

BDNF Regulates Primary Dendrite Formation in Cortical Neurons via the PI3-Kinase and MAP Kinase Signaling Pathways

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Received 15 February 2004; accepted 2 May 2004

ABSTRACT: Neurotrophins are known to regulate dendritic development, but the mechanisms that mediate neurotrophin-dependent dendrite formation are largely unknown. Here we show that brain-derived neurotrophic factor (BDNF) induces the formation of primary dendrites in cortical neurons by a protein synthesis-independent mechanism. BDNF leads to the rapid activation of PI3-kinase, MAP kinase, and PLC- γ in cortical neurons, and pharmacological inhibition of PI3-kinase and MAP kinase in dissociated cell cultures and cortical slice cultures suppresses the ability of BDNF to

induce dendrite formation. A constitutively active form of PI3-kinase, but not MEK, is sufficient to induce primary dendrite formation in cortical neurons. These observations indicate that BDNF induces primary dendrite formation via activation of the PI3-kinase and MAP kinase pathways and provide insight into the mechanisms that mediate the morphological effects of neurotrophin signaling. © 2004 Wiley Periodicals, Inc. *J Neurobiol* 62: 278–288, 2005

Keywords: neurotrophin; dendrite; cortex; pyramidal neuron; PI3-kinase; MAP kinase

INTRODUCTION

The dendritic morphology of neurons plays a critical role in the specification of neural circuits. Due to their characteristic morphologies and well-studied function, cortical neurons have long served as a model for investigating dendritic morphogenesis. Investigations

of cortical dendrite development have revealed that extracellular signals exert considerable influence over the specification of dendritic morphology. The initial growth of pyramidal neuron apical dendrites towards the pial surface is regulated by Sema3A, which acts as a chemoattractant for the developing dendrite (Polleux et al., 2000). The subsequent emergence and growth of basal dendrites is regulated by a number of factors, including neurotrophins, Notch1, and Slit1 (McAllister et al., 1995; Baker et al., 1997; Redmond et al., 2000; Whitford et al., 2002). Of these, the effects of neurotrophins have been examined most extensively. In particular, brain-derived neurotrophic factor (BDNF) plays an important role in regulating the growth and branching of cortical dendrites (McAllister et al., 1995; Niblock et al., 2000). Overexpression of either BDNF (Horch et al., 1999) or its high-affinity receptor TrkB (Yacoubian and Lo, 2000) favors addition of dendritic branches close to the soma. BDNF also appears to regulate the dynamic

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Contract grant sponsor: Netherlands Organization for Scientific Research (NWO) fellowship (P. A. D.).

Contract grant sponsor: March of Dimes Birth Defects Foundation (A. G.).

Contract grant sponsor: NIH; contract grant numbers: NS39993, MH60598 (A. G.).

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Published online 27 October 2004 in Wiley InterScience (www.interscience.wiley.com).

DOI 10.1002/neu.20100

stability of dendritic processes (Horch et al., 1999; Horch and Katz, 2002). Most of these signals have been studied with regard to their effects on dendrite extension and branching; relatively few studies have examined regulation of primary dendrite formation.

In this study, we examine the effects of BDNF on primary dendrite formation in cortical neurons and investigate the signaling pathways that mediate these effects. We find that BDNF induces primary dendrite formation via a protein synthesis-independent mechanism. The MAP kinase (MAPK), PI3-kinase (PI3-K) and PLC- γ 1 pathways are rapidly activated in response to BDNF, and inhibiting the PI3-K and MAPK pathways completely prevents BDNF-induced dendrite formation in dissociated neurons and cortical slices. Activation of the PI3-K pathway, but not the MAPK pathway, is sufficient to drive primary dendrite formation. These results identify the PI3-K and MAPK pathways as crucial for BDNF-induced dendrite formation, and together with previous studies suggest that these two signaling pathways link neurotrophin receptor activation to primary dendrite formation in developing neurons.

