Brain-derived Neurotrophic Factor Activates ERK5 in Cortical Neurons via a Rap1-MEKK2 Signaling Cascade

Received for publication, June 8, 2006, and in revised form, September 22, 2006. Published, JBC Papers in Press, September 26, 2006, DOI 10.1074/jbc.M605503200

Yupeng Wang, Bing Su, and Zhengui Xia

From the Toxicology Program in the Department of Environmental and Occupational Health Sciences and the Graduate Program in Neurobiology & Behavior, University of Washington, Seattle, Washington 98195-7234 and the M. D. Anderson Cancer Center, University of Texas, Houston, Texas 77054

The extracellular signal-regulated kinase 5 (ERK5) is activated in neurons of the central nervous system by neurotrophins including brain-derived neurotrophic factor (BDNF). Although MEK5 is known to mediate BDNF stimulation of ERK5 in central nervous system neurons, other upstream signaling components have not been identified. Here, we report that BDNF induces a sustained activation of ERK5 in rat cortical neurons and activates Rap1, a small GTPase, as well as MEKK2, a MEK5 kinase. Our data indicate that activation of Rap1 or MEKK2 is sufficient to stimulate ERK5, whereas inhibition of either Rap1 or MEKK2 attenuates BDNF activation of ERK5. Furthermore, BDNF stimulation of MEKK2 is regulated by Rap1. Our evidence also indicates that Ras and MEKK3, a MEK5 kinase in non-neuronal cells, do not play a significant role in BDNF activation of ERK5. This study identifies Rap1 and MEKK2 as critical upstream signaling molecules mediating BDNF stimulation of ERK5 in central nervous system neurons.

The extracellular signal-regulated kinase 5 (ERK5) or big MAP kinase 1 (BMK1) is a member of the mitogen-activated protein (MAP) kinase family that includes ERK1/2, p38, and N-terminal c-Jun protein kinase (JNK) (1, 2). ERK5 is comprised of 816 amino acid residues with a predicted molecular mass of 88 kDa. The sequence of the N-terminal 410 amino acids of ERK5 is homologous to ERK1/2 and to a lesser degree, p38 and JNK. However, ERK5 contains a large C terminus of ~400 amino acids not found in other MAP kinases. ERK5 is phosphorylated and activated by MEK5, but not by MEK1 or 2 (1, 3). MEK5 is specific for ERK5 and does not phosphorylate ERK1/2, JNK or p38 (1, 3, 4). A number of substrates have been identified for ERK5 including transcription factors (MEF2C, MEF2A, MEF2D, c-Myc, and Sap1a), protein kinases (Rsk2), connexin 43, and Bad (5–14). ERK5 is expressed in many tissues with the highest levels in the brain (15). We discovered that ERK5 expression in the brain is maximal during early embryonic development and declines as the brain matures (16). Interestingly, ERK5 and ERK5-regulated MEF2 gene expression contribute to BDNF-promoted survival of developing, but not mature neurons cultured from the cortex and cerebellum (16, 17).

Because ERK5 is highly expressed in developing neurons of the central nervous system and plays a critical role in their survival, it is crucial to define ERK5 signaling mechanisms in central nervous system neurons. The only known signaling molecule mediating ERK5 activation in central nervous system neurons is MEK5 (16, 17). Here, we used primary cultured cortical neurons from embryonic day (E) 16–17 rodents to investigate signaling pathways that mediate BDNF stimulation of ERK5. Our data strongly implicate Rap1 and MEKK2 in BDNF stimulation of ERK5.

EXPERIMENTAL PROCEDURES

Materials—The following plasmids have been described: the Gal4-MEF2C, Gal4-luciferase, and EF-lacZ (9); UB6lacz (18); FLAG-tagged wild-type ERK5 (5); and the HA-MEKK2 and HA-MEKK3 (19, 20). The dynamin constructs were from Dr. Rosalind Segal (Harvard Medical School, Boston, MA) (21, 22), the Rap1b constructs from Dr. Philip Stork (Oregon Health Sciences University, Portland, OR) (23), the constitutive active (V12) or dominant negative (N17) H-ras constructs from Dr. Alan Hall (Memorial Sloan-Kettering Cancer Center, NY), the HA–ERK2 construct from Dr. Melanie H. Cobb (The University of Texas Southwestern Medical Center, Dallas, TX), and GST–RalGDSRBD from Drs. Chengbiao Wu and W. C. Mobley (Stanford University) (24). GST–MEF2C and GST–RalGDSRBD fusion proteins were expressed in Escherichia coli DH5α cells and purified as described (9, 25). The polyclonal anti–ERK5 antibody was generated as described (4). BDNF and Lipofectamine 2000 were purchased from Invitrogen (Carlsbad, CA). The anti–Rab5 antibody, Poly-d-lysine and laminin were purchased from BD Biosciences (Bedford, MA). The monoclonal anti–FLAG antibody, polyclonal anti–FLAG antibody, monoclonal anti–FLAG antibody, forskolin, monodansylcadaverine (MDC), and phenylarsine oxide (PAO) were purchased from Sigma. The monoclonal anti–HA antibody was purchased from Roche Applied Science. The polyclonal anti–Rap1 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and the anti–c-Jun antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).
Mechanisms for BDNF Activation of ERK5

