 4), although the effects were weak in compared to the acute effect of BDNF. It is not surprising because acute BDNF induces phosphorylation of these molecules (8,14) and the activation of BDNF signaling is known to be sustained. However, transit time measurement suggests that enhancement of protein synthesis induced by BDNF may link to elongation step facilitation. Furthermore, transfection experiment suggests that active eEF2 levels may directly contribute basal protein synthesis at least in the neurons.

In addition to increasing the expression level of eEF2, BDNF decreased the level of phosphorylated eEF2. Thus, BDNF increased the net amount of active, unphosphorylated eEF2. This alteration in the activity of eEF2 is a consequence of both a decrease in the expression of a kinase that phosphorylates eEF2, eEF2K (38), and an increase in the expression of the PP2A phosphatase, for which eEF2 is a substrate (39,40). The enzymatic activities of eEF2K and PP2A correlated with their expression levels. It is not clear if BDNF regulates the transcription, translation and/or degradation of these proteins. Alternatively, BDNF may modulate the kinase and/or phosphatase activities as it does during acute responses. Chronic BDNF treatment, much like acute treatment (8,14), may activate the mTOR complex 1 (mTORC1) signaling cascade. mTORC1 signaling is regulated by both nutrients, such as amino acids and glucose, and growth factors, including BDNF and insulin (41-45), and eEF2 phosphorylation is regulated downstream of mTORC1 (15,41,46).

We also examined the elongation rate in cortical neurons receiving chronic BDNF treatment by measuring the ribosomal transit time, which is calculated as the ratio of ribosome-bound peptides to completed peptide chains. When elongation is enhanced, the transit time that the peptide chains are retained on the ribosome is shorter. Chronic BDNF treatment enhanced the elongation rate. We
conclude from these results that BDNF induces a sustained increase of in the level of active eEF2 and activates the elongation process to promote protein synthesis.

To confirm our findings, we examined the basal rate of protein synthesis in cortical tissue from mice lacking or overexpressing BDNF. We observed a dose-dependent reduction in the rate of protein synthesis, although this reduction was not significant in heterozygous BDNF transgenic mice. The increase in BDNF protein levels in the cortical tissues of adult BDNF transgenic mice using an actin promoter is only 9% (22). On postnatal day 2, however, the level of BDNF in the cortices of the transgenic mice is more than 2-fold higher than that in wild-type mice. Because the endogenous expression level of BDNF is low during the early postnatal days, the actin promoter may result in a higher expression level than in the adult brain. In these brains, the levels of eEF2 and phosphorylated eEF2 were also significantly altered. Transgenic mice expressed more active (unphosphorylated) eEF2, whereas and BDNF mutant mice expressed less active eEF2. Under physiological conditions, neural activity increases BDNF expression and release (5-7). Thus, sustained upregulation of BDNF signaling occurs in active neural circuits. In these neurons, translation may be enhanced and thereby contribute to synaptic plasticity as a positive feedback loop.

In conclusion, chronic changes in BDNF levels in the brain affect protein synthesis by activating eEF2. Thus, extracellular stimuli can regulate not only activity-dependent protein synthesis, but also the baseline rate of protein synthesis in response to chronic signaling.

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FOOTNOTES
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The abbreviations used are: BDNF, brain-derived neurotrophic factor; eEF2, eukaryotic elongation factor 2; eEF2K, eEF2 kinase; PP2A, protein phosphatase 2A

FIGURE LEGENDS

Figure 1. Chronic effects of various neurotrophic factors and cytokines on protein synthesis in cortical neurons.
Neurons were treated without or with BDNF (50 ng/ml), NT-4 (50 ng/ml), NT-3 (50 ng/ml), insulin (10 μg/ml), NGF (50 ng/ml), CNTF (10 ng/ml), GDNF (10 ng/ml), HGF (20 ng/ml), IL-1α (5 ng/ml), IL-1β (5 ng/ml), IL-2 (2.5 ng/ml), IL-6 (1 ng/ml), or IFN-γ (5 x 10⁵ unit/ml) for 5 days. The incorporation of [³⁵S]methionine into newly synthesized proteins was analyzed. Bars represent the means and SD (n = 4). *p < 0.05 (t-test). Similar results were obtained in three or four independent experiments.

