

Cyclin-dependent Kinase 5 (Cdk5)-dependent Phosphorylation of p70 Ribosomal S6 Kinase 1 (S6K) Is Required for Dendritic Spine Morphogenesis*

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Background: The signaling protein S6K undergoes phosphorylation at multiple serine/threonine sites, but the functional significance is unknown.

Results: Cdk5 phosphorylates S6K at Ser-411 in neuron, and loss of this phosphorylation event reduces the density of dendritic spines.

Conclusion: Cdk5-mediated phosphorylation of S6K is crucial for spine morphogenesis in neuron.

Significance: A new signaling pathway in regulating neuronal connectivity is identified.

The maturation and maintenance of dendritic spines depends on neuronal activity and protein synthesis. One potential mechanism involves mammalian target of rapamycin, which promotes protein synthesis through phosphorylation of eIF4E-binding protein and p70 ribosomal S6 kinase 1 (S6K). Upon extracellular stimulation, mammalian target of rapamycin phosphorylates S6K at Thr-389. S6K also undergoes phosphorylation at other sites, including four serine residues in the autoinhibitory domain. Despite extensive biochemical studies, the importance of phosphorylation in the autoinhibitory domain in S6K function remains unresolved, and its role has not been explored in the cellular context. Here we demonstrated that S6K in neuron was phosphorylated at Ser-411 within the autoinhibitory domain by cyclin-dependent kinase 5. Ser-411 phosphorylation was regulated by neuronal activity and brain-derived neurotrophic factor (BDNF). Knockdown of S6K in hippocampal neurons by RNAi led to loss of dendritic spines, an effect that mimics neuronal activity blockade by tetrodotoxin. Notably, coexpression of wild type S6K, but not the phospho-deficient S411A mutant, could rescue the spine defects. These findings reveal the importance of cyclin-dependent kinase 5-mediated phosphorylation of S6K at Ser-411 in spine morphogenesis driven by BDNF and neuronal activity.

Dendritic spines are specialized structures that serve as the postsynaptic sites of excitatory synapses. Spine morphogenesis

depends on neuronal activity and protein synthesis. Blockade of spontaneous neuronal activity by TTX⁴ or inhibition of dendritic protein synthesis through depletion of elongation factor kinase eEF2K leads to the appearance of immature spines (1, 2). Activity-dependent protein synthesis involves activation of the kinase mTOR, which is required for long lasting forms of synaptic plasticity, such as long term potentiation and metabotropic glutamate receptor-dependent long term depression (3), as well as dendritic spine maturation (4). One of the mTOR substrates is S6K. Upon activation, S6K phosphorylates multiple substrates that are involved in protein synthesis, including ribosomal protein S6, eEF2K, and the translation initiation factor eIF4B (5). Compared with mTOR, the function of S6K in synapse function and plasticity is less clear. S6K-deficient mice surprisingly display normal long term potentiation and metabotropic glutamate receptor-dependent long term depression (6, 7) but show impaired memory formation and hypoactive exploratory behavior (7). S6K is hyperactivated in Fragile X mental retardation protein knock-out mice, the mouse model of fragile X syndrome, and genetic removal of S6K is able to correct the phenotypes of these mice (8). Given the abnormal spine morphogenesis in Fragile X mental retardation protein deficient neurons and fragile X syndrome patients, it is conceivable that the precise control of S6K is critical to the development of dendritic spines.

The regulation of S6K appears to be highly complicated. Upon extracellular stimulation, S6K is phosphorylated by mTOR at Thr-389. S6K can also undergo phosphorylation at as many as seven other Ser/Thr sites, including four serine residues in the C-terminal autoinhibitory domain. Phosphorylation of the autoinhibitory domain was originally proposed to open up the S6K conformation, allowing phosphorylation by mTOR and subsequent S6K activation (9). Other studies, however, have challenged this hypothesis, and the importance of

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⁴ The abbreviations used are: TTX, tetrodotoxin; mTOR, mammalian target of rapamycin; S6K, p70 ribosomal S6 kinase 1; Cdk5, cyclin-dependent kinase 5; p-, phospho-; DIV, days *in vitro*; mEPSC, miniature excitatory postsynaptic current; ANOVA, analysis of variance.

Serine Phosphorylation of S6K in Spine Development

autoinhibitory domain phosphorylation remains obscure (5). It is noteworthy that virtually all previous studies on the regulatory mechanisms of S6K were limited to an *in vitro* kinase assay, and the significance of the autoinhibitory domain phosphorylation has not been explored in the cellular context.

In the present study, we examined the regulation and functional role of Ser-411 phosphorylation within the autoinhibitory domain of S6K in neuron. Multiple proline-directed serine/threonine kinases can phosphorylate S6K at Ser-411 *in vitro* (10–12). One of them is Cdk5, which is an emerging key player in regulating spine morphogenesis through phosphorylation of multiple substrates (13–17). Here we report that the phosphorylation of S6K by Cdk5 at Ser-411 is regulated by neuronal activity and the neurotrophin BDNF and that this phosphorylation event is crucial for spine morphogenesis.

Experimental Procedures

Plasmids and Antibodies—Full-length rat S6K cDNA was amplified by PCR from the expression construct (12) and subcloned into pCDNA3 with a FLAG tag at the C terminus. The target sequences for making the short hairpin RNAs (shRNAs) against rat S6K were 5'-ATCCGATCGCCTCGAAGAT-3' (shRNA1) and 5'-GCATCCCTTCATTGTGGAT-3' (shRNA2). The target sequence for making the shRNA against rat Cdk5 was 5'-CCGGGAGATCTGTC-TACTC-3'. The complementary oligonucleotides were annealed, subcloned into pSUPER vector, and expressed in cortical neurons by nucleofection to confirm the knockdown efficiencies.

The RNAi-resistant wild type, S411A and S411D S6K mutants, and constitutively active S6K construct were generated by QuikChange mutagenesis kit (Agilent Technologies). Primers for S6K-shRNA2 RNAi resistance were as follows: forward, 5'-gcggaatattctggaggaagtaaacacccttttatagtggttaattatgctttcagacc-3'; reverse, 5'-ggctgaaaggcataaataaacactataaaagggtgctttacttctccaagaatattccgc-3'. Primers for S6K S411A were as follows: forward, 5'-agaaaagttttctttgaacaaaatccgagcgctcgaagatttat-3'; reverse, 5'-ataaatcttcgaggcgtcggattttggttcaaaagaaaacttttct-3'.

Antibodies against p-S6K-Ser-411 (sc-7983R), Cdk5 (DC-17), and BDNF (N-20) were from Santa Cruz Biotechnology; GFP antibody was from Invitrogen; and antibodies against p-S6K-Thr-389, p-S6-Ser-235/236, p-eEF2-Thr-56, p-eEF2K-Ser-366, S6K, and S6 ribosomal protein were from Cell Signaling Technology. Antibodies against actin, MAP2, and FLAG (M2) were from Sigma.