FIGURE 1. Kinetics of BDNF activation of ERK1/2 and ERK5 in cortical neurons. Rat E17 cortical neurons (DIV5) were treated with 50 ng/ml BDNF for 0–12 h as indicated. Cell lysates were prepared, and 300 μg of total protein were used for immunoprecipitation with an anti-ERK1/2 or anti-ERK5 antibody. The kinase activities in the precipitates were assayed using [32P]ATP and MBP (for ERK1/2) or GST-MEF2C (for ERK5) recombinant proteins as substrates. Thirty micrograms of the same cell lysates were submitted to Western analysis to normalize for loading. A, kinetics of ERK5 activation. B, kinetics of ERK1/2 activation.

Cruz, CA). The antibodies against total ERK1/2 or ERK2, and GST-MKK4 fusion protein were purchased from Upstate (Lake Placid, NY). The anti-p-ERK5 antibody was purchased from Cell Signaling (Danvers, MA), the anti-p-ERK1/2 antibody from Promega (Madison, WI).

Cell Culture—Primary cortical neurons were prepared from embryonic day 17 (E17) Sprague-Dawley rats as described (4, 16). Briefly, dissociated neurons were plated at a density of 2 × 10^6 cells per 35-mm dish. Neurons were cultured in basal medium Eagle (BME, Sigma) supplemented with 10% heat-inactivated bovine calf serum, 35 mM glucose, 1 mM l-glutamine, 100 units/ml penicillin, and 0.1 mg/ml streptomycin and maintained in a humidified incubator with 5% CO_2 at 37 °C. Plates and glass coverslips were coated with poly-d-lysine and laminin. Neurons were cultured for 5 days in vitro (DIV5) before BDNF treatment or harvesting.

Cortical Neuron Cultures from MEKK2-deficient Mice—MEKK2-deficient mice have been described (26). The heterozygous MEKK2<sup>+/−</sup> mice were bred to generate MEKK2<sup>−/−</sup> and MEKK2<sup>+/+</sup> littermates. At E16, cortical neurons were prepared from individual single embryo using the same conditions as for rat E17 cortical neurons described above. The genotype of each embryo was determined afterward by PCR as described (26). The PCR primers used were: P1 (5′-AGGTGGTTTGTCTTATAGA-3′), P2 (5′-AGAAAAACCGAAACTTACACTTTCA-3′), and P3 (5′-TCTTATAGA-3′) for wild-type, P1 + P2 for knock-out. At DIV 5, neurons were treated with 50 ng/ml BDNF for 1 h and assayed for ERK5 kinase activity or nuclear translocation.

Transient Transfection of Primary Cortical Neurons—Cortical neurons (6 × 10<sup>6</sup> cells/60-mm dish) were transiently transfected at DIV3 using a calcium phosphate co-precipitation protocol as described (27, 28). Briefly, the DNA–calcium phosphate precipitates were prepared by mixing 1 volume of DNA in 250 mM CaCl_2 with an equal volume of 2× HEPESS-buffered saline (HBS; 274 mM NaCl, 10 mM KCl, 1.4 mM Na_2HPO_4, 15 mM d-glucose, and 42 mM HEPES, pH 7.07). The precipitates were allowed to form for 25–30 min at room temperature before addition to the cultures. The conditioned culture media were removed and saved. Cells were washed three times with BME, and 4.5 ml of transfaction media were added to each 60-mm dish. The transfection media consisted of BME supplemented with 1 mM sodium pyruvate, 10 mM MgCl_2, and 5 mM HEPES. The pH of the transfection media was kept high by incubating BME in a dish at 37 °C and 0% CO<sub>2</sub> for 30 min to degas. 180 μl of the DNA-calcium phosphate precipitates were added dropwise to each 60-mm dish and mixed gently. Plates were incubated at room temperature and ambient air for 5 min and then in a humidified incubator with 5% CO<sub>2</sub> at 37 °C for 35–45 min. The incubation was stopped 20–25 min after the