METHODS

Primary Cell Cultures

E18 cortical neurons from Long-Evans rats were cultured as described (Threadgill et al., 1997). Cells were plated at 0.9×10^6 per well (12-well plate) on poly-D-lysine and laminin coated glass coverslips, or at 3×10^6 per 60-mm plate. Cells were grown in Basal Medium Eagle (all media supplies from Gibco, Rockville, MD) supplemented with 1% N2 and 5% fetal bovine serum, or in Neurobasal Media supplemented with 2% B27. BDNF (50 ng/mL) was added to the cultures at the indicated time points before fixation at 4 days *in vitro*. For pharmacological experiments, inhibitors were added 30 min prior to addition of BDNF. LY294002, U73122, cycloheximide (Biomol, Plymouth Meeting, PA) and U0126 (Promega, Madison, WI) were used at a final concentration of 50 μ M, 5 μ M, 20 μ M and 10 μ M, respectively. Control cultures received a similar amount of DMSO, which was the organic solvent for all inhibitors.

Plasmids

The GFP expression plasmid (pEGFP-N1) was obtained from Clontech (Palo Alto, CA). The expression plasmid for the pleckstrin homology domain of Akt (PKB) fused to GFP has been described previously (Gray et al., 1999). The cDNA for constitutively active PI3-K (p110 subunit containing a CAAX membrane-targeting sequence; Wennstrom and Downward, 1999) was subcloned into pCI (Promega). The expression plasmid for constitutively active MEK (con-

taining the S218E/S222E substitutions) has been described previously (Seeger et al., 1994). The expression plasmid for BDNF was created by subcloning a full-length BDNF cDNA containing a 3' HA tag into the pEF1 α -BOS vector.

Transfections and Live Imaging of Transfected Neurons

Transfections were carried out using a calcium-phosphate precipitation method that exclusively transfects postmitotic neurons, which has been described previously (Threadgill et al., 1997). For morphological analyses, cultures were transfected at 2 days *in vitro* with either a GFP expression vector alone or in a 1:3 ratio with the expression plasmid for either constitutively active MEK or PI3-K. For imaging experiments dissociated cortical neurons were cultured on poly-D-lysine and laminin coated glass bottom dishes (MatTek Corp., Ashland, MA) and transfected after 2 days *in vitro*. The following day, Hepes pH 7.4 was added to a final concentration of 20 mM, and the dish was placed in a stage heater (Medical Systems Corp., Greenvale, NY) set at 37°C. Images were captured each minute at 60 \times with a Nikon TE300 using an Orca II camera (Hamamatsu, Japan) driven by an Openlab software automation (Improvision, Lexington, MA). BDNF was bath added to a final concentration of 50 ng/mL.

Slice Cultures and Transfections

P7 Long Evans rats were decapitated, the brains quickly removed, glued with the caudal side down to a block, and placed in a vibratome filled with ice-cold HBSS; 400- μ m thick coronal sections were collected in ice-cold HBSS and slices were bisected with a microdissection knife along the midline. Slices were plated (two slices per well) on 1.0 micron membrane inserts (Becton Dickinson, Bedford, MA) previously coated with poly-D-lysine and laminin. The culture medium consisted of 50% basal medium eagle, 25% HBSS, 25% horse serum, 10 mM Hepes pH 7.4, 35 mM glucose. Slices were transfected using a Helios Gene Gun setup (Bio-Rad, Hercules, CA) at 180 psi, 6 h after slice preparation; 25 mg of 1.6 μ m gold particles were coated with expression plasmids according to Bio-Rad protocols with 50 μ g pEGFP alone, or co-coated with 12.5 μ g pEGFP and 37.5 μ g of the BDNF-HA expression plasmid. LY294002 (50 μ M) or U0126 (10 μ M) were added to the media 14 h after transfection, and cultures were fixed 26 h later.

Western Blots

The culture medium was replaced with Neurobasal medium containing 0.2% B27 16 h prior to stimulation. Cultures were stimulated with BDNF as described under constructs and transfections. Cells were lysed in Tris-buffered saline containing (TBS) 10% glycerol, 1% Nonidet P-40, 2 mM EGTA, 1 mM sodium orthovanadate, and protease inhibitor

cocktail (Roche, Indianapolis, IN). After centrifugation, samples of the supernatant were subjected to SDS-polyacrylamide gel electrophoresis, and blotted onto nitrocellulose paper (Invitrogen, Carlsbad, CA). Blots were probed with monoclonal anti-phospho Erk1/2 (1:2500; clone E10, Cell Signaling Technology, Beverly, MA) polyclonal anti-phospho Akt S473 (1:1000; Cell Signaling Technology), polyclonal anti-phospho-PLC- γ 1 T783 (1:1000; Cell Signaling Technology), polyclonal anti-phospho Trk Y490 (1:1000; Cell Signaling Technology) or monoclonal anti- β -actin (1:10,000; Sigma, St. Louis, MO). Following incubation with a secondary antibody conjugated to HRP, protein bands were detected using chemoluminescence.

