Brain-derived Neurotrophic Factor Enhances Neuronal Translation by Activating Multiple Initiation Processes

COMPARISON WITH THE EFFECTS OF INSULIN*

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The effects of neurotrophic factors on translational activation were investigated in cortical neurons. Brain-derived neurotrophic factor (BDNF) increased protein synthesis within 30 min, whereas insulin produced a weaker enhancement of protein synthesis. BDNF-triggered protein synthesis was inhibited by LY294002, PD98059, and rapamycin, whereas the effect of insulin was unaffected by PD98059. To explore the mechanisms underlying this effect, the protein phosphorylation cascades that lead to the activation of translation initiation in neurons were examined. BDNF induced the phosphorylation of both eukaryote initiation factor (eIF) 4E and its binding protein (eIF4E-binding protein-1). The former reaction was inhibited by PD98059, whereas the latter was inhibited by LY294002 or rapamycin. In agreement, BDNF induced the phosphorylation of mammalian TOR (target of rapamycin) and enhanced its kinase activity toward eIF4E-binding protein-1. In contrast, insulin failed to activate MAPK and did not induce the phosphorylation of eIF4E. Since BDNF and insulin increased the activity of eIF2B and eIF2, the only difference between them was eIF4E phosphorylation. Thus, this may explain the lower activity of insulin in potentiating neuronal protein synthesis. These results suggest strongly that BDNF simultaneously activates multiple signaling cascades consisting of phosphatidylinositol 3-kinase, mammalian TOR, and MAPK to enhance translation initiation in neurons.

The synthesis and modification of proteins play key roles in neural plasticity, brain development, and higher brain functions such as learning and memory (1, 2). Although post-translational modification can modulate short-term neural plasticity, longer term plastic changes seem to require novel protein synthesis (3, 4). There is a certain phase of long-term potentiation and long-term depression, which are the model paradigms of the cellular basis of learning and memory, that is translation-dependent, as has been revealed by pharmacological approaches (5–7). Moreover, learning and memory processes in vivo are known to require novel protein synthesis under various conditions (8, 9). The types of external stimuli that trigger neuronal translation have not yet been fully determined. Although many pharmacological studies have suggested the involvement of novel protein synthesis in synaptic plasticity, the molecular basis of translational activation in neurons remains to be elucidated.

Many growth factors such as insulin are known to influence translation, and their signaling cascades have been studied with respect to cell growth or proliferation in several types of cell. In this study, the effects of brain-derived neurotrophic factor (BDNF)† on rapid protein synthesis and the translational control mechanisms in cortical neurons were characterized and compared with those of insulin.

BDNF is a member of the neurotrophin family, which promotes the differentiation, maturation, and survival of peripheral and central nervous system neurons (10). Recent studies have shown that BDNF and its cognate receptor TrkB may be important for synaptic plasticity in the central nervous system (11–13). For example, it has been reported that BDNF is intrinsically involved in the establishment of long-term potentiation (14). In addition, BDNF enhances basal neurotransmission in hippocampal synapses in a translation-dependent manner (15).

In eukaryotic cells, the rate of translations is regulated mainly at the initiation phase. The following reactions have been suggested as essential regulatory steps for initiation and for overall protein synthesis (16). Eukaryote initiation factor (eIF) 4E recognizes the 7-methylguanosine 5-triphosphate cap structure and binds to mRNA. The eIF4E-RNA complex then binds to eIF4G, associating with eIF4A. The complex of eIF4E, eIF4G, and eIF4A, which is called eIF4F, associates with mRNA and a 40 S ribosomal subunit, which leads to the activation of initiation. eIF4E-binding protein-1 (4EBP1) behaves as a negative regulator of initiation that binds to eIF4E and prevents the formation of the eIF4F complex in translationally silent states. The phosphorylation of 4EBP1 at serine and threonine residues makes it dissociate from eIF4E (17–19). 4EBP1 was originally identified as a protein that becomes phosphorylated after the stimulation of rat adipocytes with insulin (20). Various extracellular stimuli such as growth factors, hormones, cytokines, and mitogens induce the phosphorylation of 4EBP1 in many types of primary cultured cells or