Cell Cultures and Transfection—Primary neuronal culture was prepared from Sprague-Dawley rat embryos. Tissues were digested with trypsin and DNase I at 37 °C. Cortical neurons (4×10^6 cells) were seeded on a poly-L-lysine-coated 60-mm culture dish; hippocampal neurons (1.5×10^5 cells) were seeded on a coverslip coated with poly-D-lysine. For preparing cortical or hippocampal neurons from Cdk5 knock-out embryos, tissues were dissected from an individual mouse embryo at day 18. Hippocampal neurons at 9 DIV were transfected with different plasmids plus enhanced GFP using calcium phosphate precipitation as described previously (17). For the rescue experiment using constitutively active S6K, hip-

pocampal neurons were transfected at 7 DIV with Cdk5-shRNA and constitutively active S6K in a ratio of 1:2.

Pharmacological Treatment of Neurons—To examine dendritic spines of hippocampal neurons, half of the medium was changed the day before BDNF treatment, and neurons (13 DIV) were treated with BDNF (100 ng/ml) for 24 h. To examine the effect of TTX, transfected hippocampal neurons (16 DIV) were treated with TTX (2 μ M) for 24 h. To examine the phosphorylation of S6K and S6, cortical neurons (14–16 DIV) were starved in Neurobasal medium for 1 h followed by incubation with DMSO or roscovitine (25 μ M) for 1 h before BDNF treatment (100 ng/ml for 10 min). Alternatively, cortical neurons (14 DIV) were treated with roscovitine (25 μ M) or rapamycin (87 pM) for 6 h.

Phosphorylation Assay, Protein Extraction, and Western Blot Analysis—To examine S6K phosphorylation, FLAG-S6K construct (4 μ g) was cotransfected with Cdk5 and p35 plasmids (1 μ g each) into HEK-293T cells by Lipofectamine Plus. After 24 h, cells were lysed by radioimmune precipitation assay buffer plus various protease and phosphatase inhibitors, and lysate was collected and incubated with anti-FLAG beads (Sigma) for 2 h at 4 °C. For *in vitro* phosphorylation by recombinant proteins, the immunoprecipitated FLAG-S6K was incubated with 1 μ g of recombinant Cdk5 and p35 (Invitrogen) for 30 min at 30 °C.

To extract proteins from Cdk5 knock-out brains, tissues were homogenized in PBS supplemented with protease and phosphatase inhibitors. After adding equal volume of $2 \times$ radioimmune precipitation assay buffer, homogenate was incubated at 4 °C for 45 min and centrifuged for $16,000 \times g$ for 10 min. For biochemical studies of cortical neurons, cells were lysed by radioimmune precipitation assay buffer plus various protease and phosphatase inhibitors. Proteins were analyzed by SDS-PAGE followed by Western blotting onto nitrocellulose membranes. Signal intensity of the corresponding bands in Western blots was quantified using Photoshop software. The intensity of phospho-S6K and phospho-S6 was normalized with total S6K and total S6, respectively.

Immunocytochemistry—To examine the subcellular localization of Ser-411 phosphorylation of S6K, hippocampal neurons were fixed with 4% paraformaldehyde and 4% sucrose for 15 min. Neurons were incubated with blocking solution (Dulbecco's PBS plus 0.4% Triton X-100 and 1% BSA) for 1 h and with phospho-Ser-411 S6K antibody (1:50), total S6K antibody (1:200), or MAP2 antibody (1:1000) in blocking solution at 4 °C overnight. After washing three times with washing buffer (0.02% Triton X-100 and 0.25% BSA in Dulbecco's PBS), neurons were incubated with the corresponding Alexa Fluor-conjugated secondary antibodies (diluted in 0.02% Triton X-100 and 1% BSA in PBS) at room temperature for 1 h. To examine dendritic spines of transfected neurons, cells were incubated with anti-GFP antibody (1:2000) diluted in GDB buffer (30 mM phosphate buffer, pH 7.4 containing 0.2% gelatin, 0.5% Triton X-100, and 0.8 M NaCl) at 4 °C overnight. After washing three times with washing buffer (20 mM phosphate buffer and 0.5 M NaCl), neurons were incubated with the corresponding Alexa Fluor-conjugated secondary antibodies (diluted in GDB buffer) at room temperature for 1 h.

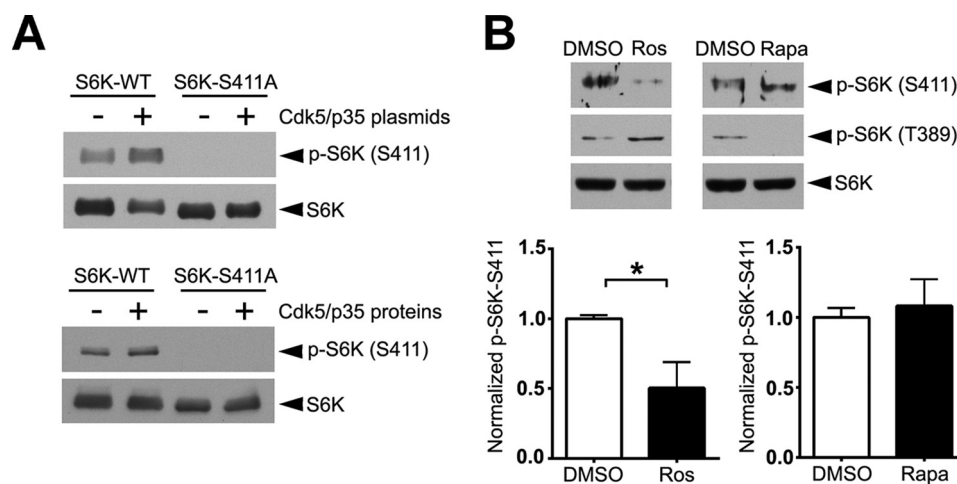


FIGURE 1. Phosphorylation of S6K at Ser-411 by Cdk5. *A*, increased phosphorylation of FLAG-S6K in 293T cells when coexpressed with Cdk5 and p35 plasmids (*upper panel*). The phosphorylation signal was eliminated when phospho-deficient S6K S411A was expressed, indicating that the antibody recognized phosphorylation of the specific site. Incubation of immunoprecipitated S6K with recombinant Cdk5 and p35 proteins also increased S6K phosphorylation at Ser-411 (*lower panel*). *B*, decreased S6K Ser-411 phosphorylation after pharmacological inhibition of Cdk5. Cortical neurons were incubated with roscovitine (*Ros*; 25 μ M) or rapamycin (*Rapa*; 87 μ M) for 6 h. Ser-411 phosphorylation was specifically reduced by roscovitine treatment (three independent experiments were performed; *, $p < 0.05$; Student's *t* test; error bars represent S.E.).