Immunofluorescence

At the appropriate times, dissociated and slice cultures were fixed overnight with 4% paraformaldehyde, 4% sucrose in phosphate-buffered saline (PBS). Following washes with PBS, cultures were incubated for 1 h in blocking solution (TBS containing 0.3% Triton X-100, 3% bovine serum albumin, 3% goat serum) followed by an overnight incubation with primary antibodies in blocking solution. Antibodies used included: polyclonal anti-GFP (1:3000; Molecular Probes, Eugene, OR), monoclonal anti-MAP-2 (1:2000; Clone HM-2, Sigma); monoclonal anti-HA (1:400; clone 12CA5, Roche), monoclonal anti-phospho-Erk1/2 (1:250; clone E10, Cell Signaling Technology), polyclonal anti-phospho-Akt Ser473 IHC (1:150, Cell Signaling Technology). Following extensive washes in TBS, cultures were incubated for 5 h with goat-anti-mouse or goat-anti-rabbit secondary antibodies conjugated to either Alexa488 or Alexa568 in blocking solution (Molecular Probes). Coverslips were mounted with Aquamount before visualization.

Analysis and Quantification

GFP-transfected dissociated neurons were captured at 20 \times or 40 \times using an OrcaII digital camera connected to a Nikon TE300 microscope driven by Openlab software. At least 20 neurons were captured per condition in each experiment, and each experiment was repeated multiple times. Primary dendrites and dendritic branches were counted using Openlab software, and dendritic length was determined by tracing all dendritic processes in IP lab software (Scanalytics, Fairfax, VA). Only neurites at least 5 μ m in length were counted as primary branches. Most cultures were double stained for the dendritic marker microtubule-associated protein-2 (MAP2), and the identity of processes as dendrites was verified based on MAP2 immunofluorescence. In slices, primary dendrites of GFP-transfected pyramidal neurons were counted using a 40 \times objective. Cortical layers were identified based on cell density, as revealed by Hoechst staining. Primary dendrites in layer 6 pyramidal neurons were analyzed in three separate experiments. All statistical analyses were performed using Statview 4.5 (SAS institute Inc., Cary, NC). Statistical significance was identified using an ANOVA and Scheffe's post-hoc test, and $p < 0.05$ was

considered statistically significant. All data are shown as mean \pm S.E.M.

RESULTS

BDNF Rapidly Alters Cortical Dendrite Morphology

To explore the time scale of BDNF-induced changes in dendritic morphology, we treated 4-day-old dissociated cortical cultures with BDNF for various durations prior to fixation, and compared dendritic morphology in untreated and BDNF-treated cultures. Dendritic morphology was visualized by transfecting the cultures with a GFP expression plasmid followed by double immunofluorescence using GFP and MAP-2 antibodies. In control cultures, as *in vivo*, neurons displayed a range of dendritic morphologies [Fig. 1(a)]. BDNF treatment induced a surprisingly rapid increase in the number of primary dendrites, while total dendritic length and number of branch-points remained unchanged [Fig. 1(b) and (c)]. Stimulation with BDNF for as short as 5 h was sufficient to induce a significant increase in the number of primary dendrites (GFP+/MAP2+ processes) [Fig. 1(c)]. This suggests that BDNF induces primary dendrite formation in cortical neurons via rapidly activated signaling events.

BDNF Signaling Activates MAP Kinase, PI3-Kinase, and PLC- γ

The observation that BDNF induces primary dendrite formation within 5 h led us to explore the signaling pathways that mediate this effect. BDNF exerts its cellular effects via activation of the TrkB receptor tyrosine kinase. Neurotrophin binding results in phosphorylation of tyrosine residues in the intracellular domain of the Trk receptors. Several of these phosphorylated tyrosines function as docking sites for downstream signaling components (reviewed in Kaplan and Miller 2000; Huang and Reichardt, 2001). Of these, the tyrosines corresponding to the sites Y490 and Y785 in the human TrkA receptor are involved in activation of the three main signaling cascades. Phosphorylation of Y490 is necessary for activation of the mitogen-activated protein kinase (MAPK) and the phosphoinositide-3 kinase (PI3-K) pathways. Y490 recruits the docking protein Shc, which in turn recruits a Grb-2/SOS complex. SOS activates Ras, leading to the activation of Raf, MEK, and MAP kinase (referred to as the MAPK pathway). In addition, Shc also recruits Gab1 through Grb-2,