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¶ The abbreviations used are: BDNF, brain-derived neurotrophic factor; eIF, eukaryotic initiation factor; 4EBP1, eIF4E-binding protein-1; PI3K, phosphatidylinositol 3-kinase; mTOR, mammalian target of rapamycin; MAPK, mitogen-activated protein kinase; GST, glutathione S-transferase; DMEM, Dulbecco’s modified Eagle’s medium; PAGE, polyacrylamide gel electrophoresis; m7GTP, 7-methyl-GTP; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase.
cell lines (16–18, 21, 22). Studies of the signal transduction cascade that leads to the phosphorylation of 4EBP1 show the importance of phosphatidylinositol 3-kinase (PI3K), Akt, and mTOR (also called FRAP/RAFT1) as upstream kinases that activate this cascade (23–28). eIF4E is also phosphorylated following stimulation by growth factors, hormones, and mitogens, which promote cell growth and global translation rates (18). The phosphorylation of eIF4E is performed by Mnk1, which is activated by MAPK or p38 (29, 30). These modifications of phosphorylation are reported to be directly related to (4EBP1) or to be suspected of being related to eIF4E). The rate of translation. However, the mechanisms underlying the regulation of cap-binding processes in central neurons remain to be established. To address this and to further our understanding of neuronal translation mechanisms, we first identified BDNF as a translational enhancer of primary cultured cortical neurons by activating the PI3K-Akt, mTOR, and MAPK pathways. We then show that BDNF induces the phosphorylation of 4EBP1 and decreases the association between 4EBP1 and eIF4E as well as the activation of mTOR. BDNF also increases the phosphorylation of eIF4E.

Another regulatory step of initiation depends on eIF2, which is required for the formation of the ternary complex eIF2-GTP-Met-tRNA, and eIF2 is essential for priming each 40S ribosomal subunit. eIF2B is a guanine nucleotide exchange factor that catalyzes the eIF2-GDP to eIF2-GTP exchange, thereby regulating eIF2 activity. eIF2B activity is regulated by the phosphorylation of its e-subunit by glycogen synthase kinase-3 (32). We therefore investigated the activities of eIF2B and eIF2 together with the phosphorylation of glycogen synthase kinase-3 in response to BDNF and insulin. Both of the factors activate eIF2B and eIF2. In contrast to the effects of BDNF, we have shown that insulin produces a weaker enhancement of protein synthesis and fails to induce the phosphorylation of eIF4E. Although it has been reported that growth factors activate each translation factor, the link between actual protein synthesis and the simultaneous activation of multiple factors is not fully understood, especially in neurons. In this study, we demonstrate that the simultaneous phosphorylation of 4EBP1 and eIF4E, which is induced by BDNF, appears to be required for the full activation of translation in neurons.

**EXPERIMENTAL PROCEDURES**

**Materials**—BDNF was a generous gift from Sumitomo Pharmaceutical Co. Anti-4EBP1 polyclonal antibody was purchased from Santa Cruz Biotechnology, and anti-eIF4E polyclonal antibody was obtained from Transduction Laboratories. Anti-Akt, anti-phospho-Akt, and anti-MAPK polyclonal antibodies and anti-phospho-MAPK monoclonal antibody were from Cell Signaling. Anti-mTOR monoclonal antibody and MAPK polyclonal antibodies and anti-phospho-MAPK monoclonal antibody were from Transduction Laboratories. Anti-Akt, anti-phospho-Akt, and anti-MAPK polyclonal antibodies and anti-phospho-MAPK monoclonal antibody were from Cell Signaling. Anti-mTOR monoclonal antibody and MAPK polyclonal antibodies and anti-phospho-MAPK monoclonal antibody were from Cell Signaling. Anti-mTOR monoclonal antibody and MAPK polyclonal antibodies and anti-phospho-MAPK monoclonal antibody were from Cell Signaling. Anti-mTOR monoclonal antibody and MAPK polyclonal antibodies and anti-phospho-MAPK monoclonal antibody were from Cell Signaling. Anti-mTOR monoclonal antibody and MAPK polyclonal antibodies and anti-phospho-MAPK monoclonal antibody were from Cell Signaling. Anti-mTOR monoclonal antibody and MAPK polyclonal antibodies and anti-phospho-MAPK monoclonal antibody were from Cell Signaling.

