

Intracellular Calcium and Calmodulin Link Brain-Derived Neurotrophic Factor to p70S6 Kinase Phosphorylation and Dendritic Protein Synthesis

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The mammalian target of rapamycin (mTOR)/p70S6 kinase (S6K) pathway plays an important role in brain-derived neurotrophic factor (BDNF)-mediated protein synthesis and neuroplasticity. Although many aspects of neuronal function are regulated by intracellular calcium ($[Ca^{2+}]_i$) and calmodulin (CaM), their functions in BDNF-induced phosphorylation of p70S6K and protein synthesis are largely unknown. Here, we report that BDNF, via TrkB-dependent activation of mTOR, induces sustained phosphorylation of p70S6K at Thr389 and Thr421/Ser424. BDNF-induced phosphorylation at Thr389 was dependent on PI3 kinase but independent of ERK-MAPK. The previously identified MAPK phosphorylation site at Thr421/Ser424 required both PI3K and MAPK in BDNF-stimulated neurons. Furthermore, we found that the reduction in $[Ca^{2+}]_i$, but not extracellular calcium, blocked the BDNF-induced phosphorylation of p70S6K at both sites. Inhibition of CaM by W13 also blocked p70S6K phosphorylation. In correlation, W13 inhibited BDNF-induced local dendritic protein synthesis. Interestingly, sustained elevation of $[Ca^{2+}]_i$ by membrane depolarization antagonized the BDNF-induced p70S6K phosphorylation. Finally, the BDNF-induced p70S6K phosphorylation did not require the increase of calcium level through either extracellular influx or PLC-mediated intracellular calcium release. Collectively, these results indicate that the basal level of intracellular calcium gates BDNF-induced activation of p70S6K and protein synthesis through CaM. © 2009 Wiley-Liss, Inc.

Key words: p70S6 kinase; BDNF; PI3 kinase; mTOR; calmodulin

Brain-derived neurotrophic factor (BDNF) is a member of the neurotrophin family and plays an essential role in regulating plasticity, such as long-term poten-

tiation (LTP; Korte et al., 1995) and long-term memory (LTM; Bekinschtein et al., 2007a). The functional relevance of BDNF in neuroplasticity at least partially is due to its ability to increase both global and local protein synthesis (Takei et al., 2001, 2004; Sutton and Schuman, 2005; Tanaka et al., 2008). It has been hypothesized that both phosphoinositide 3-kinase (PI3K)/mammalian target of rapamycin (mTOR) and ERK play important roles in activating translational machinery at multiple stages, including ribosomal biogenesis, initiation, and elongation (Takei et al., 2001, 2004; Kelleher et al., 2004; Klann and Dever, 2004; Schrott et al., 2004; Inamura et al., 2005; Sutton and Schuman, 2005; Gelinis et al., 2007), leading to an increase in mRNA translation. By activating the TrkB tyrosine kinase receptor, BDNF simultaneously stimulates multiple signaling cascades, including PI3K/mTOR and Ras/ERK pathways, to enhance neuronal protein synthesis. Consequently, the up-regulation of PI3K/mTOR and ERK correlates with, and is required for, LTP and LTM (Tang et al., 2002; Ying et al., 2002; Kelleher et al., 2004; Parsons et al., 2006; Tsokas et al., 2007).

p70S6K is a major kinase for the 40S ribosomal protein S6, whose phosphorylation often regulates the translation of 5' TOP-containing mRNA transcripts and correlates with elevation in protein synthesis (Hay and

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Sonenberg, 2004; Pfeiffer and Huber, 2006). Among many potential phosphorylation sites (Pullen and Thomas, 1997; Saitoh et al., 2002), the phosphorylation at both Thr389 and Thr421/Ser424 is required for the full activation of p70S6K (Lehman et al., 2003). Studies with nonneuronal neutrophils suggested that the phosphorylation at Thr389 is regulated by PI3K and the phosphorylation at Thr421/Ser424 by MAPK (Lehman et al., 2003). In the BDNF-stimulated neurons, the up-regulation of p70S6K phosphorylation pairs with the enhancement of protein synthesis (Takei et al., 2004). p70S6K phosphorylation and protein synthesis can be blocked by inhibiting mTOR, which is a key translational regulator in both neuronal and nonneuronal tissues (Hay and Sonenberg, 2004; Schrott et al., 2004; Bekinschtein et al., 2007b).

Given the important roles of mTOR/p70S6K in activity-dependent protein synthesis and plasticity (Tang et al., 2002; Parsons et al., 2006; Tsokas et al., 2007; Costa-Mattioli et al., 2009), this study sought to investigate the regulatory mechanisms of p70S6K phosphorylation in BDNF-stimulated neurons. We examined how distinct BDNF-activated signaling pathways function in the phosphorylation of p70S6K at distinct sites. We further tested whether the function of Ca^{2+} and its effector molecule, CaM, play a central role in regulation at these sites for the following reasons: 1) Ca^{2+} impinges on the activation of both PI3K and ERK, and 2) Ca^{2+} and Ca^{2+} -binding proteins have been shown to play a role in gene expression via translation and transcription (West et al., 2001; Atkins et al., 2004; Gong et al., 2006; Iizuka et al., 2007), though little is known about whether Ca^{2+} signaling regulates BDNF-mediated phosphorylation of translation factors. By using pharmacological manipulation on Ca^{2+} levels and CaM activity, we found that removal or elevation of intracellular Ca^{2+} ($[Ca^{2+}]_i$) leads to a decrease in BDNF-stimulated phosphorylation of p70S6K at both Thr389 and Thr421/Ser424 sites. We further show that CaM activity gates the phosphorylation of p70S6K at multiple sites and that this regulation is mediated mainly through the PI3K, rather than through CaM-dependent kinase (CaMK) signaling. Finally, we show that the CaM-dependent regulation of BDNF signaling is important for BDNF to drive local protein synthesis in dendrites.

MATERIALS AND METHODS

Cell Culture

Cortices or hippocampi from newborn (P0–P2) Sprague Dawley rats were used for neuronal culture (Zheng et al., 2008). The tissues were first digested with papain (10 units/ml; Worthington, Freehold, NJ) and DNase I (100 units/ml; Roche, Indianapolis, IN) at 37°C for 30 min and washed with Neurobasal A (Invitrogen, Carlsbad, CA). The mechanically separated neurons were plated on 12-well plates coated with PDL (50 µg/ml; Sigma, St. Louis, MO) at a density of 0.5–1 million cells/well. The cultures were maintained in

Neurobasal A with B27 supplement, 0.5 mM glutamine, and 1× penicillin/streptomycin.

Neuronal Treatments and Stimulations

Primary neuronal cultures were stimulated with recombinant human BDNF (at 5 ng/ml, or at the indicated concentrations; Calbiochem, La Jolla, CA) or KCl (50 mM). To block the receptor tyrosine kinase TrkB, neurons were preincubated for 30 min before BDNF application with 0.2 µM K252a (a general Trk receptor inhibitor) or 0.4 µg/ml TrkB-IgG (an extracellular BDNF binding domain coupled to IgG). A 30-min preincubation with 100 µM APV or 10 µM nifedipine was used to block the NMDA receptors (NMDAR) or L-type voltage-gated calcium channels (L-VGCC), respectively. A 30-min preincubation with 10 µM U0126 was used for MEK inhibition. Thirty micromolar LY294002 was used to inhibit PI3K activity. Five micromolar KN93 or ten micromolar KN62 was used to inhibit CaM K I/II/IV activity. U73122 (5, 10, or 25 µM) was used to inhibit PLC activity. Seventy micromolar W13 (or as indicated) was used for CaM inhibition. EGTA (2.5 mM) and BAPTA-AM (33 µM or as indicated) was used to chelate extracellular calcium and intracellular calcium, respectively. XeC (5 µM) was used to block IP3-mediated calcium release from the intracellular store. Thapsigargin (1 µM) or dantrolene (40 µM) was used to deplete intracellular Ca^{2+} stores. All these inhibitors were purchased from EMD Bioscience. They were added to neuronal cultures 30 min before BDNF stimulation. Unless otherwise indicated, samples were harvested 15 min after BDNF treatment.