FIGURE 2. BDNF activation of ERK5 in cortical neurons requires dynamin activity. A, expression of dn-Dynamin blocks BDNF activation of ERK5. Rat E17 cortical neurons (DIV5) were co-transfected with 6 μg each of plasmid DNA encoding wild-type, FLAG-tagged ERK5 (flag-ERK5), vector control (V), wild-type (wt), or dn-Dynamin. Forty-eight hours after transfection, cells were treated with 50 ng/ml BDNF (+) or vehicle control (−) for 1 h. The activities of transfected ERK5 were determined by an in vitro kinase assay after immunoprecipitation of FLAG-ERK5. Thirty micrograms of the same cell lysates were submitted to anti-FLAG Western analysis for loading control. B, expression of dn-Dynamin blocks BDNF activation of ERK2. Cortical neurons were transfected and treated as in A except that FLAG-ERK5 was replaced by wild-type, HA-tagged ERK2 (HA-ERK2). The ectopically expressed HA-ERK2 was immunoprecipitated using an anti-HA antibody and subject to Western analysis using an anti-p-ERK2/1 antibody to detect phosphorylated and active ERK2, and an anti-ERK2 antibody for loading control. C, expression of dn-Dynamin does not interfere with forskolin activation of ERK2. Cortical neurons were transfected as in B and treated with forskolin (10 μM, 10 min) to increase intracellular cAMP. D, expression of a temperature-sensitive dynamin mutant (tsDynamin) blocks BDNF activation of ERK5 at non-permissive temperature (39 °C). Cortical neurons were transfected with 6 μg each of plasmid DNA encoding FLASK-ERK5 and tsDynamin. Forty-eight hours after transfection, cells were placed under permissive (33 °C) or non-permissive (39 °C) temperature for 30 min, then treated with 50 ng/ml BDNF (+) or vehicle control (−) for 1 h.
layer of precipitate formed on the plates by shocking the cells for 2 min with 1/1000 HCl, 1 mM sodium kynurenate, 10 mM MgCl2 in 5 mM HEPES, pH 7.5, and 5% glycerol. Cells were then washed three times with 2 ml of BME. The saved conditioned media were added back to each plate, and cells were returned to the 5% CO2 incubator at 37 °C for 48 h before BDNF treatment or harvesting.

**Rap1 and Ras Activation Assay** — The fusion protein between glutathione S-transferase (GST) and the Rap binding domain of RalGDS (GST-RalGDSRBD) was overexpressed and purified from *Escherichia coli* DH5α cells as described (29). The GTP-bound, active Rap1 protein (Rap1GTP) was detected as described (25, 30). This assay is based on a specific binding between the GTP-bound, activated form of Rap1 (i.e., Rap1GTP) and the Rap binding domain of the RalGDS protein (RalGDSRBD). Briefly, rat E17 cortical neurons were lysed on ice for 10 min in fishing buffer (FB) which contains 10% glycerol, 1% Nonidet P-40, 50 mM Tris-HCl (pH 7.5), 200 mM NaCl, 2.0 mM MgCl2, 10 mM NaF, 1 mM Na3VO4, 250 μM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 1 μg/ml leupeptin, and 10 μg/ml soybean trypsin inhibitor. The samples were centrifuged at 14,000 rpm for 10 min at 4 °C. Ten micrograms of GST-RalGDSRBD fusion protein were prebound to agarose-glutathione conjugates, and then added to 300 μg of cortical neuron lysates. The mixture was incubated at 4 °C for 1–2 h with gentle rotation. Then, the beads were washed four times in cold FB and boiled in SDS-PAGE sample buffer. The amount of active Rap1GTP bound to GST-RalGDSRBD fusion protein that is coupled to glutathione-agarose beads was analyzed by SDS-PAGE followed by immunoblotting using an anti-Rap1 antibody. Ras activation was analyzed using Ras Activation Assay kit (Upstate) per the manufacturer’s instruction.

**Kinase Assays** — Kinase assays were performed as described (4, 31). Cell lysates were prepared and protein concentrations assayed by the Bradford method. Equal amounts of protein extracts (300 or 600 μg) were used for each kinase assay. To measure ERK5 activity, cell lysates were incubated at 4 °C for 2.5 h with either 6 μl of a polyclonal anti-ERK5 antibody (for endogenous ERK5) or 2.5 μg of a polyclonal anti-FLAG antibody (for transfected FLAG-ERK5). Protein A-Sepharose beads (60 μl) were then added, and the mixture was incubated at 4 °C for an additional hour. The activity of ERK5 in the immune precipitates was quantitated by a kinase assay using recombinant GST-MEF2C (5 μg) and [32P]ATP as the substrate (5).

Activated MEKK2 or MEKK3 can phosphorylate multiple MEKs in vitro including MKK4 and MEK5 (32). Because GST-MKK4 is commercially available, we used it as the substrate in

**FIGURE 3.** BDNF activation of ERK5 in cortical neurons is inhibited by PAO, an endocytosis blocker. **A** and **B**, PAO blocks BDNF-induced nuclear translocation of endogenous ERK5. Cortical neurons (DIV5) were treated with 5 nM PAO or vehicle control (Veh) 30 min before BDNF treatment (50 ng/ml, 1 h). **A** shows deconvolution confocal images (×400). Cells were stained with an anti-ERK5 antibody to monitor the subcellular distribution of endogenous ERK5 (red). Cells were also stained with the DNA dye Hoechst 33258 to mark nuclei which were pseudocolored to green to better visualize ERK5 nuclear staining. **B** contains the quantification of data in **A**. **C**, PAO blocks BDNF activation of endogenous ERK5. Cortical neurons were treated as in **A**. The kinase activity of endogenous ERK5 was assayed as in Fig. 1A. **D**, PAO blocks BDNF induced ERK1/2 phosphorylation. **E**, PAO does not inhibit ERK1/2 phosphorylation induced by forskolin (10 μM, 10 min).
Mechanisms for BDNF Activation of ERK5

the in vitro kinase assay to monitor the biochemical activities of MEKK2 and MEKK3. To measure the activities of transfected MEKK2 or MEKK3, cell lysates were incubated at 4 °C for 2.5 h with 2.5 μg of anti-HA antibody. Protein G-Sepharose beads (60 μl) were then added, and the mixture was incubated at 4 °C for an additional hour. The activity of MEKK2 and MEKK3 in the immune precipitates was quantitated by a kinase assay using recombinant GST-MKK4 (2 μg) and [32P]ATP as the substrates. Quantitation of kinase activity was achieved by PhosphorImager analysis (Molecular Dynamics, Sunnyvale, CA) or by using the ImageQuant program after scanning the autoradiographic images, and normalized by protein expression based on Western analysis.