**Cell Culture**—A primary culture of cortical neurons was prepared by a method of a previously described method (34). Briefly, the cerebral cortices were removed from 18–19-day-old embryonic rat fetuses and dissociated using papain/DNase I. Neurons were seeded at 2 × 10^5 cells/cm² and cultivated in Dulbecco's modified Eagle's medium (DMEM; Nissui) containing 10% fetal bovine serum (Life Technologies, Inc.). The medium was changed every 3 or 4 days, and neurons were used for each assay after 10–14 days in culture.

**Metabolic Labeling—**Cortical neurons cultured in DMEM containing 10% fetal bovine serum were washed twice with serum-free DMEM and incubated with the serum-free medium for 8–12 h to starve the neurons of serum. The medium was then changed to a 1:1 mixture of DMEM and methionine-free DMEM. Neurons were incubated with 10 μCi of [35S]methionine for 15 min. Growth factor stimulation was for 30 min, unless indicated otherwise. Inhibitors were added to the culture 10 min prior to BDNF stimulation. The labeling was stopped by three washes with ice-cold phosphate-buffered saline, and neurons were lysed in 500 μl of 0.5 M NaOH containing 10 mg/ml casein as a carrier. Samples were incubated at 37 °C for 30 min and then collected in Eppendorf tubes. An equal volume of ice-cold trichloroacetic acid-insoluble fraction. *Bars* indicate the percentage increase compared with the control. Data are presented as the means ± S.D. of four (upper panel) or six (center and lower panels) measurements.
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RESULTS

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Figs. 1 and 2. Effects of kinase inhibitors on BDNF-induced protein synthesis. Following pretreatment for 10 min with K252a (200 nm) (A) or LY294002 (10 μM), PD98059 (50 μM), rapamycin (20 ng/ml), or protein phosphatase-2 (PP2; 1 μM) (B and C), neurons were left unstimulated (Control) or were stimulated with either BDNF (100 ng/ml) (B) or insulin (1 μg/ml) (C) for 30 min. In each case, protein synthesis was determined by measuring the amount of [35S]Met incorporated into the trichloroacetic acid-insoluble fraction. Bars indicate the percentage increase compared with the control. Data are presented as the means ± S.D. of four values. The same results were obtained in three independent experiments.

Binding and then incubated overnight with primary antibodies. Protein G-Sepharose was incubated with the samples for a further 3 h. The immunocomplex was then washed five times with radioimmune precipitation assay buffer. SDS sample buffer was added to each sample and heated to 95 °C for 5 min. Each sample was subjected to SDS-polyacrylamide gel electrophoresis (PAGE), and autoradiography was performed after drying the gel. mTOR immunoprecipitation was performed essentially as described previously (30). In a co-immunoprecipitation experiment, neurons were lysed in co-immunoprecipitation buffer (10 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, and 0.5% Nonidet P-40) containing Complete[tm], 1 mM Na3VO4, and 20 mM NaF and immunoprecipitated with anti-eIF4E antibody. Samples were subjected to SDS-PAGE, and Western blotting was performed with anti-eIF4E antibody to detect the association between 4EBP1 and eIF4E. Pull-down assay using 7-methyl-GTP (m7GTP)-Sepharose (Amersham Pharmacia Biotech) was performed as described for the co-immunoprecipitation experiment.