Polyacrylamide Gel Electrophoresis and Western Blotting

After BDNF or KCl treatment, medium was removed from the culture. Neurons were lysed, harvested in SDS-PAGE loading buffer (10 mM Tris-HCl buffer, pH 6.8, 10% glycerol, 2% sodium dodecylsulfate, 0.01% bromophenol blue, and 5% β-mercaptoethanol), and boiled for 10 min. The collected samples were separated by 10% or 4–20% gradient SDS-PAGE and transferred to nitrocellulose membrane. After blocking with 5% nonfat milk in PBST, the blots were incubated with primary antibodies at 4°C in PBS with 0.1% Triton X-100 and 5% nonfat milk for 12–16 hr. The blots were then washed five times with PBST (PBS with 0.1% Triton X-100) and incubated with HRP-conjugated secondary antibodies at room temperature for 1 hour. The blots were then extensively washed and subjected to ECL detection (Super-Signal West Pico; Pierce, Rockford, IL).

Polyclonal antibodies against phosphorylated p70S6K at Thr389 (1:1,000) and at Thr421/Ser424 (1:1,000) were used to determine the phosphorylation of S6K at the corresponding sites. Polyclonal antibodies against phosphorylated-Akt at Ser473 (1:1,000) and phosphorylated-ERK1/2 at Thr202/Tyr204 (1:1,000) were used to determine the phosphorylation of Akt and ERK1/2, respectively. Polyclonal antibodies against total S6K (1:1,000), total Akt (1:1,000), and total ERK1/2 (1:2,000) were used to determine protein loading. Monoclonal antibody against β-actin (1:10,000) was also used

to determine protein loading. Anti- β -actin was purchased from Sigma. The secondary HRP-conjugated antibodies (1:5,000) were from Pierce. All other antibodies were from Cell Signaling (Beverly, MA).

To avoid signal saturation, the X-ray films were exposed to the blots with several exposure durations. The signals within the linear range were chosen for further density analysis by Scion Image software (Scion Corp., Frederick, MD).

Analysis of Global Protein Synthesis by Metabolic Labeling

The incorporation of ^{35}S -methionine was used to trace new protein synthesis as described elsewhere (Takei et al., 2001), with minor modifications. Neurons were maintained in Neurobasal A/B27 medium. ^{35}S -methionine (10 μCi , with the specificity of 10 mCi/mmol; Perkin Elmer, Norwalk, CT) was added directly to the culture for 15 min. Then, the neurons were stimulated with KCl or BDNF for 30 min. The reaction was stopped by 1 ml ice-cold PBS and subsequently washed with 1 ml PBS. The ^{35}S -labelled neurons were lysed with 500 μl 10 mg/ml casein in 0.5 M NaOH at 37°C for 30 min. The lysates were transferred to 1.5 ml microcentrifuge tubes and mixed with 500 μl 20% ice-cold trichloroacetic acid (TCA). After centrifugation (10,000g) for 10 min, the supernatants and pellets were separated. The radioactivity in the supernatant represents free ^{35}S -methionine, and the radioactivity in the pellet represents the incorporated ^{35}S -methionine. The pellets were then washed with ice-cold 5% TCA and dissolved in 0.1 M NaOH. Protein synthesis was determined by the ratio of radioactivity in the pellet to the total radioactivity (pellet plus supernatant).

Analysis of Local Dendritic Protein Synthesis

To visualize dynamic dendritic protein synthesis, cultured hippocampal neurons were prepared at a density of 230–460 cells/mm² and infected with Sindbis virus expressing a fluorescent translation reporter as previously described (Aakalu et al., 2001; Sutton et al., 2007). The Sindbis viral vector contains an expression cassette including the coding sequence for a myristoylated and destabilized green fluorescent protein (GFP) flanked by the 5' and 3' untranslated regions (UTRs) of α -CaMKII (Aakalu et al., 2001). At 8–9 hr postinfection, the growth medium was replaced by prewarmed (37°C) HEPES-buffered saline (containing, in mM: 119 NaCl, 5 KCl, 2 CaCl₂, 2 MgCl₂, 30 glucose, 10 HEPES, pH 7.4) at least 60 min prior to imaging. Neurons with a pyramidal-like morphology were imaged with an Olympus FV1000 laser scanning confocal microscope using a Plan-Apochromat $\times 40/0.95$ objective. GFP fluorescence was visualized by excitation with the 488 line of an argon ion laser, and emitted light was collected between 500 and 600 nm. After a baseline image had been acquired, neurons were immediately challenged with BDNF (20 ng/ml) or vehicle (mock-treatment) and subsequently imaged at 30-min intervals. In experiments using W13 or anisomycin, neurons were pretreated with these agents for 60 min prior to imaging. All images were acquired in 0.5- μm sections over a range that encompassed the entire dendritic volume. Image analysis was conducted on maximal

intensity z-compressed stacks. The primary dendrite from each cell was linearized using NIH Image J, and fluorescence intensity was measured as a function of both time and distance from the cell soma. The dendritic compartment was divided into proximal and distal domains, defined by distances of less or greater than, respectively, 125 μm from the soma.

Data Analysis

Student's *t*-test was used to analyze data between two groups. ANOVA was used for data between multiple groups. $P < 0.05$ was considered statistically significant.

RESULTS

BDNF Induces Phosphorylation of p70S6K in a TrkB- and mTOR-Dependent Manner

Previous studies have demonstrated that 100 ng/ml BDNF (about 4 nM for BDNF dimer) stimulates the phosphorylation of mTOR and p70S6K (Takei et al., 2004), which promotes the induction of protein synthesis. We explored this regulation in greater detail by assaying, in cultured cortical neurons, p70S6K phosphorylation at multiple sites over a range of BDNF concentrations and incubation times. Consistently with previous observations, we found that 100 ng/ml BDNF (a standard concentration used in many physiological studies) induced robust phosphorylation of p70S6K at both Thr389 and Thr421/Ser424 (Fig. 1A). Interestingly, we found that even a 200-fold lower BDNF concentration (0.5 ng/ml) stimulated mild but significant phosphorylation at both Thr389 and Thr421/Ser424 (Fig. 1A). The full-scale up-regulation of p70S6K phosphorylation was achieved by BDNF treatment at 5 ng/ml (0.2 nM), a concentration ~ 20 -fold lower than that typically used. Consistently, 5 ng/ml BDNF also stimulated significant mTOR phosphorylation (data not shown).

Because BDNF-stimulated protein synthesis may lead to long-lasting functional modification on neurons (e.g., long-term potentiation; Ying et al., 2002; Kanhema et al., 2006), we tested whether low concentrations of BDNF could induce sustained p70S6K phosphorylation. We found that the up-regulation of p70S6K, at both Thr389 and Thr421/Ser424, by 5 ng/ml BDNF persisted for at least 9 hr, with maximal phosphorylation observed between 15 min and 3 hr (Fig. 1B). To test whether BDNF-induced p70S6K phosphorylation depends on the function of Trk receptor tyrosine kinases, we pretreated neurons with the Trk inhibitor K252a before BDNF stimulation. We also pretreated neurons with TrkB-IgG, which is an extracellular BDNF binding domain fused to IgG, to block BDNF binding to TrkB specifically. We found that both K252a and TrkB-IgG significantly blocked BDNF-stimulated p70S6K phosphorylation at both Thr389 and Thr421/Ser424 (Fig. 1C).