Image Acquisition and Quantitation—Images were acquired by a Nikon A1 or Leica SP8 confocal microscope with 40 \times , 60 \times (Nikon), or 63 \times (Leica) oil immersion objectives. Eight to 12 serial individual optical sections were collected (z interval of 0.4 μ m). Dendritic spines were quantified by Metamorph software: three isolated dendritic segments (60–70 μ m long) that were about 20 μ m away from the cell body were analyzed for each neuron, and the head and neck widths of individual dendritic protrusion were measured. The protrusion was defined as a mature dendritic spine if the head width to neck width ratio was greater than 1.5. The quantification of spine density described in Figs. 6 and 7 was performed in blinded fashion.

Electrophysiology—Whole cell recordings were performed on dissociated hippocampal neurons using a MultiClamp 700A amplifier (Axon Instruments, Foster City, CA) as described in our previous study (18). The pipettes used typically had a resistance of 3–5 megaohms when filled with an internal solution consisting of 130 mM potassium gluconate, 10 mM KCl, 10 mM HEPES, 1 mM EGTA, 2 mM MgCl₂, 2 mM Na₂ATP, and 0.4 mM Tris GTP, and pH was adjusted to 7.3. The cells were continuously superfused with an external solution of the following composition: 125 mM NaCl, 4.0 mM KCl, 1.2 mM MgSO₄, 2.5 mM CaCl₂, 1.2 mM KH₂PO₄, 11 mM glucose, and 26 mM NaHCO₃ at a flow rate of 1.5–2 ml/min. The external solution was bubbled with carbogen and maintained at 34 \pm 1 $^{\circ}$ C. Tetrodotoxin (1 μ M) and bicuculline (10 μ M) were included to block action potentials and GABA transmission, respectively. Once a whole cell recording was achieved, the cell was held at -70 mV to record the miniature excitatory postsynaptic currents (mEPSCs), filtered at 3 kHz, for 5–15 min. The mEPSCs were analyzed by the commercial software MiniAnalysis (Synapse-soft, Decatur, GA).

Statistical Analysis—Statistical significance of experiments involving two experimental conditions was assessed by Student's *t* test, whereas that of experiments involving three or more experimental conditions was assessed by one-way

ANOVA followed by Tukey's or Bonferroni's post hoc test. All experiments were performed at least three times except those specifically indicated.

Results

Cdk5 Is Required for S6K Phosphorylation at Ser-411 in Neuron—The BDNF-induced mTOR pathway has been implicated in spine development and maturation (19). Given that Cdk5 activity is increased by BDNF (20), we asked whether Cdk5 might regulate substrates in the mTOR pathway. Toward this end, we focused on S6K, which is one of the major signaling proteins downstream of mTOR. S6K can be phosphorylated *in vitro* by Cdk5 at Ser-411 within the C-terminal autoinhibitory loop (12). Using an antibody that specifically recognized S6K phosphorylation at Ser-411, as indicated by the complete elimination of signal when Ser-411 was substituted by Ala (S411A), we confirmed that coexpression of Cdk5 and p35 in 293T cells increased phosphorylation of S6K at Ser-411 (Fig. 1*A*, *upper panel*). Moreover, incubation of S6K with recombinant Cdk5 and p35 proteins also increased Ser-411 phosphorylation of S6K (Fig. 1*A*, *lower panel*).

Kinases other than Cdk5, including Cdc2 and MAPK, can induce Ser-411 phosphorylation of S6K (10, 11). mTOR also phosphorylates Ser/Thr sites (Ser-424/Thr-421) in the autoinhibitory domain of S6K *in vitro* (21). It is therefore important to determine whether Cdk5 is the major kinase that phosphorylates S6K at Ser-411 in neuron. Incubation of cortical neurons with the Cdk5 inhibitor roscovitine significantly reduced Ser-411 phosphorylation. In contrast, the mTOR inhibitor rapamycin caused complete elimination of Thr-389 phosphorylation without affecting Ser-411 phosphorylation (Fig. 1*B*). To confirm the importance of Cdk5 in phosphorylating Ser-411 of S6K in the brain, Western blotting with antibody against phospho-Ser-411 was performed on brain homogenate derived from wild type or Cdk5-null mice. Ser-411 phosphorylation of S6K was drastically reduced in the absence of Cdk5. The loss of Ser-411 phosphorylation of S6K was accompanied with reduced phos-