Electrophoresis and Western Blotting—SDS-PAGE and Western blotting were performed as described previously (35). Cells were collected and sonicated in TN buffer at pH 7.5, and protein concentrations were determined. Protein samples of the same size (25–40 μg/lane) were subjected to SDS-PAGE and transferred to polyvinylidene difluoride membranes. The membranes were incubated with primary antibodies and then exposed to horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG (1:2000 dilution; Cappel). Peroxidase activity was visualized on x-ray film after being reacted with Renaissance (PerkinElmer Life Sciences).

Purification of eIF2 and Measurement of eIF2B and eIF2 Activities—eIF2 was partially purified for the measurement of eIF2B activity according to a previously reported method (36). Briefly, rat brains (~50 g) were homogenized and centrifuged. The supernatant was precipitated with ammonium sulfate. The dialyzed sample was then applied to heparin-Sepharose, SP-Sepharose, and DEAE-Sepharose (HiTrap™, Amersham Pharmacia Biotech) and eluted by stepwise salt solutions. Activity and protein concentrations were checked in each elute. The specific activity of partially purified eIF2 was >1000-fold greater than that of the initial homogenate.

eIF2B activity was assayed as reported by Quevedo et al. (37). A lysate of cultured neurons (100 μg) was incubated with the binary complex eIF2-3[H]GDP, which had been prepared by a method previously reported (36). The radioactivity of [3H]GDP remaining bound to eIF2 during 3 min of the GDP exchange reaction was counted.

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**Suppression of BDNF- and Insulin-induced Protein Synthesis by Protein Kinase Inhibitors**—The effects of kinase inhibitors on BDNF-induced protein synthesis were investigated to improve our understanding of its signaling pathways. Pretreatment with K252a, a relatively selective Trk kinase inhibitor, improved our understanding of its signaling pathways. Pretreatment also suppressed the effect of BDNF-induced protein synthesis through TrkB signaling. Therefore, the downstream signaling cascades from TrkB (a MAPK pathway and a PI3K-Akt pathway) were examined further. BDNF-induced protein synthesis was completely inhibited by either LY294002, which is a potent inhibitor of PI3K, or PD98059, which is an MEK inhibitor. Rapamycin, an immunosuppressant that inhibits the kinase activity of mTOR, also suppressed the effect of BDNF-induced protein synthesis. In contrast, pp2, an inhibitor of Src family kinases, had no effect on BDNF-induced protein synthesis (Fig. 2B). These results suggest strongly that BDNF requires the PI3K-Akt, MAPK, and mTOR pathways to trigger translational up-regulation in neurons.

To compare the signaling pathways activated by BDNF and insulin, the effects of kinase inhibitors on insulin-induced protein synthesis were also examined. Although the action of insulin was completely blocked by either LY294002 or rapamycin, it was not affected by PD98059, suggesting that the MAPK pathway is not involved in the insulin-induced weak enhancement of protein synthesis.

**BDNF Induces the Phosphorylation of 4EBP1 and Decreases the Association between 4EBP1 and eIF4E**—Rates of protein synthesis are regulated mainly by the initiation step of translation. The phosphorylation of 4EBP1 and the resulting dissociation of 4EBP1 and eIF4E are key steps that lead to the activation of initiation. Therefore, the phosphorylation of 4EBP1 by BDNF was examined. Serum-starved neurons were labeled with [32P]orthophosphate (100 Ci/ml) for 2 h and stimulated with BDNF for 5 min. Equal amounts of cell lysate protein were immunoprecipitated with anti-4EBP1 antibody. Samples were then subjected to SDS-PAGE, followed by autoradiography. BDNF increased the phosphorylation of 4EBP1, which was inhibited by LY294002 or rapamycin (Fig. 3A). The radioactivity of each band of 32P-labeled 4EBP1 was quantified using a Fuji BAS 2000 image analyzer. Data are presented as means ± S.D. of three measurements.