We next pretreated neurons with rapamycin, a well-known inhibitor of mTOR, which is an upstream regulator of p70S6K in the PI3K pathway. Interestingly, we observed that BDNF-mediated phosphorylation of

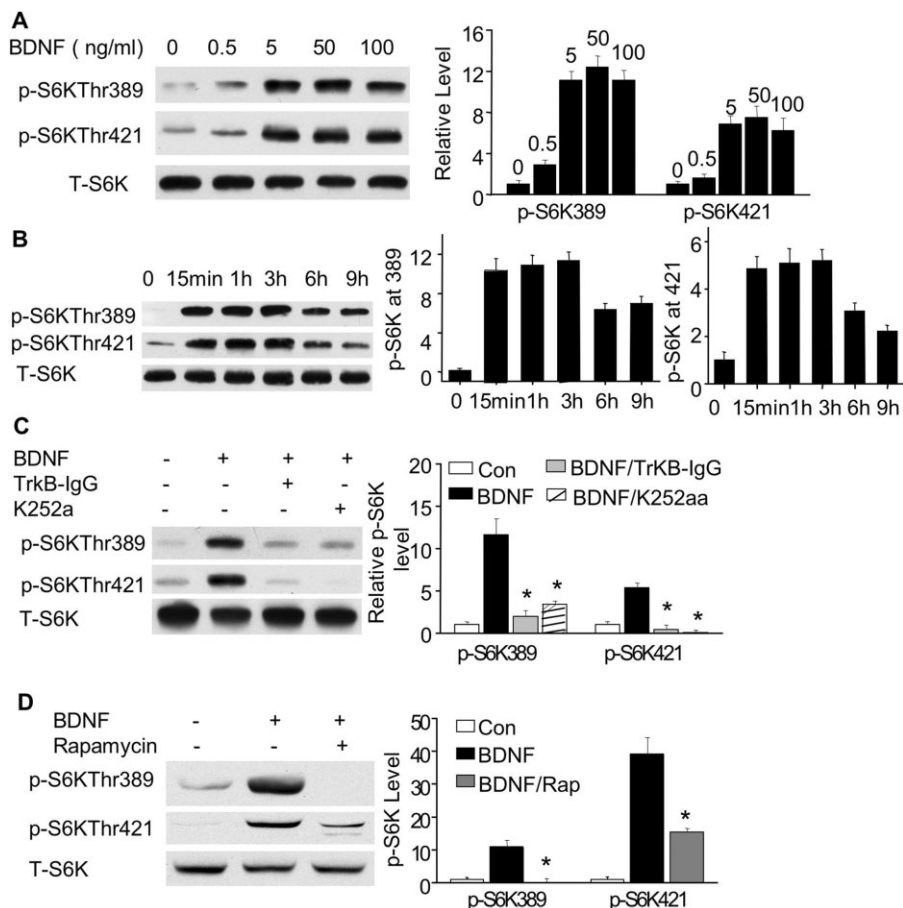


Fig. 1. Activation of p70S6K phosphorylation by BDNF depends on TrkB and is inhibited by rapamycin. **A**: Cortical neurons were treated with (in ng/ml) 0.5, 5, 50, and 100 BDNF for 15 min. The phosphorylation of p70S6K at Thr389 and Thr421/Ser424 was measured by Western blot analysis using phosphospecific antibodies. The level of total p70S6K (T-p70S6K) was used for normalization. **B**: Cell lysates were collected 15 min, 1 hr, 3 hr, 6 hr, and 9 hr after BDNF treatment and analyzed for p70S6K phosphorylation. **C**: To address whether the Trk tyrosine kinase receptors mediate p70S6K phosphorylation, cortical neurons were pretreated with K252a or

TrkB IgG for 30 min before BDNF stimulation (5 ng/ml). The level of p-p70S6K at Thr389 and Thr421/Ser424 was analyzed by Western blots. **D**: Rapamycin blocked BDNF-induced p70S6K phosphorylation. Rapamycin was added to the neuronal culture 30 min before BDNF (5 ng/ml) treatment. The representative images are shown in the left panels, and quantifications are shown in the right panels ($n = 3$ from separate experiments). Relative intensity of p-p70S6K was normalized to T-p70S6K. *Significant inhibition of BDNF-induced p70S6K phosphorylation ($P < 0.05$).

p70S6K at both Thr389 and Thr421/Ser424 was significantly blocked by rapamycin (Fig. 1D). Tsokas and colleagues (2007) previously reported that rapamycin pretreatment prevented phosphorylation at Thr389 induced by high-frequency synaptic stimulation (HFS), but the HFS-induced phosphorylation at Thr421/Ser424 was not affected by rapamycin. Likewise, we found that rapamycin completely prevented BDNF-induced phosphorylation at Thr389, whereas the inhibition of BDNF-induced phosphorylation at Thr421/Ser424 was incomplete. Still, however, the ability of BDNF to drive Thr421/Ser424 phosphorylation was partially dependent on mTOR, suggesting that the regulation of Thr389 and Thr421/Ser424 phosphorylation by mTOR may be tailored to different neuronal stimuli, such as BDNF vs. HFS.

BDNF-Induced p70S6K at Thr389 and Thr421/Ser424 Is Differentially Regulated by PI3K and MAPK

In BDNF-stimulated neurons, the activation of TrkB stimulates both Ras-Raf-MAPK and PI3K-Akt-mTOR signaling (Reichardt, 2006), and each of these pathways plays a role in the activation of neuronal protein synthesis induced by BDNF (Takei et al., 2001). Previous work has shown that phosphorylation at Thr389 and Thr421/Ser424 depends on PI3K and MAPK, respectively. Here, we found that BDNF drove parallel activation of MAPK and PI3K pathways with little cross-talk (Fig. 2A). Inhibiting MEK by U0126 did not block BDNF-stimulated Akt phosphorylation, and inhibiting PI3K by LY294002 did not block BDNF-stimulated ERK phosphorylation (Fig. 2A). Consistently

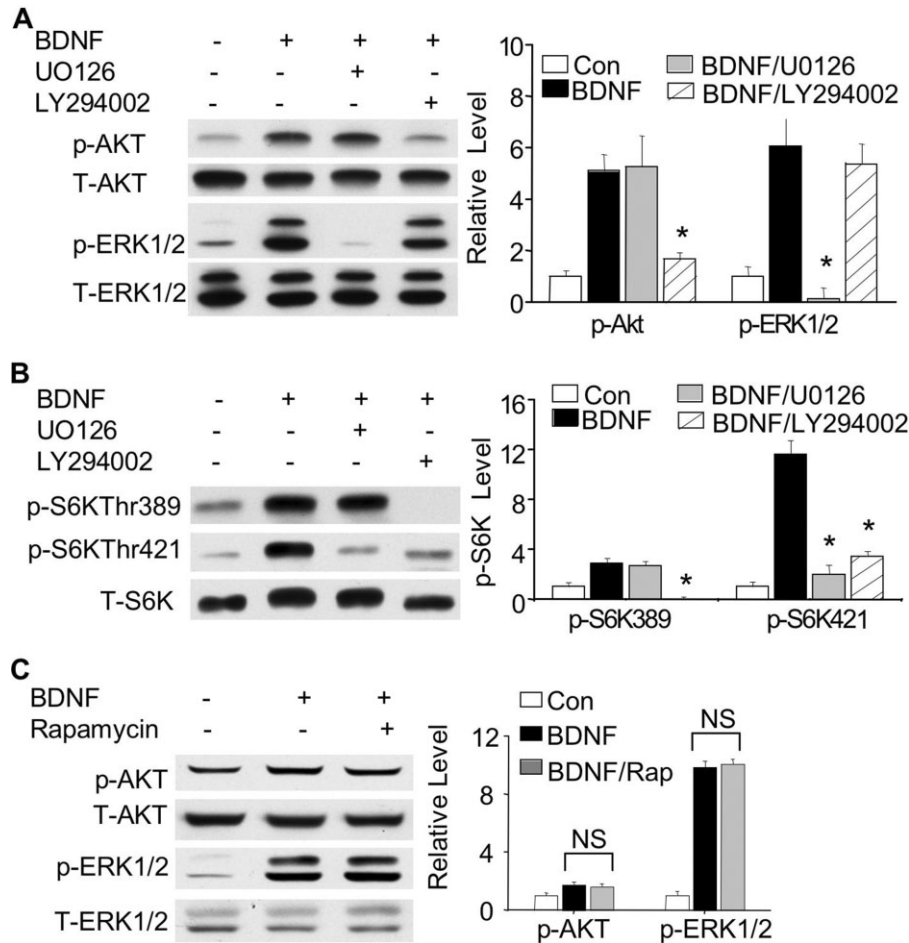


Fig. 2. PI3K and MAPK pathway differentially regulate p70S6K phosphorylation at Thr389 and Thr421/Ser424. **A:** LY294002 inhibits BDNF-stimulated p-Akt but not p-ERK; U0126 inhibits BDNF-stimulated p-ERK but not p-Akt. **B:** Western blots show effects of LY294002 and U0126 on p-p70S6K. **C:** Western blots show effects of rapamycin on p-ERK and p-Akt. Representative images are

shown in the left panels and quantification in the right panels ($n = 3$ from separate experiments). The relative intensity of p-p70S6K, p-Akt, and p-ERK was normalized to T-p70S6K, T-Akt, and T-ERK. * $P < 0.05$ when comparison was made between BDNF-stimulated neurons and inhibitor-treated neurons. NS, not statistically different.