Figure 2. Protein synthesis rate in the cortices of BDNF mutant mice.
The incorporation of [³⁵S]methionine into newly synthesized proteins was analyzed in the homogenates of cortical tissue from wild-type (wt) and BDNF mutant (+/−, heterozygous; −/−, homozygous) mice (A) or wt and transgenic (Tg) mice (B). Bars represent the means and SD; n = 10, *p < 0.05 (ANOVA) (A) and (n = 6), *p < 0.05 (t-test) (B).

Figure 3. Effects of chronic BDNF treatment on the levels of translation factors.
Western blotting analysis was performed to examine the protein levels of initiation factors (eIFs), elongation factors (eEFs), and release factors (eRFs) in neurons following BDNF treatment (50 ng/ml) for 5 days. Bands were analyzed using densitometry. Only the increase in the level of eEF2 is significant. n = 4-9 for each protein.

Figure 4. Effect of chronic BDNF treatment on the levels and phosphorylation status of eEF2.
Conventional and quantitative RT-PCR was performed to examine eEF2 mRNA levels in neurons following BDNF treatment for 5 days (A). A single band of predicted size (236bp) was observed (n=4 in each group). Quantitative RT-PCR indicates there is no difference between control and BDNF-treated neurons (n=4, p=0.931 (t-test)). Neurons were pulse labeled with [³⁵S]methionine for 1h, and then washed. eEF2 was immunoprecipitated after 1h and 6h and analyzed in neurons at 4 days in culture and quantified by Image analyzer (BAS 5000. Fuji Film) (B). Bars represent means ± SD (n=3). *p<0.01 (t-test). Western blotting (D, E) and densitometric analysis (D,E) were performed to examine the total and phosphorylated (inactive) protein levels of eEF2 in neurons following BDNF treatment (50 ng/ml) for 5 days. Bars represent the means ± SD (n = 8). *p < 0.001 (t-test). The phosphorylation ratio of eEF2 is presented in panel F.

Figure 5. The levels and phosphorylation status of eEF2 in the cortices of BDNF mutant mice.
Western blotting (A) and densitometric analysis (B) were performed to examine the total and phosphorylated (inactive) protein levels of eEF2 in cortical tissues of wild-type (wt), BDNF mutant [KO(−/−)], and BDNF transgenic (Tg) mice. Bars represent the means and SD; n = 4, *p < 0.05 (ANOVA). The phosphorylation ratio of eEF2 was calculated and is shown in panel C.

Figure 6. Effects of chronic BDNF treatment on the levels and activities of eEF2K and PP2A.
Western blotting (A) and densitometric analysis (B) were performed to examine the protein levels of eEF2K and PP2A in neurons treated with BDNF (50 ng/ml) for 5 days. Bars represent the means and SD (n = 6). *p < 0.01 and **p < 0.005 (t-test). eEF2K kinase activity and PP2A phosphatase activity
were measured (C). Bars represent the means and SD [n = 4 (eEF2K) and n = 8 (PP2A)]. ***p < 0.001 (t-test).

Figure 7. Effects of chronic BDNF treatment on the ribosomal transit time in cortical neurons.
(A) A representative example of the transit times from control neurons or neurons treated with BDNF (50 ng/ml) for 5 days. Open squares (control) and rectangles (BDNF) represent the total synthesized protein. Filled diamonds (control) and circles (BDNF) represent completed peptides at each time point. Solid lines for ‘total’ (nascent plus completed) protein and dashed lines for ‘completed’ peptides were obtained using linear regression analysis. To calculate the transit time, the difference between the lines along the time axis was measured and doubled. Quantitative data is shown in panel B. Bars represent means and SD (n = 8; *p < 0.01, t-test).

Figure 8. Overexpression of eEF2 in cortical neurons.
cDNA encoding rat eEF2 or EGFP (as a control) was transfected into primary cortical neurons. (A) Expression and phosphorylation of eEF2 were analyzed by Western blotting and quantified using densitometry. Actin was used as a standard. Bars represent the means and SD (n = 4; *p < 0.05, t-test). The incorporation of [35S]methionine into newly synthesized protein was determined using sister transfectants to the cells used to determine the level of eEF2 expression. Bars represent the means and SD (n = 12; *p < 0.01, t-test).
Fig. 1

$[^{35}\text{S}]\text{met} \text{ incorporation}$

(\% \text{ increase of control})