Serine Phosphorylation of S6K in Spine Development

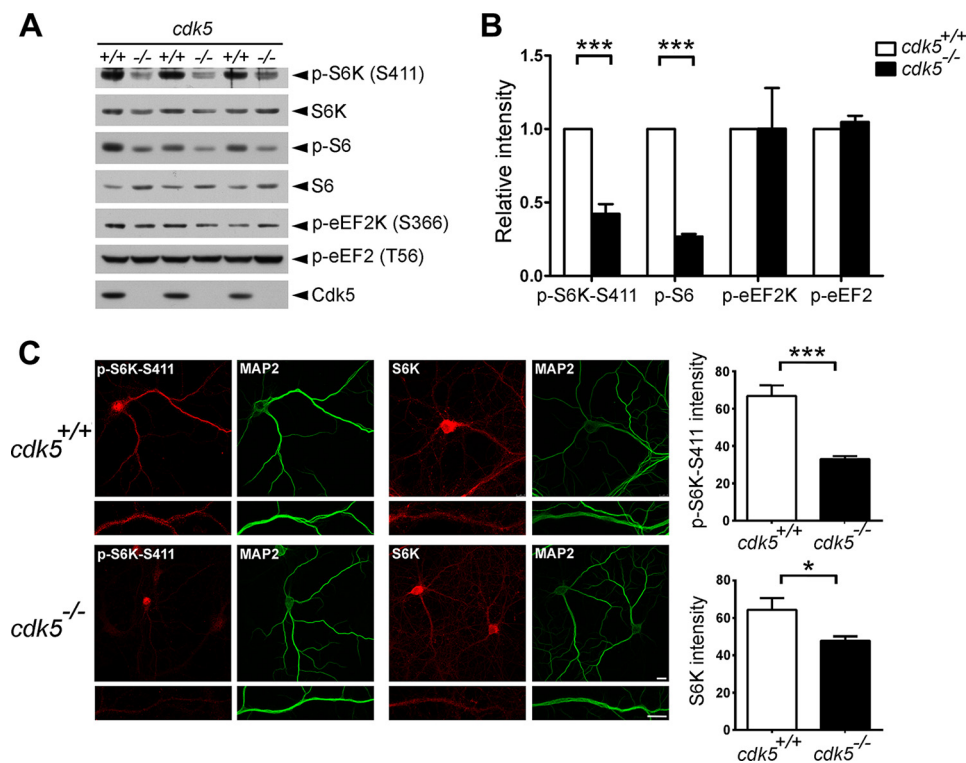


FIGURE 2. Reduced Ser-411 phosphorylation of S6K in neurons lacking Cdk5. *A*, Ser-411 phosphorylation of S6K was reduced in *Cdk5*^{-/-} brains at embryonic day 18 as compared with *Cdk5*^{+/+} brains. Phosphorylation of S6 ribosomal protein, but not eEF2K and eEF2, was also decreased. *B*, quantification of the Western blots. The intensity of phospho-S6K and phospho-S6 was normalized with total S6K and total S6, respectively. Data were pooled from five mice for each genotype (pooled from three mice for eEF2K phosphorylation; ***, $p < 0.001$; one-way ANOVA, Bonferroni's multiple comparison test; error bars represent S.E.). *C*, S6K phosphorylation at Ser-411 (left panels, red) and total S6K (right panels, red) was detected in the dendrites of hippocampal neurons. MAP2 staining (green) located the cell bodies and dendrites. Greater reduction of phospho-S6K than total S6K immunoreactivity was observed in *Cdk5*^{-/-} neurons as compared with *Cdk5*^{+/+} neurons ($n = 13$ –23 dendrites from seven to eight neurons for each genotype; ***, $p < 0.001$; *, $p < 0.05$; Student's *t* test; error bars represent S.E.). Scale bars, 10 μm .

phorylation of S6 ribosomal protein (Fig. 2, *A* and *B*). Interestingly, another S6K substrate, eEF2K, was unaffected in *Cdk5* knock-out brain as phosphorylation of eEF2K and its downstream target elongation factor eEF2 was similar between wild type and *Cdk5* knock-out brains (Fig. 2, *A* and *B*). To verify the role of *Cdk5* in mediating Ser-411 phosphorylation of S6K in neurons rather than glial cells, primary hippocampal neurons were cultured from wild type and *Cdk5* knock-out mice. Phospho-Ser-411 immunoreactivity was detected not only in the neuronal cell soma but also in dendrites, and the dendritic phospho-S6K immunoreactivity was significantly reduced in *Cdk5*^{-/-} neurons (Fig. 2*C*). These findings together indicate that *Cdk5* is the major kinase that phosphorylates S6K at Ser-411 in neuronal dendrites and the brain.

If Ser-411 phosphorylation of S6K by *Cdk5* is an important signaling event in neurons, we reasoned that a constitutively active S6K should, at least in part, rescue some of the phenotypes in neurons lacking *Cdk5*. Toward this end, we made a constitutively active S6K construct by deleting the C-terminal inhibitory domain and substituting the Thr-389 with the phosphomimetic Glu as indicated by previous study (22). Consistent with previous studies reporting a role of *Cdk5* in dendrite development, we found that knockdown of *Cdk5* in hippocampal neurons led to impaired dendritic growth. Notably, coexpression of the constitutively active but not wild type S6K could partially rescue the dendritic defects in *Cdk5*-depleted neurons

(Fig. 3). S6K therefore acts downstream of *Cdk5* in neurons to control dendrite development.

Regulation of S6K Ser-411 Phosphorylation in Neuron by BDNF and Neuronal Activity—Most studies that examine the induction of S6K activity focus on Thr-389, whereas the regulation of S6K phosphorylation at other sites is not clear. We therefore examined how the Ser-411 phosphorylation of S6K is regulated in neuron. One major extracellular stimulus that triggers the mTOR signaling pathway and is relevant to the regulation of synaptic function is the neurotrophin BDNF. Consistent with a previous study (23), BDNF stimulation led to increased Thr-389 phosphorylation of S6K and phosphorylation of S6 ribosomal protein. BDNF also induced phosphorylation of S6K at Ser-411 (Fig. 4*A*). Inhibition of *Cdk5* by roscovitine significantly attenuated the induction of Ser-411, but not Thr-389, phosphorylation of S6K (Fig. 4, *A* and *B*). Despite the normal induction of Thr-389 phosphorylation, the BDNF-induced S6 ribosomal protein phosphorylation was significantly reduced by roscovitine (Fig. 4, *A* and *B*). These findings suggest that *Cdk5*-mediated phosphorylation at Ser-411 is crucial for S6K activation by BDNF; however, the induced Ser-411 phosphorylation does not prime the subsequent induction of Thr-389 phosphorylation.

The synthesis of BDNF is regulated by neuronal activity (2). Given that Ser-411 phosphorylation was induced by BDNF, we asked whether the S6K phosphorylation also depends on neu-

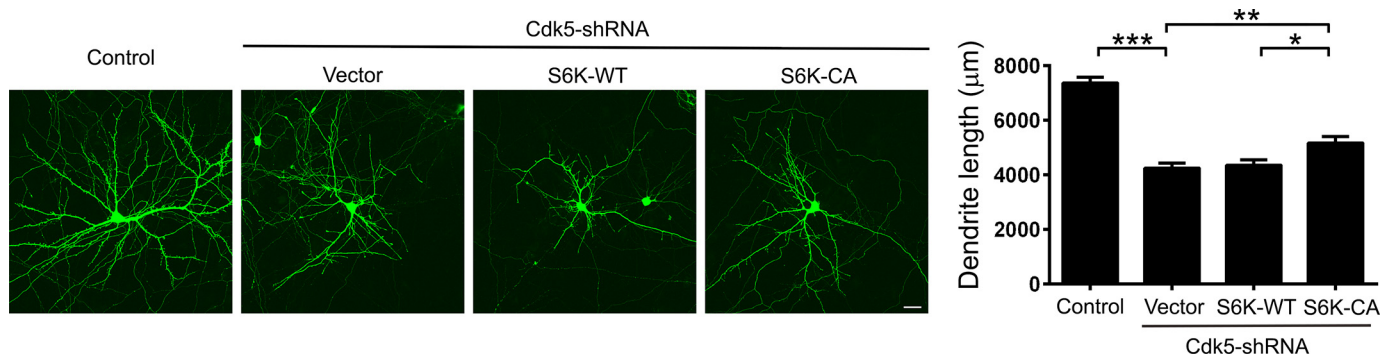


FIGURE 3. Expression of constitutively active S6K rescues the defects of dendrite morphology in Cdk5-depleted neurons. Hippocampal neurons (7 DIV) were cotransfected with GFP and control vector or shRNA targeting Cdk5 in the absence or presence of wild type (WT) or constitutively active (CA) S6K. Quantification revealed that the dendrite length of transfected neurons was significantly reduced by knocking down Cdk5, and the impaired dendritic growth was significantly reversed by coexpressing constitutively active S6K but not WT S6K (results were pooled from three independent experiments; 43–45 neurons were quantified for each condition; ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$; one-way ANOVA, Tukey's multiple comparison test; error bars represent S.E.). Scale bar, 25 μm .