with the previous reports, blocking MEK by U0126 inhibited BDNF-stimulated phosphorylation at Thr421/Ser424 but not Thr389 (Fig. 2B). Surprisingly, blocking PI3K by LY294002 inhibited phosphorylation at both sites (Fig. 2B). These results suggest that, whereas PI3K controls phosphorylation at Thr389 independently of MAPK, signaling through both pathways is necessary for maximal regulation at Thr421/Ser424. Finally, we found that rapamycin did not block BDNF-induced up-regulation of p-ERK or p-Akt (Fig. 2C), despite the fact that rapamycin blocks BDNF-induced phosphorylation at both Thr389 and Thr421/Ser424 (Fig. 1D). These results are consistent with the notion that mTOR acts downstream of PI3K to promote activation of p70S6K in response to BDNF, insofar as coactivation of MAPK and PI3K without mTOR activity is not sufficient to stimulate pS6K phosphorylation.

BDNF-Induced p70S6K Phosphorylation Depends on Intracellular Calcium and CaM

Previous observations have indicated an important role of calcium and CaM in the regulation and activation of intracellular pathways related to neuronal plasticity. BDNF can alter intracellular calcium in a variety of ways, via, for example, enhancement of glutamate release, modulation of NMDA and calcium channels, and stimulating calcium release from internal stores (Levine et al., 1998; Lin et al., 1998; Rose et al., 2004; Reichardt, 2006). We thus investigated whether calcium-dependent processes impinge on BDNF-induced p70S6K activation.

We first tested the role of NMDA receptors (NMDAR) and L-type voltage-gated calcium channels (L-VGCC), both of which are strongly implicated in regulating synaptic plasticity. Blocking NMDAR and L-

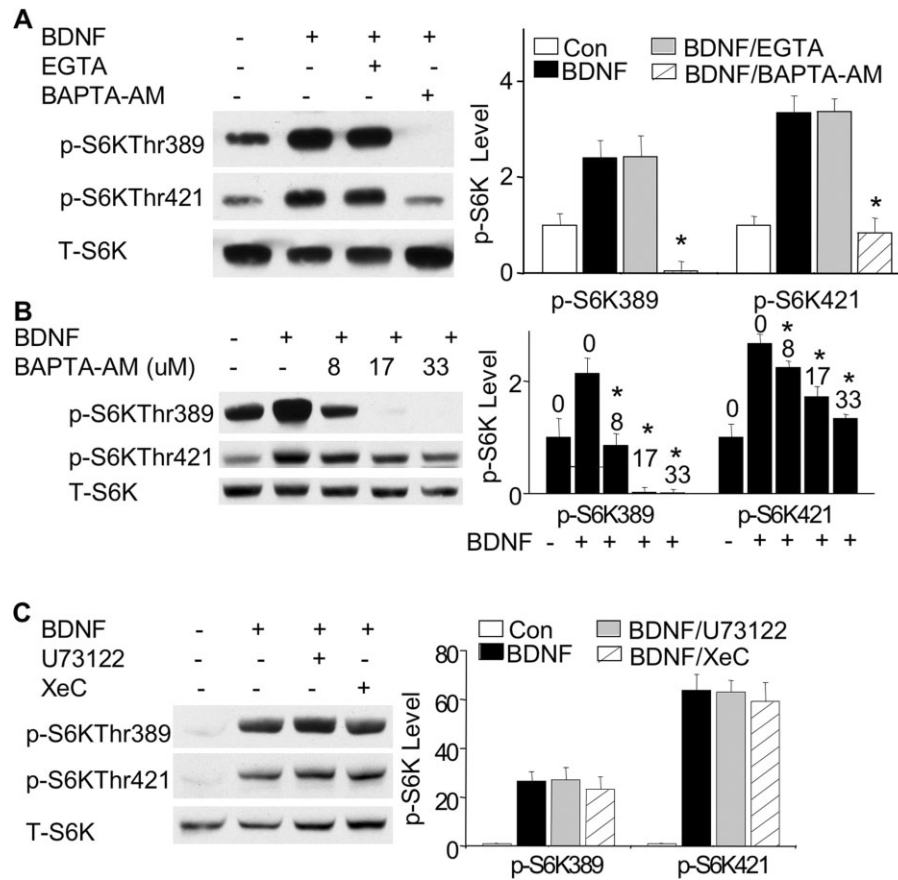


Fig. 3. Intracellular, but not extracellular, calcium is required for BDNF-induced p70S6K phosphorylation. **A**: The BDNF-induced phosphorylation of p70S6K is blocked by BAPTA-AM but not EGTA. Cultured neurons were pretreated with BAPTA-AM (33 μ M) to chelate intracellular calcium or with EGTA (2.5 mM) to chelate extracellular calcium for 30 min before BDNF stimulation. The samples were collected 15 min after BDNF stimulation and analyzed by Western blot. **B**: Neurons were pretreated with BAPTA-AM at different concentrations as indicated (in μ M) for 30 min and then

stimulated by BDNF. Samples were collected 15 min after BDNF stimulation, and p-p70S6K was analyzed by Western blots. **C**: Inhibition of PLC by U73122 or inhibition of Ca^{2+} release from intracellular Ca^{2+} stores by XeC does not block BDNF-induced p-p70S6K. Representative images are shown in the left panels and quantification (normalized to T-p70S6K) in the right panels ($n = 3$ from separate samples). * $P < 0.05$ when comparison was made between BDNF-stimulated neurons and BAPTA-AM-treated neurons.

VGCC by APV and nifedipine, respectively, did not affect BDNF-induced p70S6K phosphorylation at the two sites (data not shown). To inhibit overall calcium influx, we used EGTA (2.5 mM) to chelate extracellular calcium. Although the same concentration of EGTA blocked NMDA-induced ERK phosphorylation (data not shown), there was no effect of EGTA on BDNF-induced phosphorylation of p70S6K at either Thr389 or Thr421/Ser424 (Fig. 3A). In contrast, chelation of intracellular calcium by BAPTA-AM (33 μ M) ablated BDNF-stimulated p70S6K phosphorylation at both Thr389 and Thr421/Ser424 (Fig. 3A). Further analysis determined that BDNF-stimulated p70S6K phosphorylation could be blocked by BAPT-AM at as low as 8 μ M, and the phosphorylation at Thr389 was more sensitive to BAPTA-AM than Thr421/Ser424 (Fig. 3B). Our previous results showed that pretreatment with BAPTA-AM suppressed only BDNF-stimulated PI3K activity but

not ERK (Zheng et al., 2008, 2009). Thus, we propose that intracellular calcium may regulate BDNF-induced p70S6K phosphorylation through PI3K rather than ERK.