Reporter Gene Assays—The Gal4-MEF2C-driven, Gal4-luciferase reporter gene expression was assayed as described (4). Cortical neurons were transfected using Lipofectamine 2000 (33). Briefly, 0.5 × 10⁶ cells were plated onto each well of a 24-well plate coated with poly-L-lysine and laminin. At DIV3, cells were co-transfected with the Gal4-luciferase reporter construct (1.2 μg DNA/4 wells), Gal4-MEF2C fusion protein construct (0.9 μg DNA/4 wells), and EF1a.LacZ DNA (0.55 μg DNA/4 wells). Where indicated, cortical neurons were also co-transfected with various expression vectors for the ERK5 signaling pathways. Cells were treated 40 h after transfection with 50 ng/ml BDNF for 6 h when indicated. Cell lysates were prepared, and the activities of luciferase and β-galactosidase were measured as described (33). The reporter gene luciferase activity was normalized to β-galactosidase activity and expressed as the fold induction relative to control.

Assay of Apoptosis—Apoptosis was determined by nuclear condensation and/or fragmentation after Hoechst staining (28). Healthy cells have evenly and uniformly stained nuclei. To facilitate detection of apoptosis in transfected cells, cortical cultures were cotransfected with an expression vector encoding β-galactosidase (UB6-LacZ) as a marker for transfected cells. To obtain unbiased counting, slides were coded, and cells were scored blindly without prior knowledge of treatment.

Data Analysis—Data were either the averages or representatives of at least three independent experiments. Statistical analysis of data were performed using one-way analysis of variance. Error bars represent S.E. *, p < 0.05; **, p < 0.01; ***, p < 0.001; n.s., not statistically significant (p > 0.05).

RESULTS

BDNF Induces Sustained Activation of ERK5 in E17 Cortical Neurons—To investigate signaling pathways responsible for BDNF stimulation of ERK5, we prepared primary neurons from embryonic day (E) 17 rat cortex. In initial experiments, we examined the kinetics for ERK5 activation after BDNF treatment and compared it to ERK1/2 activation. The kinase activities of ERK1/2 and ERK5 were directly measured by in vitro kinase assays using MBP or GST-MEF2C fusion protein as substrates, respectively (Fig. 1). BDNF activated both ERK1/2 and ERK5 in E17 cortical neurons. Like ERK1/2 activation, ERK5 activation was prolonged and sustained for at least 12 h after BDNF treatment. However, the peak of ERK5 activity occurred later than ERK1/2 and did not reach a maximum until 1–2 h after BDNF treatment. In contrast, ERK1/2 was maximally activated by BDNF 10 min after BDNF treatment. The kinetics for activation of ERK1/2 and ERK5 in E17 cortical neurons is similar to that in postnatal cortical neurons (4).

BDNF Activation of ERK5 Is Mediated by Receptor-mediated Endocytosis—Receptor-mediated endocytosis has been implicated in sustained neurotrophin signaling (34–37). Upon BDNF treatment for 20 min, we detected co-immunostaining of phospho-ERK5 and Rab5 (supplemental Fig. S1), a marker for endosomes (38). These data are consistent with the notion that ERK5 is found in endosomes following neurotrophin stimulation (39).

Dynamin, a large G protein, is involved in clathrin-dependent and -independent receptor-mediated endocytosis (40). Moreover, mutation of dynamin inhibits internalization of neurotrophin receptors (10, 21). To determine if BDNF activation of ERK5 depends on receptor-mediated endocytosis, cortical neurons were transiently transfected with a dominant negative (dn) dynamin mutant (K444A) to block receptor-mediated endocytosis (10, 21). The wild-type dynamin and cloning vector were used as controls. Cortical neurons were also co-transfected with FLAG-tagged, wild-type ERK5 to facilitate detection of ERK5 in transfected cells. In contrast to the wild-type dynamin, expression of the dnDynamin completely abolished BDNF stimulation of ERK5 (Fig. 2A). As a control for specificity, neurons were co-transfected with the dynamin constructs and wild-type, HA-tagged ERK2. Although dnDynamin attenuated BDNF stimulation of ERK2 (Fig. 2B), it had no effect on ERK2 activation by forskolin (Fig. 2C). Forskolin activates adenyl cyclases which increase intracellular cAMP, thereby activating ERK2 (41). Our results are consistent with the reports that neurotrophin but not cAMP regulation of ERK1/2 signaling in neurons is regulated by receptor-mediated endocytosis (36). These results also demonstrate that expression of the dnDynamin specifically inhibits receptor-mediated endocytosis, rather than nonspecifically interfering with MAP kinase signaling.
Cortical neurons were also co-transfected with ERK5 and a temperature-sensitive (ts) dynamin mutant (G273D) (10, 21). The G273D Dynamin protein functions as a wild-type protein at the permissive temperature (33 °C), but as a dominant-negative protein at the non-permissive temperature (39 °C). Cells were incubated at 37 °C for 48 h after transfection to allow expression of the transgenes, at which time the expression of tsDynamin can be detected by Western analysis. The cells were then incubated at 33 °C or 39 °C for 30 min, followed by BDNF treatment for 1 h. BDNF stimulated ERK5 in the presence of co-transfected tsDynamin at the permissive temperature (33 °C), but not at the non-permissive temperature (39 °C) (Fig. 2D). The fact that transfection of tsDynamin did not affect the ability of BDNF to stimulate ERK5 at the permissive temperature suggests that overexpression of tsDynamin per se does not interfere with endocytosis. In the absence of co-transfected tsDynamin, BDNF activated ERK5 at 39 °C, excluding the possibility that BDNF activation of ERK5 is inhibited at 39 °C. Moreover, we did not observe any morphological changes or cell death of cortical neurons during the 1.5-h incubation at 33 or 39 °C. Thus, expression of the tsDynamin selectively blocks BDNF activation of ERK5, presumably because it blocks receptor-mediated endocytosis.