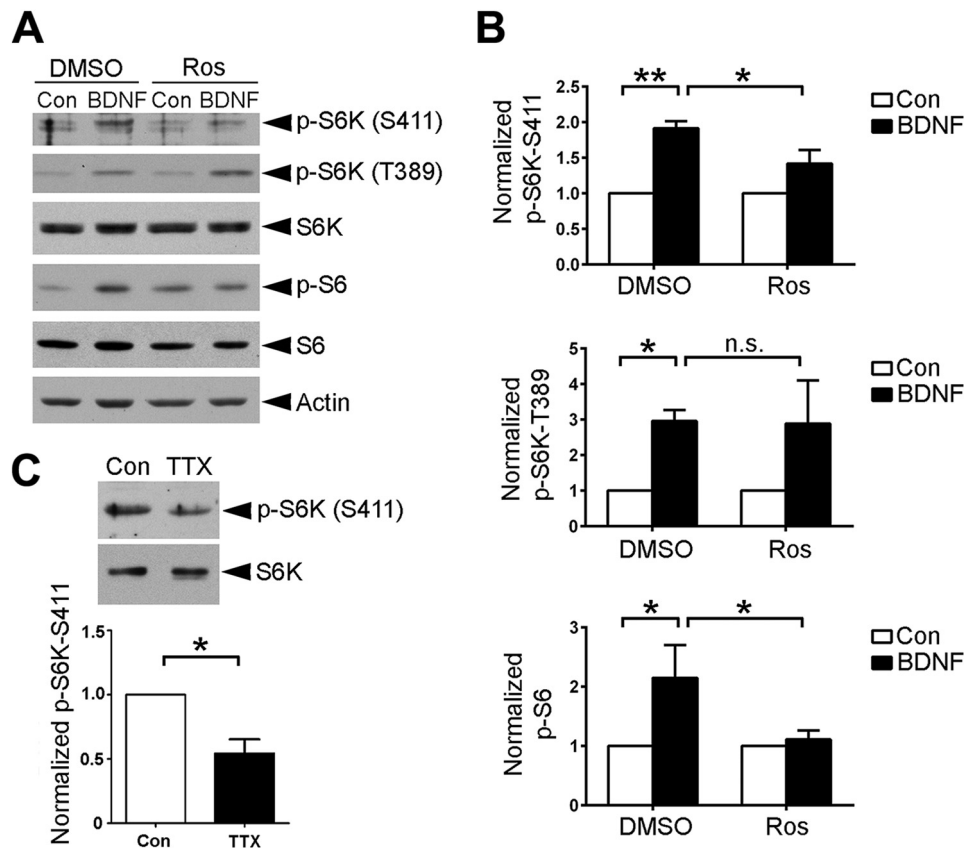


FIGURE 4. Regulation of S6K Ser-411 phosphorylation by BDNF and neuronal activity. *A*, S6K phosphorylation at Ser-411 was induced by BDNF in cortical neurons, and the induction of S6K Ser-411 phosphorylation and S6 ribosomal protein phosphorylation was attenuated by roscovitine (*Ros*). Roscovitine did not affect the induced S6K phosphorylation at Thr-389. *B*, quantification of the Western blots. The intensity of phospho-S6K and phospho-S6 was normalized with total S6K and total S6, respectively. Three independent experiments were performed (*, $p < 0.05$; **, $p < 0.01$; one-way ANOVA, Tukey's or Bonferroni's multiple comparison tests; error bars represent S.E.). *C*, treatment of cortical neurons (14–17 DIV) with TTX (2 μM for 24 h) significantly reduced Ser-411 phosphorylation of S6K (three independent experiments were performed; *, $p < 0.05$; n.s., not significant; Student's *t* test; error bars represent S.E.). Con, control.

ronal activity. Indeed, blockade of spontaneous neuronal activity by TTX significantly reduced S6K phosphorylation at Ser-411 (Fig. 4C) as well as S6 phosphorylation (data not shown). This raises the possibility that the Ser-411 phosphorylation of S6K might be involved in mediating neuronal responses to synaptic activity.

S6K Regulates Dendritic Spine Morphogenesis in Response to BDNF and Neuronal Activity—Either BDNF or spontaneous neuronal activity promotes spine morphogenesis (1, 2, 17, 19).

The regulation of S6K Ser-411 phosphorylation by BDNF or neuronal activity therefore prompted us to investigate whether S6K regulates dendritic spine density and whether it depends on Ser-411 phosphorylation. Two different shRNAs were used to knock down S6K expression (Fig. 5A). Compared with control neurons transfected with vector, the depletion of S6K by either of the two shRNAs significantly reduced the dendritic spine density (Fig. 5, B and C). Interestingly, despite leading to a significant loss of dendritic spines, S6K knockdown did not