Stimulation of TrkB by BDNF can induce IP₃-mediated calcium release from internal stores via activation of the PLC- γ pathway (Finkbeiner et al., 1997; Reichardt, 2006). Therefore, it is possible that PLC- γ signaling is involved in BDNF-induced phosphorylation of p70S6K. To examine this possibility, we pretreated neurons with a potent PLC inhibitor, U73122 (5 μ M), and an IP₃ receptor inhibitor, XeC, to prevent Ca^{2+} release from intracellular Ca^{2+} stores. Surprisingly, neither U73122 nor XeC blocked BDNF-induced p70S6K phosphorylation (Fig. 3C). Moreover, at a higher concentration, U73122 (25 μ M) also failed to suppress p70S6K phosphorylation (data not shown). When neurons were pretreated with thapsigargin (1 μ M) or dan-

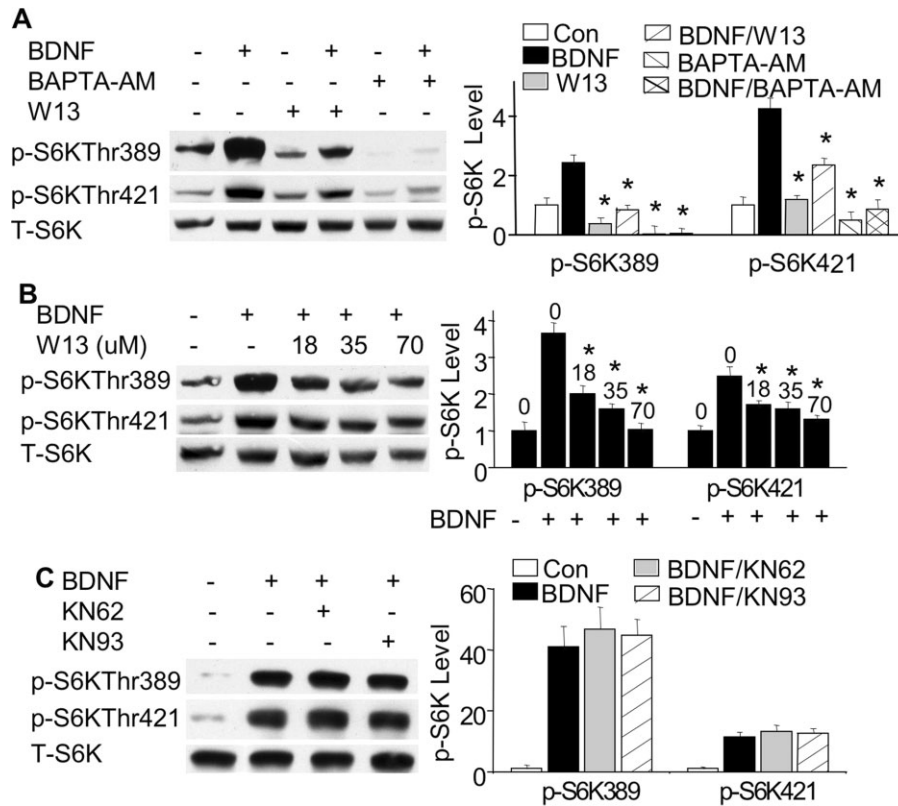


Fig. 4. CaM activity is required for BDNF-induced p70S6K phosphorylation. **A:** Neurons pretreated with CaM inhibitor W13 (70 μ M) or BAPTA-AM (33 μ M) show significant reduction of BDNF-stimulated phosphorylation at both Thr389 and Thr421/Ser424. **B:** Neurons were pretreated with W13 at different concentrations as indicated (in μ M) for 30 min and then stimulated by BDNF. Samples were collected 15 min after BDNF stimulation, and p-p70S6K was

analyzed by Western blots. **C:** Inhibition of CaM kinases I/II/IV by KN62 or KN93 does not inhibit BDNF-stimulated p70S6K phosphorylation. Representative images are shown in the left panels and quantification (normalized to T-p70S6K) in the right panels ($n = 3$ from separate samples). * $P < 0.05$ when comparison was made between BDNF-stimulated neurons and W13- or BAPTA-treated neurons.

tolerene (40 μ M), which deplete intracellular Ca^{2+} stores, no inhibition of BDNF-induced p70S6K phosphorylation was observed (data not shown). These results indicate that PLC- γ -mediated intracellular calcium release is not required for p70S6K activation. Given that BDNF-stimulated extracellular calcium influx (presumably through NMDAR and L-VGCC) is not necessary for p70S6K activation, we conclude that the basal level of intracellular calcium may gate BDNF-induced p70S6K phosphorylation.

Because CaM is a major effector of intracellular calcium and plays a key role in regulating neuronal function (Soderling, 2000), we investigated the effects of CaM inhibition on p70S6K phosphorylation. Neurons were pretreated for 30 min with W13 (70 μ M), a specific CaM inhibitor. As shown in Figure 4A, BDNF-induced phosphorylation of p70S6K at Thr389 was dramatically reduced by W13. A milder, but significant, suppression was observed for the phosphorylation at Thr421/Ser424 (Fig. 4A). In contrast to that at Thr389, the basal level of phosphorylation at Thr421/Ser424 was not affected by W13 in unstimulated neurons (Fig. 4A).

Further analysis showed that phosphorylation at both Thr389 and Thr421/Ser424 could be blocked by W13 at as low as 18 μ M (Fig. 4B).

Previous reports indicate that CaM may regulate p70S6K through CaM-dependent protein kinases (CaMKs; Soderling, 2000; Griffith, 2004). In this study, we also observed that the phosphorylation of α -CaMKII was ablated in W13 (at 30 μ M)-treated neurons (data not shown). To determine more precisely the role of CaMKs in BDNF-induced p70S6K phosphorylation, neurons were pretreated for 30 min with the CaMKI/II/IV inhibitors KN62 (10 μ M) and KN93 (5 μ M). We found that inhibition of CaMKs by either KN62 or KN93 had no effects on BDNF-induced p70S6K phosphorylation (Fig. 4C), although the two inhibitors effectively blocked the phosphorylation of α -CaMKII at Thr286 (data not shown). At higher concentration, KN62 (50 μ M) and KN93 (25 μ M) also failed to suppress BDNF-induced p70S6K phosphorylation (data not shown). These data suggest that the regulatory function of intracellular calcium and CaM on the phosphorylation of p70S6K is not mediated through CaMKI, -II, or -IV.