To further investigate the role of receptor-mediated endocytosis in BDNF signaling to ERK5, we utilized phenylarsine oxide (PAO), an inhibitor widely used to inhibit receptor-mediated endocytosis (42-44). We pretreated cortical neurons with 5 nM PAO, a concentration specific for inhibition of endocytosis (45). The endogenous ERK5 is normally a cytoplasmic protein but translocates to the nucleus upon BDNF stimulation (Fig. 3A and B). PAO inhibited BDNF-induced nuclear translocation of endogenous ERK5, suggesting PAO suppression of ERK5 activation. Furthermore, treatment with PAO greatly attenuated BDNF stimulation of ERK5 kinase activity (Fig. 3C). Similar results were obtained using monodansylcadaverine (MDC) (Fig. 4A), another endocytosis blocker (35). Treatment with PAO or MDC attenuated BDNF stimulation of ERK5.

Mechanisms for BDNF Activation of ERK5

Cortical neurons were also co-transfected with ERK5 and a temperature-sensitive (ts) dynamin mutant (G273D) (10, 21). The G273D Dynamin protein functions as a wild-type protein at the permissive temperature (33 °C), but as a dominant-negative protein at the non-permissive temperature (39 °C). Cells were incubated at 37 °C for 48 h after transfection to allow expression of the transgenes, at which time the expression of tsDynamin can be detected by Western analysis. The cells were then incubated at 33 °C or 39 °C for 30 min, followed by BDNF treatment for 1 h. BDNF stimulated ERK5 in the presence of co-transfected tsDynamin at the permissive temperature (33 °C), but not at the non-permissive temperature (39 °C) (Fig. 2D). The fact that transfection of tsDynamin did not affect the ability of BDNF to stimulate ERK5 at the permissive temperature suggests that overexpression of tsDynamin per se does not interfere with endocytosis. In the absence of co-transfected tsDynamin, BDNF activated ERK5 at 39 °C, excluding the possibility that BDNF activation of ERK5 is inhibited at 39 °C. Moreover, we did not observe any morphological changes or cell death of cortical neurons during the 1.5-h incubation at 33 or 39 °C. Thus, expression of the tsDynamin selectively blocks BDNF activation of ERK5, presumably because it blocks receptor-mediated endocytosis.

To further investigate the role of receptor-mediated endocytosis in BDNF signaling to ERK5, we utilized phenylarsine oxide (PAO), an inhibitor widely used to inhibit receptor-mediated endocytosis (42-44). We pretreated cortical neurons with 5 nM PAO, a concentration specific for inhibition of endocytosis (45). The endogenous ERK5 is normally a cytoplasmic protein but translocates to the nucleus upon BDNF stimulation (Fig. 3A and B). PAO inhibited BDNF-induced nuclear translocation of endogenous ERK5, suggesting PAO suppression of ERK5 activation. Furthermore, treatment with PAO greatly attenuated BDNF stimulation of ERK5 kinase activity (Fig. 3C). Similar results were obtained using monodansylcadaverine (MDC) (Fig. 4A), another endocytosis blocker (35). Treatment with PAO or MDC attenuated BDNF stimulation of ERK5.

![FIGURE 5. BDNF induces activation of endogenous Rap1 in cortical neurons in an endocytosis-dependent manner. A, kinetics of Rap1 activation. Rat E17 cortical neurons (DIV5) were treated with 50 ng/ml BDNF for 0–4 h. To reduce background activity, serum was removed from the culture 2 h before BDNF treatment. 300 μg of total protein were used to analyze for the amounts of active Rap1GTP. Anti-Rap1 Western blotting was used as a loading control. B, quantification of the data in A. Shown are pooled data from three independent experiments. C, effect of PAO on BDNF activation of Rap1. Cortical neurons were pretreated with 5 μM PAO for 30 min before BDNF stimulation (50 ng/ml, 1 h). D, quantification of the data in C.](image-url)
ERK1/2 (Fig. 3D and Fig. 4B) but had no effect on forskolin stimulation of ERK1/2 (Fig. 3E and Fig. 4C), demonstrating the specificity of PAO and MDC for endocytosis. Together, data in Figs. 2, 3, and 4 suggest that BDNF activation of ERK5 in cortical neurons depends on receptor-mediated endocytosis.