Serine Phosphorylation of S6K in Spine Development

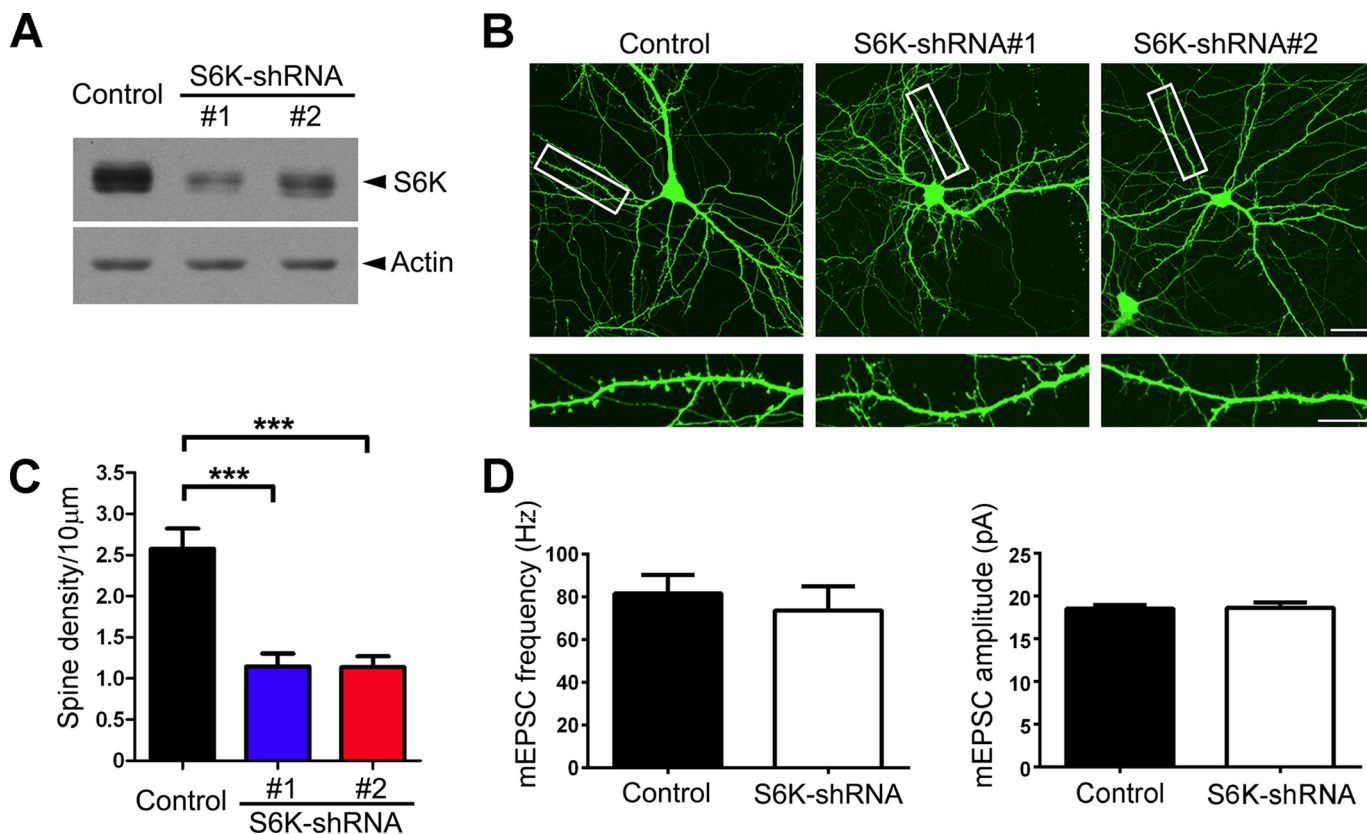


FIGURE 5. Depletion of S6K leads to dendritic spine loss, but basal synaptic transmission is normal. *A*, knockdown efficiency of two different shRNAs for S6K as revealed by a Western blot of lysate from primary cortical neurons collected 5 days after transfection. *B*, representative images of dissociated hippocampal neurons (16 DIV) transfected with GFP and pSUPER vector (control) or either of the two shRNAs are shown. *C*, quantification revealed reduction of dendritic spine density after S6K knockdown (15 dendrites from five neurons were quantified for each condition; ***, $p < 0.001$; one-way ANOVA, Tukey's multiple comparison test; error bars represent S.E.). Scale bars, 20 (upper) or 10 μm (lower). *D*, dissociated hippocampal neurons (9 DIV) were transfected with GFP and pSUPER vector (control) or S6K-shRNA2, and mEPSC was measured at 16 DIV. No significant difference in mEPSC frequency or amplitude was observed between the two conditions (mean \pm S.E.; results were pooled from three independent experiments; $n = 34$ for control and $n = 27$ for S6K-shRNA; $p > 0.05$; Student's *t* test; error bars represent S.E.).

affect synaptic transmission as indicated by the comparable frequency and amplitude of mEPSC (Fig. 5D). To rule out the possibility of off-target effects of the shRNA on spine morphogenesis, rescue experiments were performed in which neurons were cotransfected with shRNA plus the RNAi-resistant S6K. The spine defects upon depletion of S6K were rescued by the coexpression of S6K (see Fig. 7, *B* and *C*). These findings therefore indicate that S6K is essential for dendritic spine formation and/or stability.

Next, we investigated whether S6K mediates the effect of BDNF on spine formation. Consistent with previous studies (17, 24), treatment of dissociated hippocampal neurons with BDNF significantly increased spine density in neurons transfected with scrambled control RNA. Conversely, introduction of S6K-shRNA completely abolished the promotive effect of BDNF (Fig. 6, *A* and *B*). These findings together support the notion that activation of S6K is required for BDNF-induced spine morphogenesis. To address whether S6K is also required for spine maintenance in response to neuronal activity, we compared the effect of TTX on spine density in control or S6K-depleted neurons. Activity blockade by TTX significantly reduced the spine density in neurons transfected with the scrambled control RNA. In contrast, knockdown of S6K by shRNA induced spine loss and occluded the effect of TTX (Fig.

6, *C* and *D*). This suggests that the spine maintenance by spontaneous activity involves activation of S6K.

S6K Function in Spine Morphogenesis Depends on Cdk5-mediated Ser-411 Phosphorylation—Finally, we addressed whether Cdk5-mediated phosphorylation of S6K at Ser-411 is required for the function of S6K in promoting spine morphogenesis. We created the RNAi-resistant, phospho-deficient S411A mutant, which showed an expression level similar to that of wild type S6K (Fig. 7, *A* and *B*). The wild type or S411A S6K mutant was cotransfected with the S6K-shRNA into hippocampal neurons, and any rescue effect on spine morphogenesis was examined. The coexpression of wild type S6K, but not the S411A mutant, significantly rescued the spine loss triggered by the S6K-shRNA (Fig. 7C). In contrast, coexpressing the phosphomimetic S411D mutant could rescue the spine loss to an extent similar to that of the wild type (Fig. 7D). Taken together, our findings collectively indicate that phosphorylation of S6K at Ser-411 within the autoinhibitory loop is a crucial signaling event for dendritic spine morphogenesis in neuron.

Discussion

Dysregulation of mTOR and S6K is implicated in neurodevelopmental disorders, such as autism (8, 25), and absence of S6K in rodents is associated with abnormal behavior (7). None-