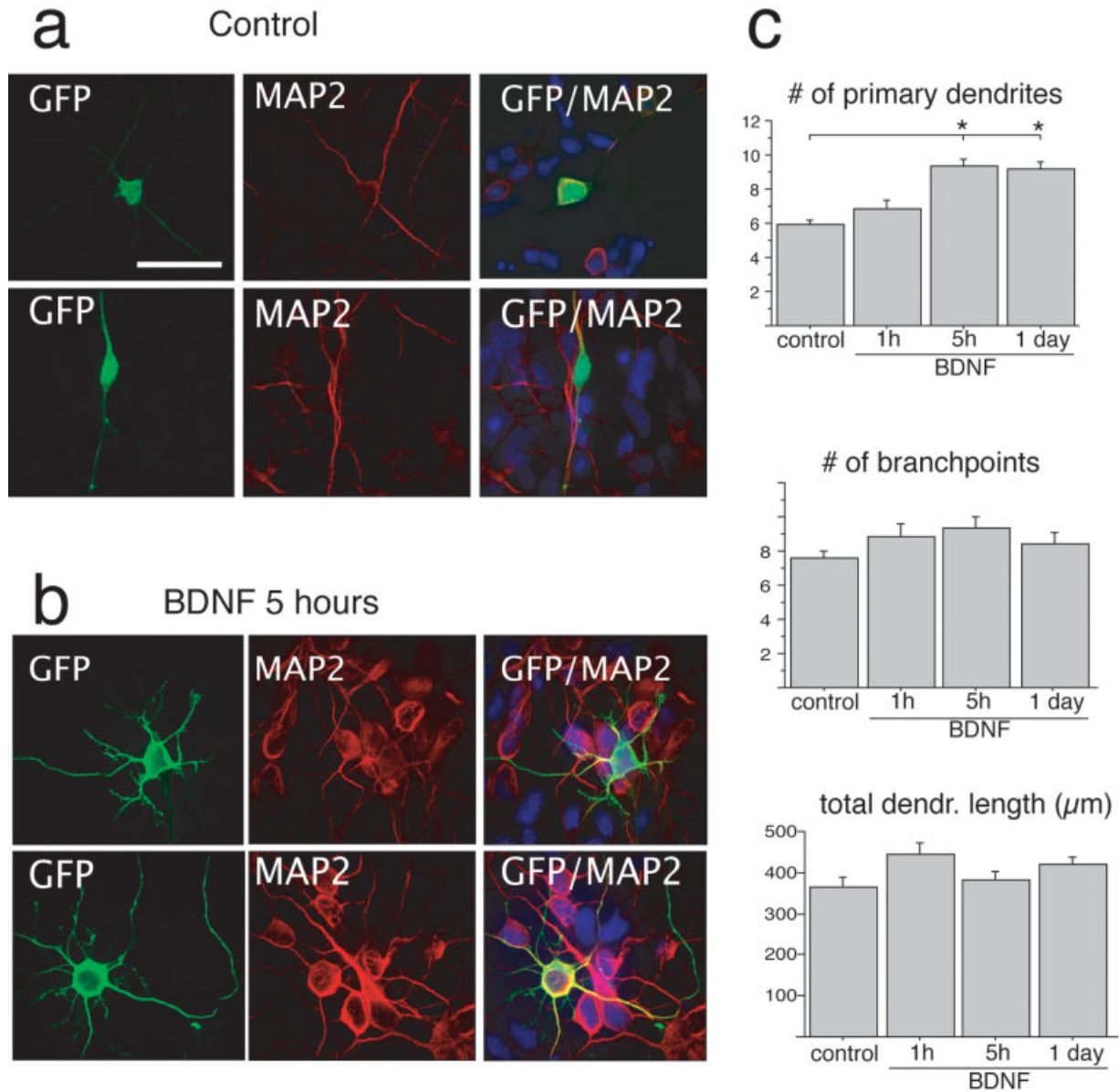


Figure 1 BDNF stimulation rapidly alters dendritic morphology of cortical neurons. Dendritic morphology in dissociated cultures is revealed by GFP immunostaining (green) of GFP-transfected cortical neurons. Dendritic structures are confirmed by co-immunostaining for the dendritic marker MAP-2 (red). Nuclei are visualized by Hoechst staining (blue). (a) Examples of GFP-transfected cortical neurons in culture under control conditions. (b) Examples of GFP-transfected cortical neurons following 5-h stimulation with BDNF. Note that BDNF treatment induces a marked increase in the number of primary dendrites emerging from the soma. (c) Quantification of various parameters of dendritic morphology following different periods of BDNF exposure. Scale bar [for (a) and (b)] = 50 μm .