**BDNF Activates Rap1**—Rap1 belongs to the small monomeric GTP-binding proteins of the Ras family. It is involved in endocytosis and in the sustained, but not the initial transient activation of ERK1/2 in PC12 cells after NGF treatment (23, 30, 36). We hypothesized that Rap1 may also contribute to the sustained activation of ERK5 by BDNF. To test this hypothesis, we determined if BDNF activates Rap1, and as a control, if it also activates Ras. Ras activation by BDNF was rapid and transient; it peaked in 2 min and returned to baseline 30 min after BDNF treatment (supplemental Fig. S2). This is consistent with the published literature, which reports that neurotrophins activate Ras rapidly and transiently (46). In contrast, Rap1 was maximally activated by BDNF at 20 min with sustained activation for at least 4 h (Fig. 5, A and B). Pretreatment with 5 nM of PAO abolished BDNF stimulation of Rap1 (Fig. 5, C and D). Thus, BDNF induces a persistent Rap1 activation in cortical neurons that requires receptor-mediated endocytosis.

**Rap1 but Not Ras Mediates BDNF Stimulation of ERK5**—To determine if Rap1 is sufficient to stimulate ERK5, cortical neurons were transfected with constitutive active (ca) Rap1 (Rap1bV12) to activate Rap1 signaling (23). The wild-type FLAG-ERK5 was co-expressed to monitor ERK5 activity in transfected cells. Expression of caRap1 stimulated ERK5 more than 5-fold (Fig. 6, A and C). To ascertain if Rap1 is necessary for BDNF stimulation of ERK5, cortical neurons were transfected with dnRap1 (Rap1bN17) to block Rap1 signaling (23). Expression of dnRap1 almost completely inhibited BDNF stimulation of co-transfected ERK5 (Fig. 6, B and D).

Although Ras is required for neurotrophin stimulation of ERK1/2, its role in neurotrophin activation of ERK5 has not been examined. Therefore, we performed a similar experiment to determine if Ras is required for BDNF activation of ERK5. In contrast to Rap1, expression of constitutive active Ras (RasV12) did not stimulate ERK5, although it clearly activated the co-transfected wild type HA-ERK2 (Fig. 6, A and C). Accordingly, expression of the dominant negative Ras (RasN17) attenuated BDNF activation of ERK2 but had little effect on ERK5 (Fig. 6, B and D). These data suggest that BDNF stimulation of ERK5 requires Rap1 but not Ras.

In addition to the ectopically expressed ERK5, we also examined the role for Rap1 in BDNF stimulation of endogenous ERK5 in cultured cortical neurons. Expression of dnRap1 blocked BDNF-induced nuclear translocation of the endogenous ERK5 (Fig. 7). These data suggest a critical role for Rap1 in BDNF stimulation of endogenous ERK5 in neurons.

**Rap1 Provides Neuronal Survival Signals to Cortical Neurons**—We have implicated ERK5 in mediating BDNF neuroprotection in cortical neurons (16). To investigate the physiological significance of Rap1 in BDNF signaling, we transiently transfected cortical neurons with caRap1 or dnRap1 and exam-
Mechanisms for BDNF Activation of ERK5

Serum withdrawal

A

B

FIGURE 8. Rap1 provides a neuronal survival signal. A, expression of 
caRap1 is sufficient to protect E17 cortical neurons from serum 
w withdrawal-induced apoptosis. E17 cortical neurons were transfected with plasmid DNA 
encoding caRap1 or vector control (V). Cells were also co-transfected with 
plasmid DNA encoding β-galactosidase (UB6-LacZ) as a marker to identify 
transfected cells. One day after transfection, cells were placed in serum-free 
medium and apoptosis in the transfected cell population (β-galactosidase-
positive neurons) was scored 24 h later. B, expression of dnRap1 blocks BDNF 
protection against serum withdrawal. Cells were transfected and treated as in 
A except that cells were placed in serum-free medium supplemented with 50 
g/ml BDNF 1 day after transfection.

BDNF Strongly Activates MEKK2 but Not MEKK3 in Cortical 
Neurons—Active MEKK2 or MEKK3 can phosphorylate and 
stimulate MEK5 in vitro; both have been implicated as ERK5 
kinase kinases (MAPK/ERK) in response to EGF stimulation in 
non-neuronal cells (47–51). To determine whether MEKK2 or 
MEKK3 contribute to BDNF stimulation of ERK5 in primary 
neurons, we monitored their activities after BDNF treatment. 
Cortical neurons were transfected with HA-tagged, wild-type 
MEKK2 or MEKK3. BDNF strongly activated MEKK2, and this 
activation was blocked by pretreatment with PAO or by 
expression of the tsDynamin at the non-permissive temperature 
(39 °C) (Fig. 9, A, B, and D). Although BDNF also activated 
MEKK3 somewhat, its activation was much weaker 
compared with MEKK2 at both 37 and 33 °C (Fig. 9, A, C, and 
E). These data suggest that BDNF preferentially stimulates 
MEKK2 through a mechanism depending on receptor-
mediated endocytosis.