**BDNF Increases the Phosphorylation of mTOR and Increases Rates of Protein Synthesis**—Rates of protein synthesis are regulated mainly by the initiation step of translation. The phosphorylation of mTOR leads to increased rates of protein synthesis. BDNF increases the phosphorylation of mTOR, which is an important regulator of protein synthesis. Therefore, the phosphorylation of mTOR was examined. Serum-starved neurons were labeled with [32P]orthophosphate (100 Ci/ml) for 2 h and stimulated with BDNF for 5 min. Equal amounts of cell lysate protein were immunoprecipitated with anti-mTOR antibody. Samples were then subjected to SDS-PAGE, followed by autoradiography. mTOR immunoprecipitated with anti-mTOR antibody was subjected to Western blotting with anti-phospho-Akt antibody and immunoprecipitated with anti-4EBP1 antibody. The bands represent the amount of 4EBP1-bound eIF4E (first row), Akt (second row), and mTOR (third row). Furthermore, mTOR immunoprecipitated with anti-mTOR antibody was subjected to Western blotting with anti-phospho-Akt antibody and immunoprecipitated with anti-4EBP1 antibody. The bands represent the amount of 4EBP1-bound eIF4E (first row), Akt (second row), and mTOR (third row).
BDNF induces the phosphorylation of mTOR and increases the kinase activity of mTOR toward 4EBP1 in cortical neurons. Neurons were treated without (Control) or with BDNF (100 ng/ml) for 5 min. Before stimulation with BDNF, neurons were treated for 10 min with LY294002 (10 μM) or rapamycin (20 ng/ml). A, neurons were labeled with [32P]orthophosphate and then stimulated either with BDNF (100 ng/ml) for 5 min or with BDNF (100 ng/ml) + LY294002 (10 μM). Cell lysates were immunoprecipitated with anti-mTOR antibody. Samples were then subjected to SDS-PAGE, followed by autoradiography (upper panel). Neurons were stimulated with BDNF or with BDNF + LY294002 as described for the phosphorylation assay, and cell lysates were immunoprecipitated and Western-blotted with anti-mTOR antibody (lower panel). B, the radioactivity of each band was quantified using a Fuji BAS 2000 image analyzer. Bars represent the relative increase in the signal. Data are presented as the means ± S.D. of three measurements. C, neurons were stimulated with BDNF (100 ng/ml) or with BDNF (100 ng/ml) + rapamycin (20 ng/ml) and then immunoprecipitated with anti-mTOR antibody. The immunoprecipitates were incubated with GST-4EBP1 and [γ-32P]ATP. Samples were then subjected to SDS-PAGE, followed by autoradiography. D, the radioactivity of each band was quantified using a Fuji BAS 2000 image analyzer. Bars represent the relative increase in the signal. Data are presented as the means ± S.D. of three measurements.

BDNF Induces the Phosphorylation of eIF4E and Increases m7GTP Binding in Parallel with MAPK Phosphorylation—Since PD98059 inhibited the protein synthesis induced by BDNF, translation initiation enhanced by BDNF is likely to be affected by the MAPK cascade. Neurons were labeled with [32P]orthophosphate (100 μCi/ml) for 2 h and stimulated with BDNF (100 ng/ml) for 5 min. Immunoprecipitation was carried out with anti-eIF4E antibody, and then SDS-PAGE was performed. BDNF increased the phosphorylation of eIF4E within 5 min (Fig. 5) without affecting the amount of eIF4E (data not shown). The BDNF-induced phosphorylation of eIF4E was completely inhibited by PD98059, but not by LY294002. The pattern of phosphorylation of eIF4E was similar to that of MAPK, as revealed by immunoblotting with anti-phospho-MAPK antibody (Fig. 5). In addition, pull-down assay showed that BDNF increased the amount of m7GTP-bound eIF4E and that the binding was inhibited by PD98059. These results, the phosphorylation of eIF4E and its association with m7GTP, are consistent with the results of the pharmacological analysis of BDNF-induced protein synthesis.