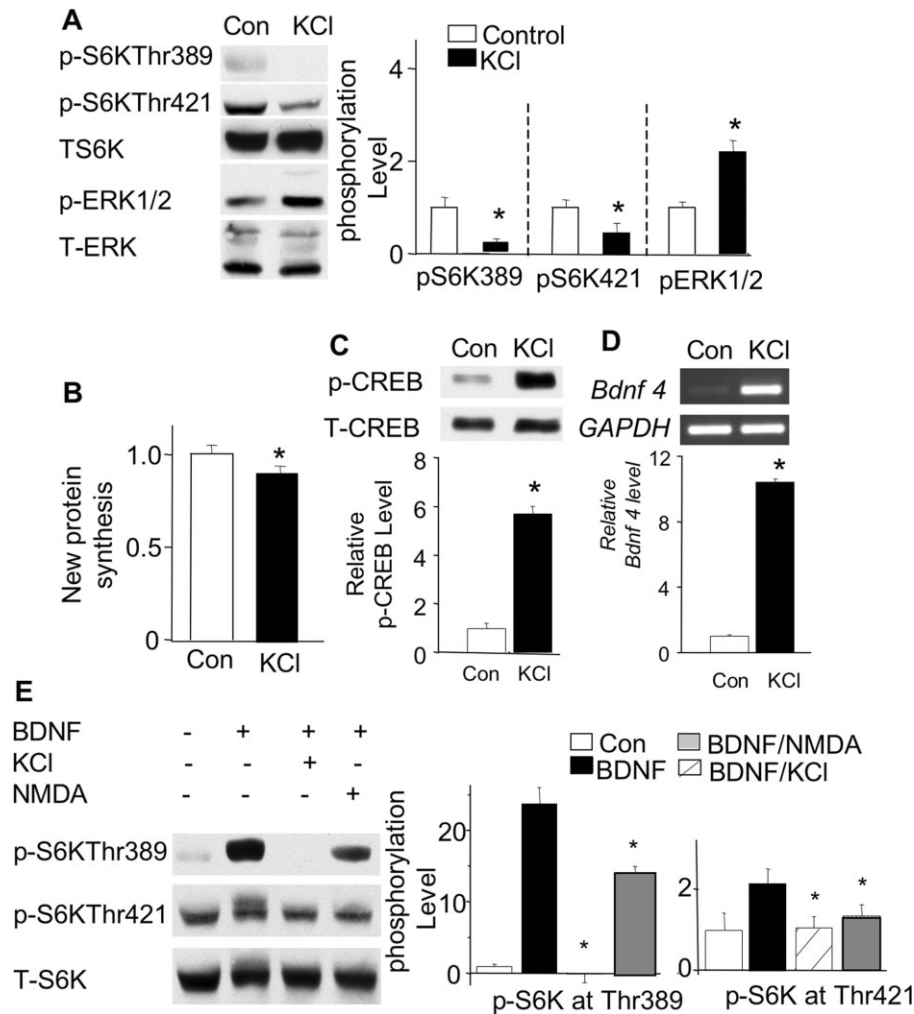


Fig. 5. Increase in intracellular calcium antagonizes the BDNF-stimulated p70S6K phosphorylation. To increase the level of intracellular calcium, neurons were depolarized by KCl. **A**: Membrane depolarization by KCl (50 mM) for 15 min suppresses p70S6K phosphorylation at both Thr389 and Thr421/Ser424 ($n = 3$). **B**: Membrane depolarization causes a reduction in global protein synthesis. New protein synthesis was determined by ^{35}S labeling as described in Materials and Methods ($n = 5$). **C**: Membrane depolarization activates transcription factor CREB ($n = 3$). **D**: Membrane depolariza-

tion activates the transcription of the immediate early gene *Bdnf* ($n = 3$). The relative level of exon 4-containing *Bdnf* mRNA was determined by semiquantitative RT-PCR using gene-specific primers. **E**: Coapplication of KCl or NMDA (50 μM) with BDNF causes a significant reduction of p70S6K phosphorylation at both Thr389 and Thr421/Ser424 ($n = 3$). Neurons were treated with either BDNF alone or with BDNF plus KCl (or NMDA) for 15 min and then analyzed by Western blots. Quantifications are shown in the right (A,E) and lower (C,D) panels. * $P < 0.05$.

The Increases in Intracellular Calcium Level and MAPK Activation Are Not Sufficient To Induce p70S6K Phosphorylation

To obtain further experimental support, we next tested whether elevation in intracellular calcium is sufficient to cause p70S6K phosphorylation. To trigger activity-dependent calcium influx, we depolarized neurons by elevating the extracellular K^+ concentration to 50 mM (with KCl; Dolmetsch et al., 2001). Consistently with other studies, we observed significant up-regulation of p-ERK1/2 (Fig. 5A). However, p70S6K phosphorylation at Thr421/Ser424, the previously identified MAPK site, was down-regulated in KCl-treated neurons

(Fig. 5A). This result implies that the activation of ERK alone is not sufficient to stimulate phosphorylation at Thr421/Ser424. The phosphorylation at Thr389 was also dramatically decreased in KCl-treated neurons (Fig. 5A). Consistently with the decrease in p70S6K phosphorylation, membrane depolarization resulted in a mild, but significant, reduction ($11.4\% \pm 3\%$; $P < 0.05$) in global protein synthesis (Fig. 5B). In contrast, membrane depolarization activated the transcription factor CREB (Fig. 5C) and transcriptional up-regulation of *Bdnf* exon 4 (Fig. 5D).

We next examined how membrane depolarization-induced intracellular calcium elevation affects BDNF-

stimulated p70S6K phosphorylation. We coapplied KCl (50 mM) and BDNF to cultured neurons. We found that the BDNF-induced phosphorylation of p70S6K at Thr389 and Thr421/Ser424 was completely blocked in costimulated neurons (Fig. 5E). Because NMDA also can trigger a sustained $[Ca^{2+}]_i$ elevation by stimulating NMDA receptors (Dolmetsch et al., 2001), we next coapplied NMDA (50 μ M) and BDNF to cultures. NMDA significantly suppressed BDNF-induced p70S6K phosphorylation at Thr389 and Thr421/Ser424 (Fig. 5E). Although these results do not identify the function of specific KCl- and NMDA-responsive molecules, they demonstrate that elevation of intracellular calcium decreases the phosphorylation of p70S6K, further supporting the idea that the basal level of intracellular calcium is required for gating BDNF-induced p70S6K phosphorylation.

CaM Is Required for BDNF-Induced Protein Synthesis

In addition to a stimulatory role in overall neuronal translation (Takei et al., 2001), BDNF is known to enhance local protein synthesis in dendrites (Aakalu et al., 2001; Yin et al., 2002). First, we confirmed that BDNF stimulated global protein translation by metabolic labeling with 35 S-methionine. At 5 ng/ml, BDNF caused a mild but significant increase in overall protein synthesis ($11.7\% \pm 4\%$ increase compared with untreated neurons; $P < 0.05$). A larger degree of overall protein synthesis ($29\% \pm 7\%$ increase; $P < 0.05$) was observed in neurons treated with BDNF at 20 ng/ml. To examine whether the requirement for CaM-dependent regulation of p70S6K phosphorylation by BDNF applies to dendritic synthesis, we examined regulation of a fluorescent translation reporter known to be responsive to BDNF (Aakalu et al., 2001). This construct uses a destabilized, myristoylated GFP that is flanked by the 5' and 3' UTRs of α -CaMKII. Using this same construct, Aakalu et al. (2001) demonstrated that BDNF induced rapid reporter synthesis at specific hot spots along dendrites. Consistently with these earlier observations, we similarly found that BDNF application (20 ng/ml) rapidly stimulated reporter synthesis in dendrites (Fig. 6A–C), with particularly strong increases in reporter expression at specific hot spots (Fig. 6A,B). In contrast, reporter expression in mock-treated control neurons typically remained stable or even decreased over the course of imaging. These differential effects of BDNF were prevented by pretreatment with the protein synthesis inhibitor anisomycin (40 μ M; data not shown). To control for the effects of viral delivery and any potential posttranslational processing that could alter GFP fluorescence, we also examined a control construct encoding GFP that lacked α -CaMKII UTRs. BDNF did not alter GFP expression of this control construct (Fig. 6D–F). Finally, pretreatment with the calmodulin inhibitor W13 (30 μ M) abolished the increase in reporter synthesis induced by BDNF but did not alter reporter expression in control

neurons (Fig. 6G,H). Taken together, these results suggest that local dendritic synthesis induced by BDNF is CaM dependent.

DISCUSSION

In this study, we examined the regulatory mechanisms for BDNF-induced phosphorylation of p70S6K in cultured neurons. As summarized in Figure 7, we identified novel functions of PI3K, which regulates p70S6K phosphorylation at both Thr389 and Thr421/Ser424 through mTOR. Activation of MEK-ERK1/2 alone is not sufficient to support the phosphorylation at Thr421/Ser424, which may also require coactivation of mTOR in BDNF-stimulated neurons. The basal level of intracellular calcium, rather than elevation of the intracellular calcium, is required for the TrkB- and mTOR-dependent phosphorylation at both Thr389 and Thr421/Ser424. Furthermore, CaM activity is a key component for p70S6K phosphorylation and the BDNF-stimulated local protein synthesis in dendrites. Finally, we suggest that basal level of intracellular calcium and CaM may function through PI3K rather than CaM kinases.