Rap1 Regulates MEKK2 but Not MEKK3—To determine if 
Rap1 regulates MEKK2 or MEKK3, cortical neurons were co-
transfected with HA-MEKK2 or HA-MEKK3 together with 
caRap1 to activate Rap1 signaling, or with dnRap1 to inhibit 
Rap1 signaling. Expression of caRap1 was sufficient to activate 
MEKK2 but not MEKK3 (Fig. 10A). Expression of dnRap1 sup-
pressed BDNF stimulation of MEKK2 but had no effect on 
MEKK3 (Fig. 10B). These data suggest that Rap1 mediates 
BDNF stimulation of MEKK2 but not MEKK3.

MEKK2 Is Required for BDNF Stimulation of ERK5 and 
MEF2C-mediated Gene Transcription in Cortical Neurons—To 
determine if MEKK2 or MEKK3 is required for BDNF stimula-
tion of ERK5, cortical neurons were transfected with dnMEKK2 
or dnMEKK3 to block MEKK2 or MEKK3 signaling, respec-
tively (52). Inhibition of MEKK2 almost completely abolished 
BDNF stimulation of co-transfected ERK5 (Fig. 11, A and B). 
Although expression of dnMEKK3 caused a small inhibition of 
BDNF stimulation of ERK5, it was not statistically significant 
(p > 0.5).

The transcription factor MEF2C is one of the best character-
ized downstream targets of ERK5 (5). ERK5 is required for 
BDNF stimulation of MEF2C-mediated gene expression in 
cortical neurons (4, 16). To evaluate the contribution of Rap1 and 
MEKK2 to ERK5 regulation of transcription, cortical neurons 
were transfected with dnRap1 or dnMEKK2. Cells were also 
co-transfected with a Gal4-MEF2C expression vector and a 
Gal4-luciferase reporter to monitor MEF2C activity. As shown

FIGURE 9. BDNF preferentially activates MEKK2 over MEKK3 in cortical 
neurons. A, BDNF preferentially activates MEKK2 in an endocytosis-depend-
ent manner. Rat E17 cortical neurons were transfected with 6 μg each of 
plasmid DNA encoding HA-MEKK2 or HA-MEKK3. 48 h after transfection, cells 
were treated with 5 nm PAO (+) or vehicle control (−) for 30 min, followed by 
0 (−) or 50 ng/ml BDNF (+) for 1 h. 600 μg of total protein were used for 
immunoprecipitation with an anti-HA antibody. The kinase activities of 
HA-MEKK2 or HA-MEKK3 in the precipitates were assayed using GST-MKK4 
recombinant protein and [γ-32P]ATP as substrates. Anti-HA Western analysis 
was used to normalize for expression of the transfected HA-MEKK2 or 
HA-MEKK3. B, kinase activities of HA-MEKK2 or HA-MEKK3 in A were quantified 
and normalized by their expression. C, kinase activity of HA-MEKK3 relative to 
that of HA-MEKK2 from data in A. D, tsDynamin mutant blocks BDNF stimula-
tion of MEKK2 at non-permissive temperature. Cortical neurons were 
co-transfected with tsDynamin and HA-MEKK2 or HA-MEKK3. Two days later, 
cells were placed under permissive (33 °C) or non-permissive (39 °C) temper-
atume for 30 min, then treated with 50 ng/ml BDNF (+) or vehicle control (−) 
for 1 h at the respective temperatures. The kinase activities of HA-MEKK2 or 
HA-MEKK3 in D were normalized by their expression. E, relative kinase activi-
ties of HA-MEKK3 and HA-MEKK2 at 33 °C from data in D.
Before (4, 16), BDNF activated MEF2C-induced transcription in cortical neurons (Fig. 11C). Expression of either dnMEKK2 or dnRap1 greatly reduced BDNF activation of MEF2C. These data support the hypothesis that BDNF activation of ERK5 depends on Rap1 and MEKK2.

**BDNF Fails to Activate ERK5 in MEKK2^{−/−} Cortical Neurons**—We used a transgenic mouse line in which the MEKK2 gene is disrupted (26) to evaluate the importance of MEKK2 for ERK5 activation. E16 primary cortical neurons from MEKK2^{+/+} and MEKK2^{−/−} mice were isolated and examined for the effect of MEKK2 ablation on BDNF stimulation of ERK5. Disruption of the MEKK2 gene did not change the expression level of endogenous ERK5 (Fig. 12A). However, it abrogated BDNF stimulation of the kinase activity of ERK5 (Fig. 12, A and B). Furthermore, BDNF failed to induce ERK5 nuclear translocation in neurons prepared from MEKK2^{−/−} cortex (Fig. 12C). Together, data in Figs. 9–12 strongly suggest a key role for MEKK2, but not MEKK3, in BDNF activation of ERK5.