which activates PI3-K. The phospholipase C- γ 1 (PLC- γ 1) signaling pathway is activated by direct recruitment of PLC- γ 1 to phosphorylated Y785.

Given their central role in Trk signaling, we decided to examine the contribution of the MAP kinase, PI3-K, and PLC- γ 1 pathways in BDNF-induced dendrite exten-

sion. To determine how effectively these pathways are activated in our cortical cultures, we treated the cultures with BDNF and examined activation of various signaling pathways by Western blotting using different phospho-specific antibodies [Fig. 2(a)]. BDNF induced rapid phosphorylation of Trk receptors in cortical neurons, as

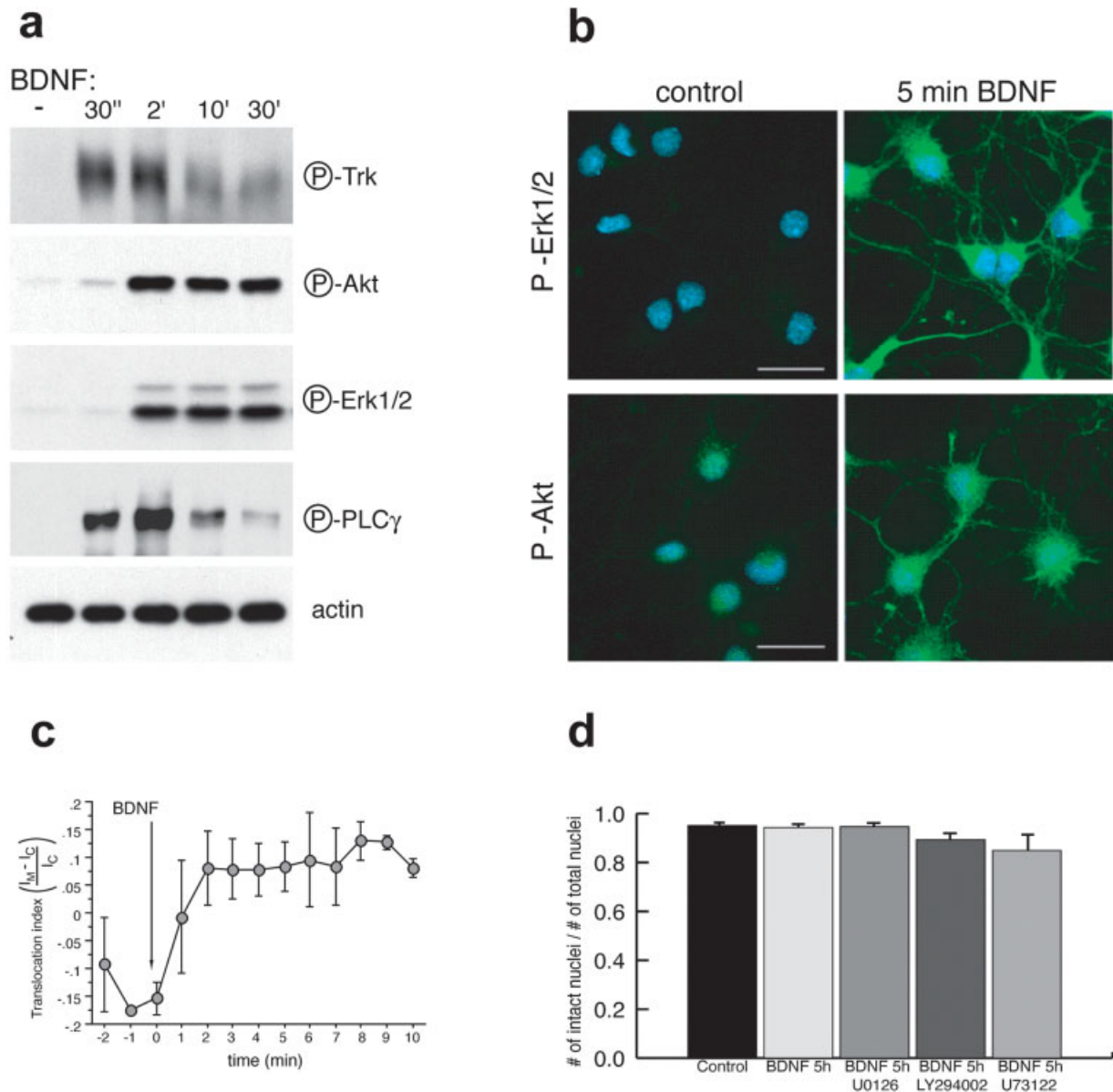


Figure 2 BDNF induces the activation of the PI3-K, MAPK, and PLC- γ 1 in cortical neurons. (a) Western blots of cortical neuron protein extracts probed with phospho-specific antibodies, as indicated. Western blotting for β -actin demonstrates equal protein content in lysates. (b) PI3-K and MAPK are activated both in dendrites and soma in response to BDNF stimulation. Cortical cultures were stimulated with BDNF for 5 min and immunostained for phosphorylated Akt and Erk1/2 (green), and counterstained with the Hoechst nuclear stain (blue). In contrast to control cultures, strong immunostaining for both p-Akt and p-Erk1/2 can be detected in the perinuclear region and in all dendrites of BDNF-treated cortical neurons. (c) Translocation of PH-Akt-GFP to the membrane following BDNF stimulation. Increase in membrane localization of PH-Akt-GFP is apparent within 1 min of BDNF application, indicating rapid activation of P13 kinase. (d) Pharmacological inhibitors LY294002, UO126, and U73122 do not affect viability of cortical neurons within at least 5 h after addition. Cortical cultures were subjected to treatment as indicated, and nuclei were stained with the Hoechst nuclear stain. The number of intact nuclei was counted and expressed as a ratio of the total number of nuclei. As shown, there is no difference in the intact nuclei/total nuclei ratio for control cultures, BDNF cultures, and cultures treated with BDNF together with LY294002, UO126, or U73122. I_m = intensity at the plasma membrane, I_c = intensity in the cytoplasm. Scale bar in (b) = 25 μ m.