The Phosphorylations at Thr389 and Thr421/Ser424 Are Differentially Regulated in BDNF-Stimulated Neurons

Previous studies have demonstrated that BDNF at 100 ng/ml (about 4 nM for BDNF dimmer) stimulates robust p70S6K phosphorylation and protein synthesis (Takei et al., 2004). Here we demonstrate that a subnanomolar concentration of BDNF (5 ng/ml, about 0.2 nM) is sufficient to induce persistent phosphorylation of p70S6K at Thr389 and Thr421/Ser424 as well as global synthesis in cultured neurons. However, 20 ng/ml BDNF does stimulate more protein synthesis than 5 ng/ml, presumably because 5 ng/ml BDNF only partially stimulates other translational components, such as 4EBP, eIF-4E, and eEF2 (Klann and Dever, 2004; Costa-Mattioli et al., 2009).

As described earlier, the phosphorylation of both sites at Thr389 and Thr421/Ser424 accounts for full strength of p70S6K activity. Previous studies with non-neuronal cells have indicated that Thr389 is the target of PI3K and that Thr421/Ser424 is mainly phosphorylated by ERK (Lehman et al., 2003). Because PI3K and ERK are the two major signaling pathways activated by BDNF/TrkB and are involved in many aspects of plasticity, the activity of p70S6K is potentially tightly coupled to neuronal stimulation. It is apparent that the regulatory function of PI3K and ERK on these two sites depends on how neurons are stimulated. For example, when neurons are stimulated by the adenylyl cyclase activator forskolin, inhibiting PI3K blocks only Thr389 and not Thr421/Ser424 phosphorylation (Gobert et al., 2008). Inhibiting MEK suppresses phosphorylation at both Thr389 and Thr421/Ser424 (Gobert et al., 2008). In addition, rapamycin blocks phosphorylation only at Thr389 but not at Thr421/424 (Gobert et al., 2008).

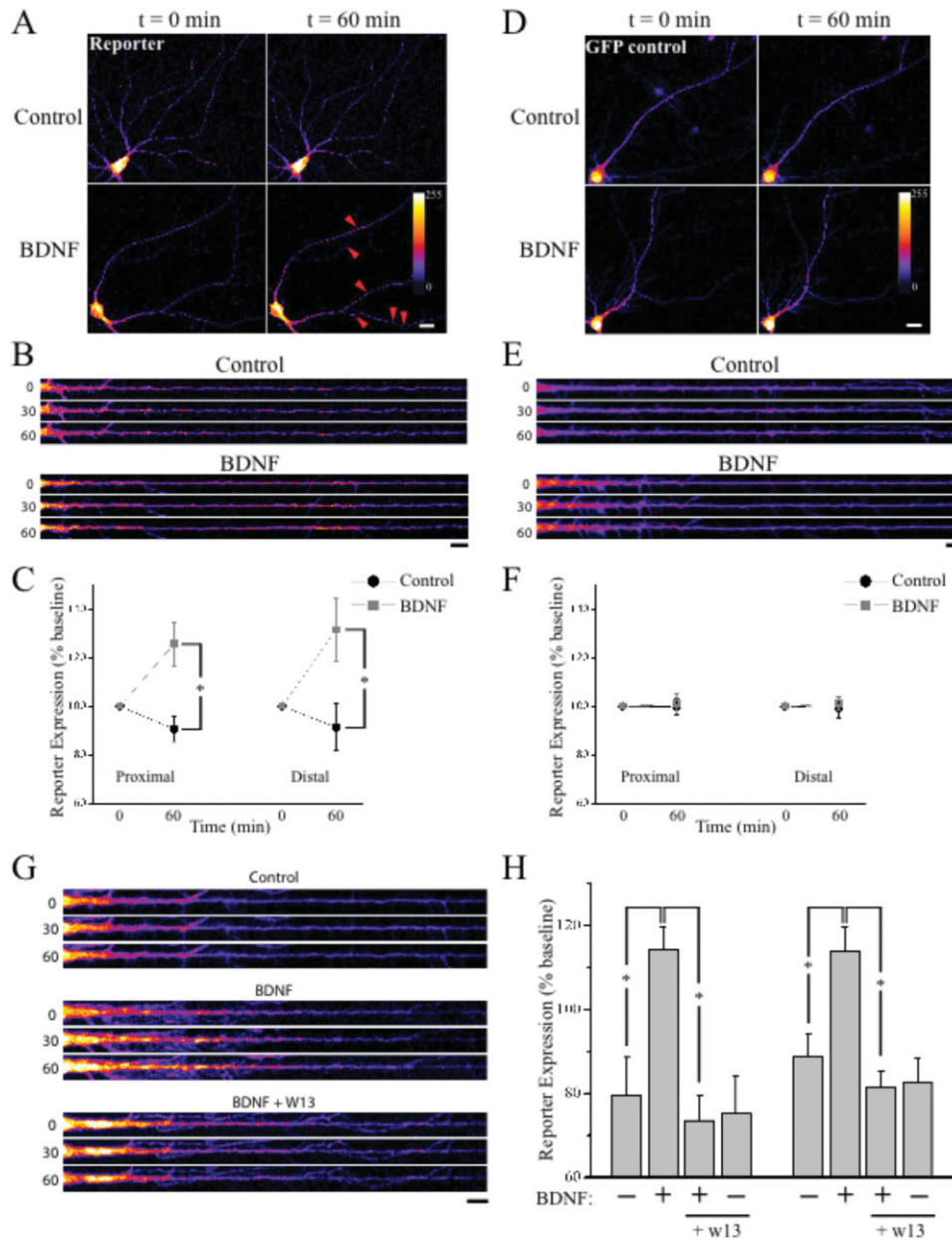


Fig. 6. BDNF-induced translation in dendrites is CaM dependent. Neurons expressing either the protein synthesis reporter (A–C) or control GFP (D–F) were either mock-treated (control) or treated with 20 ng/ml BDNF immediately following acquisition of a baseline image ($t = 0$). Representative full-frame examples (A,D) and time-lapse montages of straightened dendrites (B,E) are shown; fluorescence intensity is indicated by the color lookup table. Arrowheads in A highlight hot spots of reporter synthesis evident after BDNF treatment. C,F: Mean (\pm SEM) GFP expression (relative to baseline) in the proximal ($<125 \mu\text{m}$ from soma) and distal ($>125 \mu\text{m}$ from soma) dendritic compartment for mock-treated ($n = 10$) and BDNF-treated ($n = 8$) neurons. BDNF significantly ($*P < 0.05$ by t -test) enhanced GFP

expression from the reporter, but not from a control construct lacking the α -CaMKII UTRs ($n = 12/\text{group}$). G: Time-lapse montages of straightened dendrites from mock-treated neurons ($n = 12$) or neurons treated with 20 ng/ml BDNF ($n = 12$) either alone or after pretreatment (60 min) with 30 μM CaM inhibitor W13 ($n = 12$). Another group of neurons was treated with W13 alone ($n = 9$); fluorescence intensity is indicated by the color lookup table in A. H: Mean (\pm SEM) reporter expression (relative to baseline) in proximal (left columns) and distal dendrites (right columns) in each of the treatment conditions described for G. The increase in dendritic reporter expression induced by BDNF was blocked by W13. $*P < 0.05$ by Fisher's LSD. Scale bars = 20 μm in A,D; 10 μm in B,E,G.