**DISCUSSION**

The objective of this study was to identify signaling components that mediate BDNF activation of ERK5 in central nervous system neurons. Prior to this study, MEKK5 was the only upstream regulator of ERK5 identified in central nervous system neurons (4). Using primary neurons cultured from E16–17 rodent cortex, we investigated mechanisms regulating neurotrophin-stimulation of ERK5. We discovered that BDNF causes sustained ERK5 activation, a process that requires receptor-mediated endocytosis. We also identified Rap1 and MEKK2 as upstream components in ERK5 signaling.

Receptor-mediated endocytosis has been implicated in the sustained signaling of neurotrophins (34–37). For example, BDNF induces TrkB receptor internalization and endocytosis (10, 53). The tsDynamin mutant blocks retrograde nerve growth factor (NGF)/BDNF stimulation of ERK5 in dorsal root ganglion (DRG) neurons of the peripheral nervous system (PNS) (10). The endocytic protein Pincher mediates pinocytic endocytosis of NGF/TrkA endosomes and persistent NGF activation of ERK5 in PC12 cells (39). Furthermore, the atypical protein kinase C-interacting protein, p62, interacts with active TrkA in the endosomes and mediates NGF stimulation of ERK5 in PC12 cells (54). Using two independent dominant negative dynamin mutants and two general inhibitors for receptor-mediated endocytosis, we discovered that BDNF stimulation of ERK5 in cortical neurons is also mediated by endocytosis. Thus, receptor-mediated endocytosis may be a common mechanism underlying neurotrophin activation of ERK5 in both peripheral nervous system and central nervous system neurons.

Growth factor activation of MAP kinases requires Ras family small GTP-binding proteins. We found that BDNF activates Rap1 in cortical neurons and that expression of constitutive
The MAP kinases are organized into three-tier kinase cascades that proceed through MAP kinase kinases (MAPKK, e.g. MEKK1–4), which phosphorylate and activate dual specificity Thr/Tyr MAP kinase kinases (MAPKKK, e.g. MEK5), which then phosphorylate and activate MAP kinases (e.g. ERK5). MEK5 is not a good substrate for Raf, c-Mos, or MEKK1 (3), but is phosphorylated and activated by MEKK2, MEKK3, or Cot in vitro (47, 57, 58). Both MEKK2 and MEKK3 have been implicated in EGF activation of ERK5 in non-neuronal cells (8, 47, 48, 57). MEKK2 also regulates the activation of ERK5 in response to FGF-2 in fibroblasts (60). However, studies in non-neuronal cells suggest that MEKK2 and MEKK3 have different intracellular localizations and may function in different signaling pathways (61). In this study, we demonstrated that although BDNF activates both MEKK2 and MEKK3 via receptor-mediated endocytosis, the stimulation of MEKK2 is much more robust than MEKK3. Furthermore, Rap1 was both necessary and sufficient to mediate BDNF stimulation of MEKK2 but not MEKK3. Expression of dominant negative MEKK2 but not MEKK3 blocked BDNF stimulation of ERK5 and downstream MEF2C-mediated gene transcription. Significantly, BDNF activation of ERK5 was almost completely abrogated in cortical neurons prepared from MEKK2−/− mice. These data indicate that BDNF stimulation of ERK5 in cortical neurons is primarily mediated by MEKK2.

It is interesting to note that the relatively weak MEKK3 activation induced by BDNF requires endocytosis but does not require Rap1. In addition to Ras and Rap1, neurotrophins activate several other small G proteins including the Rac, Cdc42, and Rho family GTPases (46), many of which play a role at different steps of endocytosis (62). For example, Alsin is an activator of Rac1 and Rab5 small GTPases. Loss of function of Alsin (Als2−/−) in mice results in abnormal endosomal transport of the BDNF receptor in neurons (59). Therefore, it is conceivable that BDNF activates MEKK3 through a Rap1-independent endosome signaling.

In summary, we have elucidated signaling mechanisms regulating BDNF stimulation of ERK5 in cortical neurons. We discovered that BDNF activation of ERK5 requires receptor-mediated endocytosis and a Rap1-MEKK2-MEK5 signaling cascade (Fig. 12D). Our data demonstrate the specificity of Rap1 for MEKK2 activation and the specificity of both Rap1 and MEKK2 in ERK5 signaling in central nervous system neurons upon BDNF treatment. Considering the important role ERK5 plays in neuronal survival, the elucidation of these signaling components may provide potential drug targets for disease treatment against neuronal cell death.

Acknowledgments—We thank Dr. R. A. Segal for providing dynamin plasmids, Dr. Philip Stork for Rap1b constructs, and Drs. C. Wu and W. C. Mobley for the GST-RalGDSRBD plasmid.

REFERENCES
1. Zhou, G., Bao, Z. Q., and Dixon, J. E. (1995) J. Biol. Chem. 270, 12665–12669
2. Lee, J. D., Ulevitch, R. J., and Han, J. (1995) Biochem. Biophys. Res. Commun. 213, 715–724
3. English, J. M., Vanderbilt, C. A., Xu, S., Marcus, S., and Cobb, M. H. (1995)