Differences and Similarities between BDNF and Insulin Signaling in the Initiation Processes in Neurons—Both BDNF and insulin enhanced protein synthesis in neurons, but the degree of enhancement differed. To elucidate the cause of the difference, the effects of BDNF and insulin on the activities of eIF2B and eIF2 were examined. As shown in Fig. 6 (A and B), both factors increased eIF2B activity as well as eIF2 activity. BDNF and insulin also induced the phosphorylation of glycogen syn-

FIG. 4. BDNF induces the phosphorylation of mTOR and increases the kinase activity of mTOR toward 4EBP1 in cortical neurons. Neurons were treated without (Control) or with BDNF (100 ng/ml) for 5 min. Before stimulation with BDNF, neurons were treated for 10 min with LY294002 (10 μM) or rapamycin (20 ng/ml). A, neurons were labeled with [32P]orthophosphate and then stimulated either with BDNF (100 ng/ml) for 5 min or with BDNF (100 ng/ml) + LY294002 (10 μM). Cell lysates were immunoprecipitated with anti-mTOR antibody. Samples were then subjected to SDS-PAGE, followed by autoradiography (upper panel). Neurons were stimulated with BDNF or with BDNF + LY294002 as described for the phosphorylation assay, and cell lysates were immunoprecipitated and Western-blotted with anti-mTOR antibody (lower panel). B, the radioactivity of each band was quantified using a Fuji BAS 2000 image analyzer. Bars represent the relative increase in the signal. Data are presented as the means ± S.D. of three measurements. C, neurons were stimulated with BDNF (100 ng/ml) or with BDNF (100 ng/ml) + rapamycin (20 ng/ml) and then immunoprecipitated with anti-mTOR antibody. The immunoprecipitates were incubated with GST-4EBP1 and [γ-32P]ATP. Samples were then subjected to SDS-PAGE, followed by autoradiography. D, the radioactivity of each band was quantified using a Fuji BAS 2000 image analyzer. Bars represent the relative increase in the signal. Data are presented as the means ± S.D. of three measurements.

FIG. 5. BDNF induces the phosphorylation of eIF4E and increases the binding to m7GTP along with MAPK phosphorylation. Neurons were treated without (Control) or with BDNF (100 ng/ml) for 5 min. In the case of inhibitor experiments, they were pretreated for 10 min with LY294002 (10 μM), PD98059 (50 μM), or rapamycin (20 ng/ml). Neurons were labeled with [32P]orthophosphate and stimulated with BDNF or with BDNF + inhibitors. Cell lysates were immunoprecipitated with anti-eIF4E antibody. Samples were then subjected to SDS-PAGE, followed by autoradiography. Control, BDNF-treated, or (BDNF + inhibitor)-treated neurons were subjected to Western blotting with anti-phospho-MAPK (P-MAPK) or anti-MAPK antibody. BDNF- or inhibitor-treated neurons were lysed and pulled down with m7GTP-Sepharose, followed by Western blotting with anti-eIF4E antibody.
BDNF-induced Translational Activation in Neurons

Effects of Various Neurotrophic Factors on Protein Synthesis—Among the several neurotrophic factors tested, BDNF showed the strongest effect on protein synthesis. Insulin, a well-known stimulator of translation in other cell types, is less potent even at its highest dose in cortical neurons, although its receptor(s) are widely expressed in and induce biological and biochemical effects on these cells (38, 39). Likewise, although their receptors are expressed and their biological effects have been reported (40–42), epidermal growth factor and ciliary neurotrophic factor had no effects and neurotrophin-3 had little effect (from 1 to 300 ng/ml) on protein synthesis in cortical neurons. The action of BDNF was dose-dependent and mediated by TrkB receptors. The culture medium used in this study contained 10% serum, so a considerable proportion (10–20%) of astrocytes was present. Although astrocytes have a basal level of protein synthesis, they do not express full-length TrkB (43). Indeed, BDNF increased neither the protein synthesis nor the phosphorylation of signaling molecules in a pure astrocyte culture (data not shown). Thus, the effects of BDNF shown in this study must be attributable to neuronal responses. Rather, the net increase in neuronal protein synthesis induced by BDNF may be higher than is suggested by the present data because it included the basal protein synthesis of astrocytes, which is not affected by BDNF. Actinomycin D, an mRNA synthesis inhibitor, had no effect on BDNF-induced protein synthesis (data not shown), suggesting strongly that the process involves transcription-independent translational control.