revealed by an antibody that recognizes Trk receptors phosphorylated at position Y490. Activation was maximal within 30 s of stimulation, and persisted for up to 30 min [Fig. 2(a)]. Activation of the MAPK pathway was analyzed by probing the blot with an antibody that specifically recognizes dually phosphorylated Erk1 and 2 (pErk1/2). The MAPK pathway was maximally activated 2 min after BDNF treatment and remained strongly activated for at least 30 min [Fig. 2(a)]. The PI3-K pathway was also maximally activated 2 min after stimulation as revealed by phosphorylation of Akt (pAkt), a well-known target of PI3-K [Fig. 2(a)]. Western blotting using an antibody against phosphorylated PLC- γ 1 showed that the PLC- γ 1 pathway was robustly activated within 30 s of BDNF application, and began to decline within 10 min. Thus, BDNF stimulation leads to the robust activation of the MAP kinase, PI 3-kinase, and PLC- γ pathways in cortical neurons. The distinct time courses of activation and inactivation of these pathways is consistent with the view that these pathways are regulated by different receptor-linked biochemical events.

To determine whether the MAPK and PI3-K pathways were activated within dendrites, cortical neurons were treated with BDNF and processed for pErk1/2 or pAkt immunofluorescence. In untreated cultures, pErk1/2 and pAkt immunofluorescence was virtually undetectable [Fig. 2(b)]. BDNF stimulation led to marked increases in pErk1/2 and pAkt immunofluorescence in the cell body and dendrites within 5 min of stimulation [Fig. 2(b)]. Thus activation of the PI3-K and MAPK pathways in response to BDNF stimulation occurs in both the somatic and dendritic compartments.

PI3-K phosphorylates phosphatidylinositol lipids at the D3 position to generate PI-(3,4)P2 and PI-(3,4,5)P3, which serve as docking sites for proteins containing PH domains (Fruman et al., 1998). Therefore, the PH domain of Akt fused to GFP can serve as a sensitive probe to study the localization of PI3-K activation. Cortical neurons transfected with Akt-GFP showed uniform distribution of GFP fluorescence in the cytoplasmic compartment. Stimulation with BDNF led to a rapid redistribution of some of the GFP signal to the membrane [Fig. 2(c)], suggesting that BDNF stimulation leads to activation of PI3-K near the membrane.