-20 0 20 40 60 100

Control
BDNF
NT-4
NT-3
Insulin
NGF
CNTF
GDNF
HGF
IL-1\text{\textalpha}
IL-1\text{\beta}
IL-2
IL-6
IFN-\gamma
Fig. 2

A

[\text{[3S]}\text{Met incorporation (\% of wild type)}]

\begin{table}
\begin{tabular}{c c c}
\hline
 & w.t. & +/- & -/- \\
\hline
\text{[3S]}\text{Met incorporation} & 100 & 50 & 150 \\
\hline
\end{tabular}
\end{table}

B

[\text{[3S]}\text{Met incorporation (\% of wild type)}]

\begin{table}
\begin{tabular}{c c}
\hline
 & w.t. & Tg \\
\hline
\text{[3S]}\text{Met incorporation} & 100 & 150 \\
\hline
\end{tabular}
\end{table}

\*
Fig. 3

Control  BDNF

eIF2α

eIF2β

eIF2Bβ

eIF2Bε

eIF4E

eIF4AII

eIF4G

eIF5

eIF6

eEF1A

eEF2

eEF1

eRF3

actin

GAPDH

NSE
Fig. 4
**Fig. 6**

Panel A:
- eEF2K (Control vs. BDNF)
- PP2A C-subunit (Control vs. BDNF)

Panel B:
- eEF2K activity: Control 100%, BDNF 60% (p < 0.01)**
- PP2A activity: Control 100%, BDNF 120% (p < 0.05)*

Panel C:
- eEF2K activity: Control 100%, BDNF 30% (**p < 0.001**)
- PP2A activity: Control 100%, BDNF 150% (**p < 0.001**)

Legend:
- *p < 0.05
- **p < 0.01
- ***p < 0.001

Note: The images and data in the figure suggest that BDNF has a significant effect on the activity of eEF2K and PP2A, with eEF2K activity decreasing and PP2A activity increasing in the presence of BDNF compared to controls.
A

Control

BDNF

Transit time (% of Control)

B

Fig. 7

Control BDNF

*
Fig. 8

A

Total eEF2 (% of Control)

EGFP  eEF2

EGFP  eEF2

P-eEF2

Actin

B

[\textsuperscript{35}S]Met incorporation (% of Control)

0  50  100  150

EGFP  eEF2

19

Fig. 8
Legends to Supplemental Figures

Supplemental Fig. 1. Densitometric analysis of translation factor levels.
Western blotting analysis was performed to examine the protein levels of translation factors in neurons following BDNF treatment (50 ng/ml) for 5 days as shown in Fig.3. Bands were analyzed using densitometry. Open (white) bars indicate control and filled (black) bars indicate BDNF-treated group. Bars represent the means and SD n=4-6. There are no statistical significance in all proteins listed by t-test. *p*-values are shown in the figure.

Supplemental Fig. 2. Immunocytochemistry with anti-eEF2, P-eEF2 and MAP-2.
Neurons treated without (A,B,E,F) or with BDNF (50ng/ml) (C,D,G,H) for 5 days were immunostained with anti-eEF2 (A and C), anti-P-eEF2 (E and G) and anti-MAP-2 (B,D,F,H) antibodies. Note that BDNF had no effects on MAP-2 immunocytochemistry in their number, shapes and intensities.

Supplemental Fig. 3. Effects of chronic BDNF treatment on the levels of eEF1A and S6 proteins.
Western blotting and densitometric analysis were performed to examine the protein levels of eEF1A (A) and S6 (B), both have 5’TOP sequences in their mRNA, in neurons following BDNF treatment (50 ng/ml) for 5 days. Bars represent the means ± SD (n = 9). There are no statistical significances (*t*-test). *p*-values are shown in the figure.

Supplemental Fig. 4. Effects of chronic BDNF treatment on the phosphorylation status of several initiation factors and the modulator.
Western blotting and densitometric analysis were performed to examine the phosphorylated protein levels of eIF2a, eIF4E and eIF4G in neurons following BDNF treatment (50 ng/ml) for 5 days. 4EBP and phospho-4EBP (T37/46) levels were also examined. Bars represent the means ± SD (n = 3-6). *p*-values are shown in the figure.
Supplemental Fig. 3

A. eEF1A

- Cont
- BDNF

B. rpS6

- Cont
- BDNF

n=9, p=0.794

n=9, p=0.896