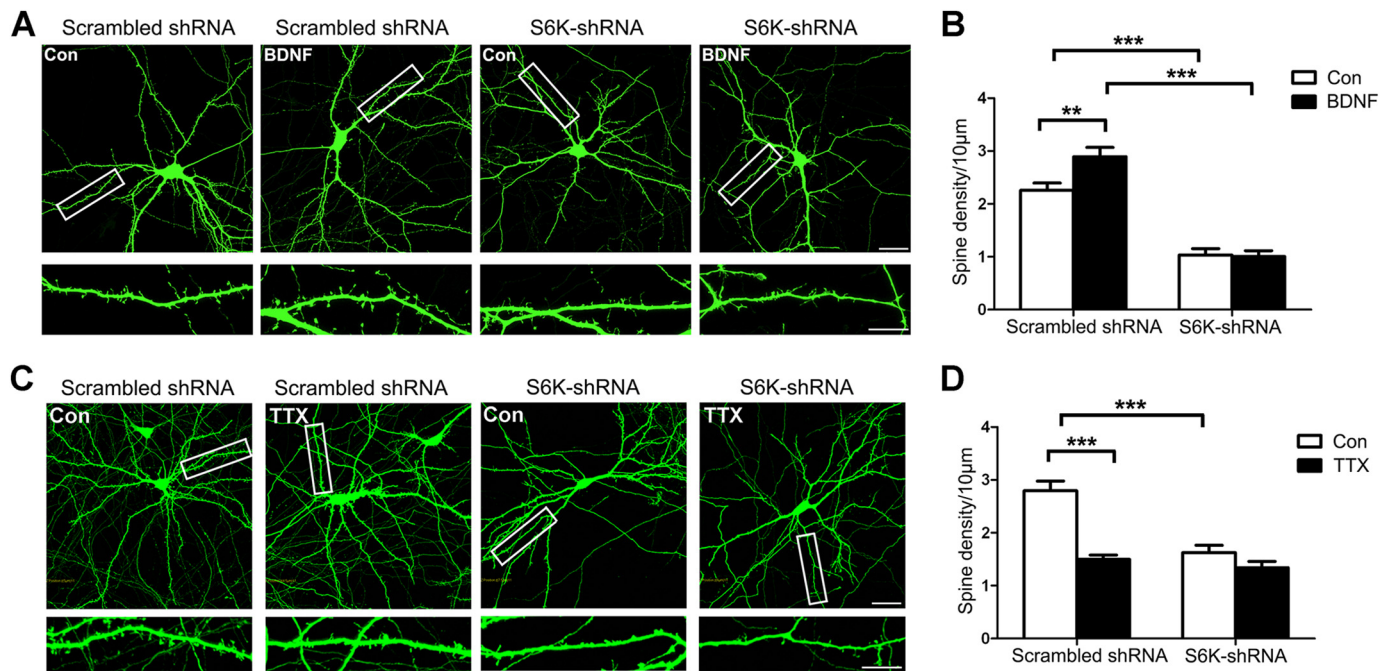


FIGURE 6. S6K is required for spine morphogenesis driven by BDNF or neuronal activity. *A*, knockdown of S6K blocked the BDNF-induced increase in spine density. Dissociated hippocampal neurons (13 DIV) were treated with BDNF (100 ng/ml) or vehicle control (Con) for 24 h, and representative images are shown. *B*, quantification of spine density (results were pooled from two independent experiments; 54 dendrites from 18 neurons were analyzed; **, $p < 0.01$; ***, $p < 0.001$; one-way ANOVA, Tukey's multiple comparison test; error bars represent S.E.). *C*, knockdown of S6K attenuated the effect of TTX on spine loss. Dissociated hippocampal neurons (16 DIV) were treated with TTX or vehicle control for 24 h, and representative images are shown. *D*, quantification of spine density (results were pooled from two independent experiments; 48 dendrites from 16 neurons were analyzed; ***, $p < 0.001$; one-way ANOVA, Tukey's multiple comparison test; error bars represent S.E.). Scale bars in *A* and *C*, 20 (upper) or 10 μ m (lower).

theless, the regulation and cellular function of S6K in neuron remain unclear. Our study showed that S6K is crucial for activity-dependent spine development. We further found that Ser-411 of S6K is phosphorylated by Cdk5 in response to neuronal activity or BDNF, and this phosphorylation event is required for dendritic spine morphogenesis. Our study therefore suggests that, in addition to the well known mTOR pathway that phosphorylates S6K at Thr-389, a parallel pathway that involves Ser-411 phosphorylation by Cdk5 is also required for S6K function in neuron.

Although phosphorylation of S6K in the autoinhibitory loop, including Ser-411, has been reported for a long time, its functional significance remains unresolved. One study showed that S6K that is completely deficient in phosphorylation of the autoinhibitory domain retains comparable kinase activity after insulin stimulation (26). Others have reported reduced S6K activity while lacking phosphorylation of the autoinhibitory domain, but the kinase activity of the mutant can still be induced by serum, suggesting that the autoinhibitory domain phosphorylation is not a prerequisite for kinase activation (27, 28). The importance of the Ser-411 phosphorylation therefore has not been resolved. More importantly, those studies only examined the phospho-deficient SA mutant function using *in vitro* kinase assay, and it is essential to address the function of the S6K SA mutant in a cellular context. Our observations in the RNAi rescue experiments represent the first demonstration that Ser-411 phosphorylation is indeed crucial for S6K function in a cellular context. Inhibition of Cdk5 specifically blocked the BDNF-induced Ser-411 phosphorylation without affecting the induction of Thr-389 phosphorylation, suggesting that Ser-411

does not serve as the priming phosphorylation event for subsequent Thr-389 phosphorylation by mTOR. One possible role of Ser-411 phosphorylation might be the regulation of substrate specificity of S6K. In this context, it is noteworthy that although both S6 ribosomal protein and eEF2K are downstream substrates of S6K only phosphorylation of S6, but not eEF2K, is reduced in the brains of Cdk5-null mice. With the identification of more S6K substrates (5), further studies on the interaction between different substrates and the wild type *versus* various phospho-deficient mutants of S6K should provide important insights into how S6K function is precisely regulated by multiple phosphorylation events.

S6K promotes dendritic arborization in young neurons (29). Using shRNA to knock down S6K expression in mature neurons, we observed a significant reduction of dendritic spine density. The spine defects could be rescued upon coexpression of RNAi-resistant S6K, indicating that the impaired spine morphogenesis is specifically attributed to the depletion of S6K. Interestingly, dendritic arborization and spine morphogenesis appear to be normal in S6K knock-out mice (8). It is likely that other proteins, such as the closely related S6K2 and p90RSK, can compensate for the absence of S6K in the knock-out mice, and the role of S6K in dendritic arborization and spine morphogenesis can only be revealed upon acute knockdown of the protein.

Knockdown of S6K mimicked and occluded the effect of TTX on spine loss and abolished the effect of BDNF on spine formation. Our findings therefore suggest that S6K is required for spine formation and maintenance in response to neuronal activity. BDNF signaling has been implicated in activity-depen-