BDNF-Induced Dendrite Formation in Cortical Cultures Requires MAP Kinase and PI3-Kinase Activation

We next asked whether activation of known intracellular targets of TrkB signaling was required for BDNF-induced primary dendrite formation. To this end, dissoci-

ated cortical cultures were treated with BDNF for 5 h, while the different signaling pathways were blocked using specific pharmacological inhibitors. Activation of the MAPK, PI3-K, and PLC- γ 1 pathways was blocked using U0126 (10 μ M), LY294002 (50 μ M), and

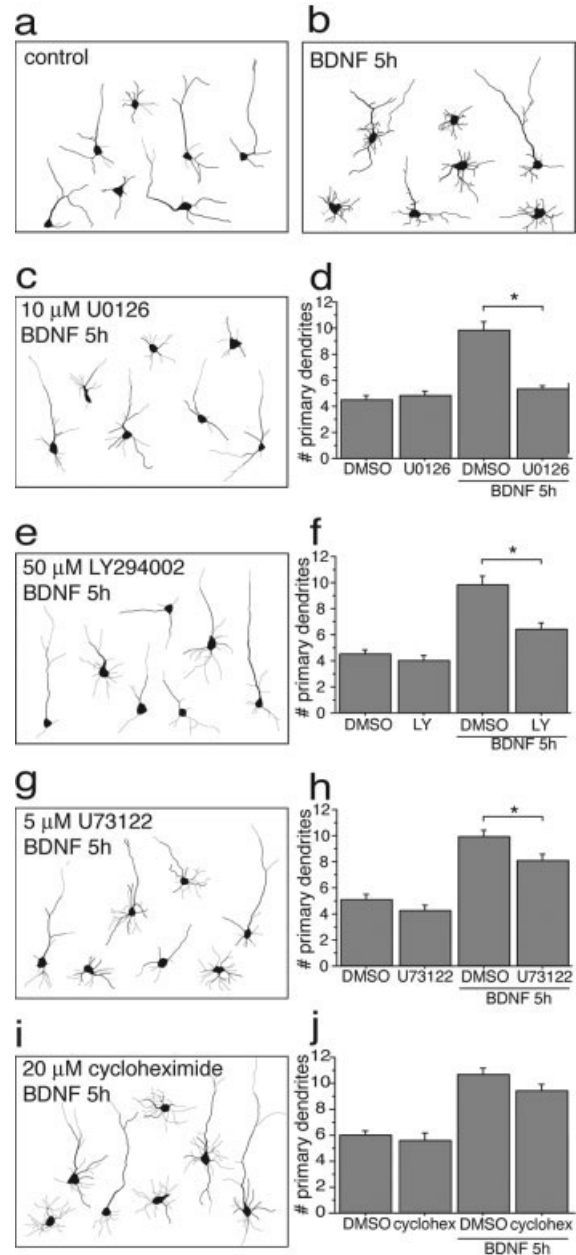


Figure 3 BDNF-induced primary dendrite formation requires MAPK and PI3-K signaling. Camera lucida drawings (a,b,c,e,g,i) and quantitative analysis (d,f,h,j) of GFP-immunostained cortical neurons treated with BDNF under control conditions or in the presence of pharmacological inhibitors indicated. Note that inhibition of MAPK (with U0126) or PI3-K (with LY294002) prevents BDNF-induced increase in primary dendrite formation.