Pharmacological Analysis of BDNF-induced Protein Synthesis—Pharmacological analysis showed that the BDNF effect...
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action of insulin was unaffected by PD98059, suggesting that it is independent of MAPK pathway activation.

It has yet to be determined why PD98059 or LY294002 causes a complete block of BDNF-induced protein synthesis. There may be a complex synergy between these signaling pathways.

4EBP1 Phosphorylation and Association between 4EBP1 and eIF4E—In addition to the pharmacological approaches, the phosphorylation of 4EBP1 was examined to further our understanding of the molecular mechanisms underlying BDNF-induced translational activation. Upon stimulation by serum or insulin, 4EBP1 is phosphorylated and liberated from eIF4E (17–19). We have shown that BDNF triggers the phosphorylation of 4EBP1 together with the dissociation of 4EBP1 from eIF4E. The results that 4EBP1 phosphorylation and dissociation from eIF4E induced by BDNF are events that occur downstream of the PI3K pathway (but not of the MAPK pathway) are in good agreement with the results of previous studies performed using mitotic cells. Although 4EBP1 is phosphorylated by MAPK in vitro (44), insulin induces 4EBP1 phosphorylation via the PI3K pathway without activating the MAPK pathway in HEK293 and Swiss 3T3 cells (45). Furthermore, there is direct evidence that PI3K and Akt are the upstream signaling molecules for 4EBP1 (27, 28): 4EBP1 is phosphorylated in HEK293 cells and L6 myotubes carrying constitutive active forms of PI3K or Akt.

Phosphorylation and Activation of mTOR—The inhibitory effects of rapamycin on both protein synthesis and 4EBP1 phosphorylation suggest a role for mTOR in BDNF-induced translational activation. Rapamycin, which was reported to inhibit protein synthesis in aplysia neurons (46), is an immunosuppressant that binds to FKBP12. The complex then binds to and inhibits the activity of mTOR. mTOR has been reported to have a kinase activity that relays the signal from PI3K and Akt to 4EBP1 and that regulates 4EBP1 phosphorylation in several cells (47–49). Although it is not clear whether the role of mTOR in the regulation of 4EBP1 is direct or indirect (50–52), the activation of mTOR by BDNF seems to be essential for BDNF-triggered protein synthesis. We found that BDNF induced mTOR phosphorylation, which was inhibited by LY294002. In addition, in vitro kinase assay using mTOR immunoprecipitates and GST-4EBP1 showed that BDNF increased the kinase activity of mTOR toward 4EBP1. Although it remains to be established whether mTOR phosphorylates 4EBP1 directly, mTOR immunoprecipitates, which may contain mTOR-associated kinases, do phosphorylate 4EBP1 in vitro. These results suggest strongly that the BDNF-induced phosphorylation of 4EBP1 in neurons is mediated by the mTOR pathway. mTOR is reported to interact with gelsolin, a molecule that is necessary for the clustering of glycine and γ-aminobutyric acid receptors (53). Thus, mTOR may play a predominant role in BDNF-induced translation at synapses.

eIF4E Phosphorylation and Its Binding to m7GTP—Although the MAPK pathway was found not to be involved in either the BDNF-induced phosphorylation of 4EBP1 or the association between 4EBP1 and eIF4E, the enhancement of protein synthesis by BDNF was blocked by PD98059, which is an MEK inhibitor. This suggests that another signaling pathway contributes to BDNF-induced protein synthesis. To characterize that pathway, the phosphorylation of eIF4E was examined in detail since previous reports have shown that MAPK activation leads to eIF4E phosphorylation (54) and an increase in protein synthesis (55). A role of eIF4E phosphorylation in translation has been examined in NIH3T3 and Rat-2 fibroblasts: overexpression of wild-type eIF4E (but not of a negative mutant of eIF4E) results in a high rate of protein synthesis and occurred downstream of PI3K pathway activation. The finding that wortmannin, another inhibitor of PI3K, blocked the effect of BDNF on protein synthesis confirmed this conclusion (data not shown). PD98059 and U0126 (data not shown), both of which are MEK inhibitors, also inhibited BDNF-induced protein synthesis. These results suggest the additional involvement of MAPK signaling in BDNF-induced protein synthesis. BDNF activates both the PI3K-Akt and MAPK signaling cascades; the link between these cascades and the translation machinery in neurons has not yet been determined. The inhibition of each cascade is enough to block the effect of BDNF on protein synthesis. These observations suggest strongly that simultaneous activation of both of these pathways is required for BDNF-induced protein synthesis. The importance of cova lation of these cascades also seems to be supported by the weaker effect of insulin on protein synthesis in neurons. The