Another study has reported similar regulation by ERK and rapamycin (Tsokas et al., 2007). In their report, Tsokas and colleagues stimulated the CA1 hippocampal

neurons with high-frequency stimulation (HFS) and observed that rapamycin only blocked phosphorylation only at Thr389 but not at Thr421/Ser424 and that

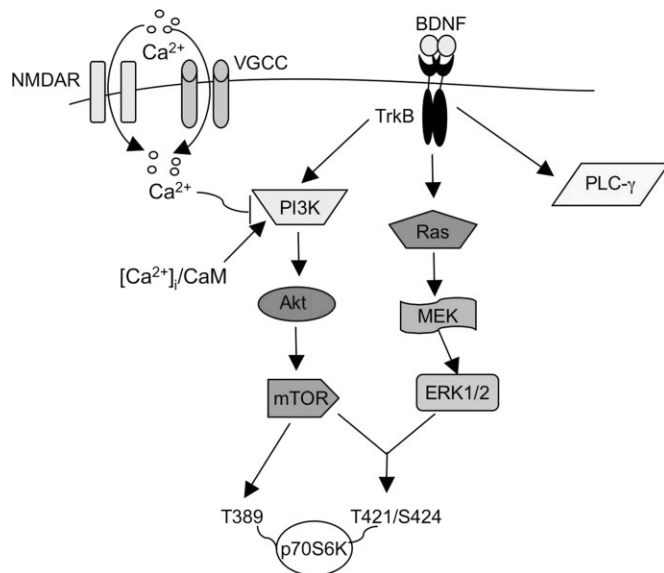


Fig. 7. Regulation of BDNF/TrkB-dependent p70S6K phosphorylation. Stimulation of receptor tyrosine kinase TrkB by BDNF leads to activation of Ras-MEK-ERK1/2, PI3K-Akt-mTOR, and PLC- γ pathways. It appears that intracellular calcium and CaM impinge on PI3K-Akt-mTOR signaling and regulate p70S6K phosphorylation at both Thr389 and Thr421/Ser424. Phosphorylation at Thr421/Ser424, the previously identified MEK-ERK1/2 site, requires coactivation of Ras-MEK-ERK1/2 and mTOR. Extensive calcium influx, presumably through NMDA receptors and VGCC, may antagonize BDNF-stimulated PI3K signaling and in turn suppress p70S6K phosphorylation.

U0126 blocked phosphorylation at both sites. Because MEK inhibition not only blocked p-ERK but also blocked HFS-stimulated p-Akt, the MEK inhibition on Thr389/Ser424 may be indirect and mediated through PI3K (Tsokas et al., 2007). In our study, we show that PI3K and MEK/ERK do not cross-talk in BDNF-stimulated neurons. Inhibition of PI3K did not block p-ERK, and inhibition of MEK did not block p-Akt (Fig. 2A). We found that, in BDNF-stimulated neurons, the phosphorylation of Thr389 was regulated only by PI3K and that the phosphorylation of Thr421/Ser424 was regulated by both PI3K and MEK. Consistently, an inhibitor (rapamycin) of mTOR, which is downstream of PI3K and upstream of S6K, blocked the phosphorylation of both sites in BDNF-stimulated neurons. Moreover, the regulatory role of PI3K and ERK in p70S6K phosphorylation is reflected in protein synthesis. Inhibition of PI3K and MEK blocks activity-induced protein synthesis (Takei et al., 2001; Kelleher et al., 2004). It has been reported that inhibition of MEK impairs BDNF-mediated LTP in the dentate gyrus (Ying et al., 2002). However, the functional relevance of PI3K in BDNF-mediated LTP has not been addressed.

The Function of CaM in p70S6K Phosphorylation and Local Protein Synthesis

Although BDNF stimulates both extracellular Ca^{2+} influx and intracellular Ca^{2+} release, it is not clear how

these dynamic changes in calcium regulate the mTOR-dependent phosphorylation of p70S6K. Our data show that the BDNF-mediated Ca^{2+} elevation may not be required. We further demonstrate that removal of $[Ca^{2+}]_i$ by BAPTA-AM or persistent elevation of $[Ca^{2+}]_i$ by membrane depolarization or activation of NMDAR causes p70S6K dephosphorylation and antagonizes the effects of BDNF. Together, we suggest that the basal-level $[Ca^{2+}]_i$ is necessary and may gate p70S6K phosphorylation. It is important to note that the basal-level $[Ca^{2+}]_i$ is an outcome of calcium homeostasis, which may be handled differently under specific environmental conditions. It was documented that the basal-level $[Ca^{2+}]_i$ increases during in vitro neuronal maturation, possible as a result of significant synaptogenesis (Zhou et al., 2009). Dysregulation of calcium homeostasis and increase in basal $[Ca^{2+}]_i$ are also observed in aged animals (Kirischuk et al., 1992; Thibault et al., 2007). Further investigation is needed to clarify the cross-talk between basal $[Ca^{2+}]_i$ and BDNF in vivo. The changes of intracellular calcium level affect not only p70S6K phosphorylation by BDNF but also other translational factors, such as 4EBP and eIF4E. Previous reports show that sustained increase of $[Ca^{2+}]_i$ by glutamate (Marin et al., 1997) or KCl (Iizuka et al., 2007) leads to inhibition of eukaryotic elongation factor-2 (eEF2) activity and in turn suppresses mRNA translation. It is important to note that membrane depolarization and bath incubation of NMDA trigger massive calcium influx under our culturing conditions. The massive calcium elevation may mimic the situation during brain injury and stroke (Nishizawa, 2001; Sattler and Tymianski, 2001). However, mild elevation of calcium upon physiological activation of L-VGCC or NMDA receptors in vivo (e.g., during learning and exploration) might have different effects on BDNF-induced p70S6K activation and protein synthesis.

Further analysis of the major BDNF-stimulated pathways indicates that intracellular Ca^{2+} may regulate p70S6K phosphorylation through PI3K but not ERK and CaM kinases. First, we have observed that the BDNF-mediated phosphorylation of Akt is similarly regulated by $[Ca^{2+}]_i$ (Zheng et al., 2008). BAPTA-AM suppresses p-Akt but not p-ERK in BDNF stimulated neurons; KCl leads to p-Akt dephosphorylation and ERK phosphorylation; coapplication of KCl with BDNF causes Akt dephosphorylation without affecting BDNF-stimulated p-ERK (Zheng et al., 2008). Second, inhibition of CaM kinases by either KN62 or KN93 has no effects on BDNF-stimulated p70S6K phosphorylation.

Our results reveal that the regulatory function of $[Ca^{2+}]_i$ is mediated, at least partially, through CaM. There is evidence that CaM binds to PI3K in vitro and that W13 disrupts the binding (Fischer et al., 1998; Perez-Garcia et al., 2004). Thus, it is possible that the effects of $[Ca^{2+}]_i$ reduction and CaM inhibition on phosphorylation of p70S6K may be attributed to the reduction of PI3K signaling. Previous studies have also

suggested that the basal level of intracellular calcium and CaM plays an important role in activation of Akt (or PKB) and neuronal survival mediated by neurotrophic factors (Egea et al., 2001; Cheng et al., 2003). Although the function of protein synthesis in neuronal survival and neuronal death has not been extensively investigated, there is evidence that transient focal ischemia leads to massive glutamate release and significant calcium influx into postsynaptic neurons and causes a decrease in PI3K activity and p70S6K phosphorylation (Janelidze et al., 2001). It is conceivable that the apoptotic effects of CaM inhibition suppress BDNF-regulated elevation in protein synthesis, which may be required for survival. Interestingly, double knockout for both S6K1 and S6K2 in mice is perinatally lethal (Pende et al., 2004).

Although we demonstrate correlated responses of p70S6K phosphorylation and protein synthesis to CaM inhibition, the functional role of p70S6K in dendritic synthesis remains unclear. On one hand, partial phosphorylation of S6 and mitogen-stimulated translation of 5' TOP-containing mRNA (as demonstrated by EF1A mRNA) are still present in S6K1/S6K2 double-knockout cells (Pende et al., 2004). On the other hand, memory acquisition or retention for contextual fear conditioning, Morris water maze, and conditioned taste aversion is impaired in either S6K1 or S6K2 knockout mice (Antion et al., 2008b). Further analysis with the cellular plasticity models has demonstrated that the late-phase LTP as well as the translation- and mGluR-dependent LTD are fairly normal in the S6K1 or S6K2 knockout mice (Antion et al., 2008a,b). However, it is worthwhile to note that the basal level of EF1A was elevated in S6K knockout mice, and the activation of group 1 mGluR fails to stimulate EF1A translation further (Antion et al., 2008a). These studies suggest that the function of S6K is, at least, relevant to certain aspects of neuroplasticity. As far as we know, our data are the first to suggest that the CaM regulation of p70S6K phosphorylation may be involved in BDNF-stimulated dendritic protein translation. Future experiments with molecular approaches are needed to address the function of S6K in BDNF-mediated protein synthesis and plasticity.

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