Serine Phosphorylation of S6K in Spine Development

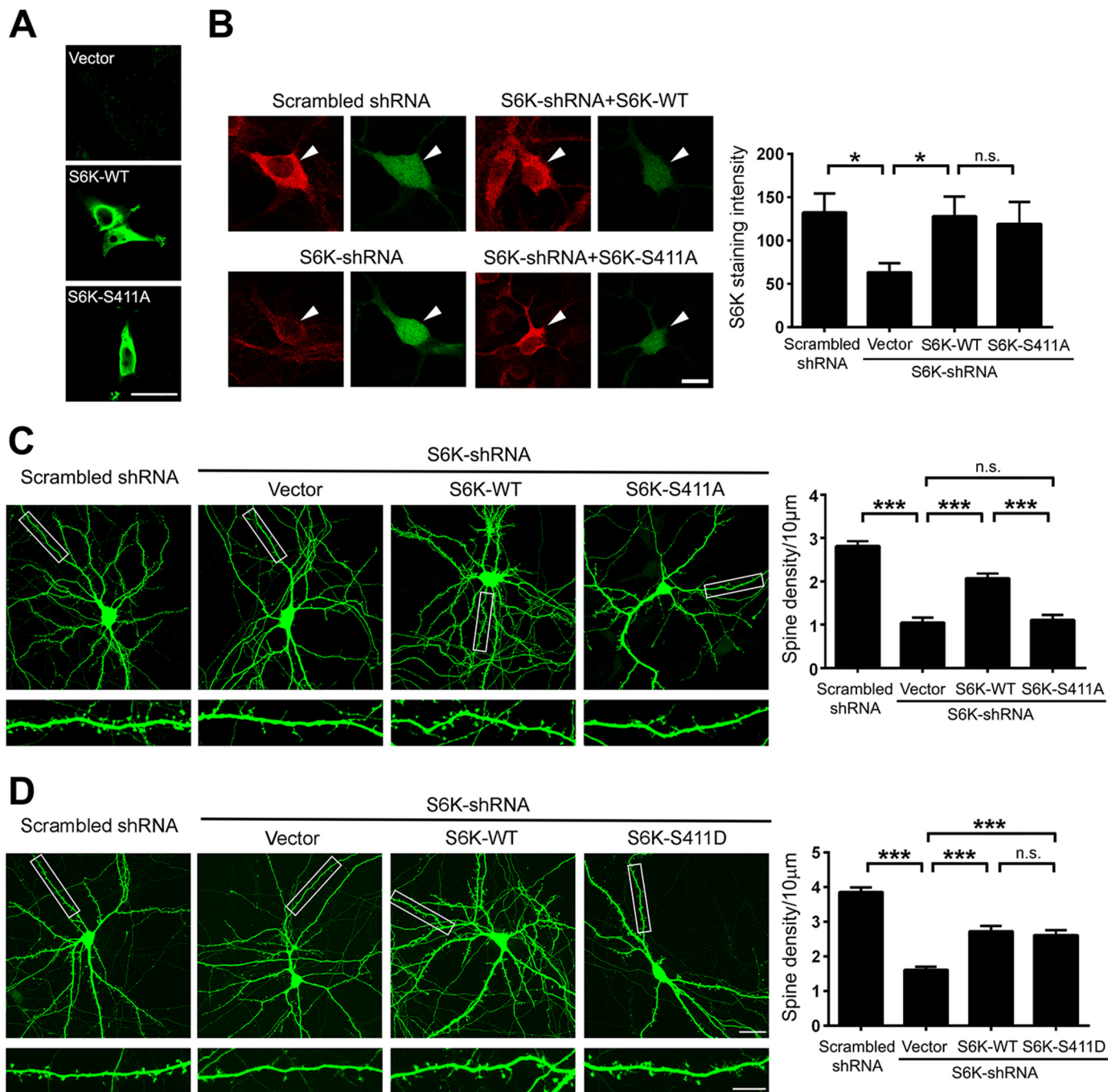


FIGURE 7. Phosphorylation of S6K at Ser-411 is required for spine morphogenesis. *A*, 293T cells were transfected with vector, FLAG-tagged WT S6K or phospho-deficient S411A mutant, and cells were stained by anti-FLAG antibody. Both wild type and S411A mutant were expressed similarly. *B*, transfecting hippocampal neurons with GFP and S6K-shRNA significantly reduced S6K staining intensity (red) in the GFP-positive neurons, and the S6K staining was reversed by coexpression of either WT or phospho-deficient S411A mutant of S6K (seven to nine neurons were analyzed for each condition; *, $p < 0.05$; n.s., not significant; one-way ANOVA, Bonferroni's multiple comparison test; error bars represent S.E.). *C*, hippocampal neurons were transfected with GFP and scrambled shRNA (control), S6K-shRNA, or S6K-shRNA plus RNAi-resistant WT or S411A mutant of S6K. Representative images are shown. Coexpression of wild type S6K rescued the reduced spine density upon knockdown of S6K, whereas the phospho-deficient mutant failed to rescue the spine loss. Quantification of spine density (results were pooled from three independent experiments; 119–120 dendrites from 30 neurons were analyzed for each condition; ***, $p < 0.001$; one-way ANOVA, Tukey's multiple comparison test; error bars represent S.E.). *D*, hippocampal neurons were transfected with GFP and scrambled shRNA (control), S6K-shRNA, or S6K-shRNA plus RNAi-resistant WT or phosphomimetic S411D mutant of S6K. Representative images are shown. Coexpression of either the wild type or S411D mutant of S6K rescued the reduced spine density upon knockdown of S6K. Quantification of spine density (results were pooled from two independent experiments; 60 dendrites from 20 neurons were analyzed for each condition; ***, $p < 0.001$; one-way ANOVA, Tukey's multiple comparison test; error bars represent S.E.). Scale bars, 25 (upper) or 10 μ m (lower).

dent spine maintenance, and we also observed reduced BDNF expression in S6K knockdown neurons (data not shown). However, in contrast to the dramatic (~50%) loss of dendritic spines after S6K knockdown or TTX treatment, neurons incubated

with TrkB-IgG for 24 h only displayed modest reduction of spine density (less than 10%; data not shown). Therefore, although S6K is crucial to mediate the effect of BDNF on spine formation, it is likely that the observed spine loss after S6K

knockdown or TTX treatment is caused by mechanisms other than reduced BDNF signaling.

Most excitatory synapses are located on dendritic spines. Whereas knockdown of S6K led to a roughly 50% reduction of spine density, surprisingly excitatory synaptic transmission remained unaffected as indicated by normal mEPSC frequency and amplitude. This suggests that excitatory synaptic transmission can be dissociated from dendritic spines. One possibility is that the mEPSCs are being generated at sites in the dendritic arbor spatially distinct from the regions chosen for quantifying spines. Alternatively the excitatory synapses might be shifted from the spines to dendritic shaft. This is consistent with a recent study showing a drastic reduction of spine density and abnormal distribution of excitatory synapses in neuron upon overexpression of the nuclear receptor Nr4a1 but no change in excitatory synaptic transmission (30). Besides simply acting as a site for excitatory synapse, dendritic spines offer additional properties for excitatory synaptic transmission, such as forming isolated compartments for Ca^{2+} signaling and allowing modulation of synaptic strength as a result of rapid changes in spine size (31). It therefore remains possible that the properties of excitatory synapses on the dendritic shaft differ from those on the spines, and the spine location of excitatory synapses might be important for specific features, such as synaptic plasticity.

How S6K promotes spine morphogenesis remains to be determined. One possibility is that S6K is crucial for global *de novo* protein synthesis in neuron. We think this is unlikely because *in situ* metabolic labeling using the azide-bearing non-canonical amino acid followed by click reaction revealed that the general protein synthesis is not affected in neurons upon knockdown of S6K by shRNA (data not shown). Conversely, it is plausible that S6K might be required for the induced synthesis of specific proteins by BDNF. For example, the mTOR-dependent synthesis of LIMK1 has been implicated in BDNF-induced spine maturation (19). BDNF also induces the expression of Arc, which can modulate phosphorylation of the actin-binding protein cofilin (32) and represents another potential candidate that promotes spine formation and maintenance. Alternatively, S6K might regulate spine morphogenesis in a protein synthesis-independent manner. S6K has been shown to interact with neurabin and the Rac guanine nucleotide exchange factor Tiam1 that are known to directly regulate spine growth (33, 34). It will be of significant interest to further elucidate how S6K affects spine morphogenesis through phosphorylation of different substrates.

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