![Fig. 7. Differential signaling of BDNF and insulin in the initiation processes in neurons.](http://www.jbc.org/)

**Fig. 7.** Differential signaling of BDNF and insulin in the initiation processes in neurons. Neurons were labeled with $^{32}$Porthophosphate and then either left unstimulated (Control) or stimulated with either BDNF (100 ng/ml) or insulin (1 μg/ml) for 5 min. Cell lysates were immunoprecipitated with anti-eIF4E or anti-4EBP1 antibody. Samples were then subjected to SDS-PAGE, followed by autoradiography. BDNF- or insulin-treated neurons were also subjected to Western blotting with anti-phospho-Akt (P-Akt), anti-phospho-MAPK (P-MAPK), and anti-phospho-Mnk1 (P-Mnk1) antibodies. Each cell lysate was pulled down with m7GTP-Sepharose and then subjected to Western blotting with anti-eIF4E antibody.
malignant transformation (56). In addition, BDNF increased the binding of eIF4E to m7GTP, and the binding was inhibited by PD98059, which was parallel to the levels of eIF4E phosphorylation. Although the direct relation between eIF4E phosphorylation and cap binding is unclear, the phosphorylation is suspected of playing a role in the regulation of the eIF4F-mRNA interaction (19). We have currently no information that would explain why rapamycin reduces eIF4E phosphorylation. There may be a novel signaling pathway that links mTOR and eIF4E, or there may be cross-talk between them.

The results of this study suggest that BDNF enhances protein synthesis through the phosphorylation of MAPK-eIF4E cascade as well as through the previously mentioned PI3K-Akt-4EBP1 and mTOR-4EBP1 cascades. Considering the results of pharmacological studies of protein synthesis (Fig. 2), the simultaneous phosphorylation of both 4EBP1 and eIF4E may be necessary for the BDNF-induced activation of translation in neurons.

**Differences in Signaling between BDNF- and Insulin-induced Translation Initiation**—Many studies have shown that insulin induces translational activation through multiple processes in various cell types (57). However, insulin exhibited somewhat different effects from BDNF on translation initiation in neurons. Although both factors equally activated eIF2B and eIF2 as well as induced the phosphorylation of 4EBP1, BDNF (but not insulin) induced the phosphorylation of eIF4E and increased its binding to the cap analog. The lack of MAPK and Mnk1 activation by insulin signaling in neurons may be responsible for the different eIF4E phosphorylation states. The fact that insulin fails to activate multiple cascades may explain its lower activity on protein synthesis.

In addition to the effects of BDNF on the initiation processes examined in this study, BDNF may influence elongation processes in neurons. Further analysis is required to clarify whether BDNF affects other initiation or elongation mechanisms in neurons.

There are numerous reports demonstrating that novel protein synthesis is necessary for activity-dependent synaptic plasticity in the central nervous system (1–9). Moreover, recent studies have suggested that BDNF plays an essential role in synaptic plasticity (11–13). Here we have demonstrated the molecular basis of BDNF-induced up-regulation of translation in central neurons. BDNF may contribute to long-term synaptic plasticity through translational activation via multiple signaling cascades. The present findings may provide the missing link between BDNF and translation-dependent synaptic plasticity.

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**REFERENCES**

33. Mank1 activation by insulin signaling in neurons may be re-
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