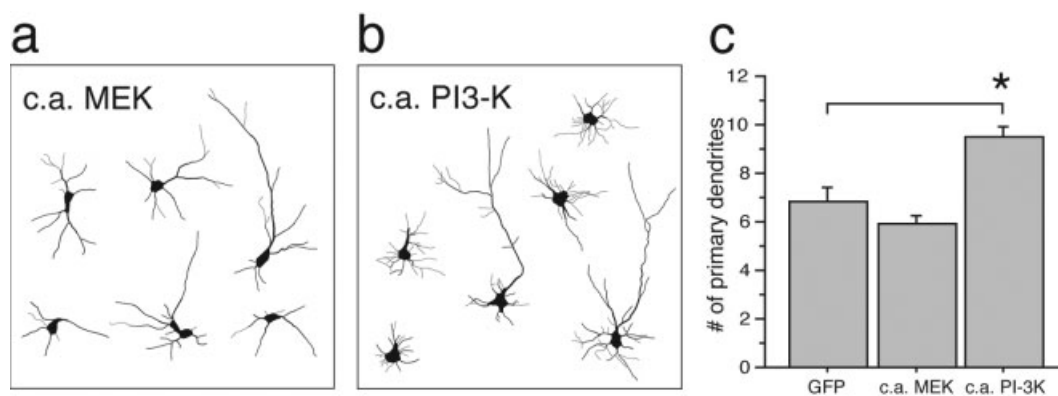


Figure 4 Overexpression of constitutively active PI3-K, but not constitutively active MEK, induces primary dendrite formation in cortical neurons. (a) Neurons transfected with constitutively active MEK do not differ significantly from control neurons [compare with Figs. 1(a) and 3(a)]. (b) Neurons expressing a constitutively active form of PI3-K show an increase in number of primary dendrites. The morphology of these neurons is similar to BDNF-treated neurons [compare with Figs. 1(b) and 3(b)]. (c) Quantitative analysis of primary dendrites in neurons transfected with a control plasmid (GFP) or with constitutively active forms of MEK or PI3-K.

U73122 (5 μ M), respectively (Jin et al. 1994; Duncia et al., 1998; Davies et al., 2000; Atwal et al., 2000). The efficacy of block was confirmed by Western blotting using phospho-specific antibodies (data not shown). In all cases, treatment of control cultures for 5 h with the inhibitors did not affect the number of primary dendrites (Fig. 3) or the viability of the cultures [Fig. 2(d)]. As previously described, BDNF stimulation of control-treated cultures resulted in formation of new primary dendrites [Fig. 3(b)]. However, blocking the MAPK pathway using U0126 completely abolished BDNF-induced dendrite formation in cortical neurons [Fig. 3(c) and (d)]. Similar results were obtained when the PI3-K pathway was blocked with the inhibitor LY294002 [Fig. 3(e) and (f)]. In contrast, inhibiting the PLC- γ 1 pathway attenuated, but did not prevent, BDNF-induced dendrite formation [Fig. 3(g) and (h)].

Since many of the cellular effects of MAPK and PI3-K activation involve signaling to the nucleus, we used pharmacological inhibitors to determine if BDNF-induced dendrite formation requires new protein synthesis. As shown in Figure 3(i, j), the protein synthesis inhibitor cycloheximide did not affect primary dendrite formation in response to BDNF. These results indicate that the rapid effects of BDNF on primary dendrite formation require MAPK and PI3-K signaling, but are protein synthesis-independent.

We next wanted to determine if activation of the MAPK or PI3-K was sufficient to induce primary dendrite formation in cortical neurons. For these experiments we transfected cortical cultures with constitutively active MEK or PI3-K after 2 days *in vitro*, and analyzed the number of primary dendrites 2 days

later. Expression of constitutively active PI3-K led to a significant increase in the number of primary dendrites [Fig. 4(b) and (c)]. In contrast, a constitutively active MEK construct did not alter the number of primary dendrites compared to control neurons [Fig. 4(a) and (c)]. Therefore, constitutive activation of the PI3-K pathway, but not the MAPK pathway, is sufficient to induce primary dendrite formation in cortical neurons.

BDNF-Induced Dendrite Formation in Layer 6 Cortical Neurons Requires MAP Kinase and PI3-Kinase Activity

In the final set of experiments, we examined the role of various signaling pathways in regulating dendritic development in a more physiological context. Since most of cortical dendritic growth and remodeling occurs postnatally, we used organotypic slice cultures to examine the signaling mechanisms that mediate BDNF-induced primary dendrite formation in postnatal cortical slices. Pyramidal neurons in the deeper cortical layers have achieved their basic dendritic morphology by postnatal day 7 (P7), but the dendritic tree is still quite immature (Miller, 1981; Juraska and Fifikova, 1979). To visualize the dendritic morphology of these neurons, we transfected P7 cortical slices with GFP using particle-mediated gene transfer (Lo et al., 1994). Slices were fixed 40 h after transfection, and neurons were visualized by anti-GFP immunofluorescence. GFP-expressing layer 6 pyramidal neurons had a single apical dendrite and several distinct basal dendrites of varying lengths [Fig. 5(a)]. BDNF trans-

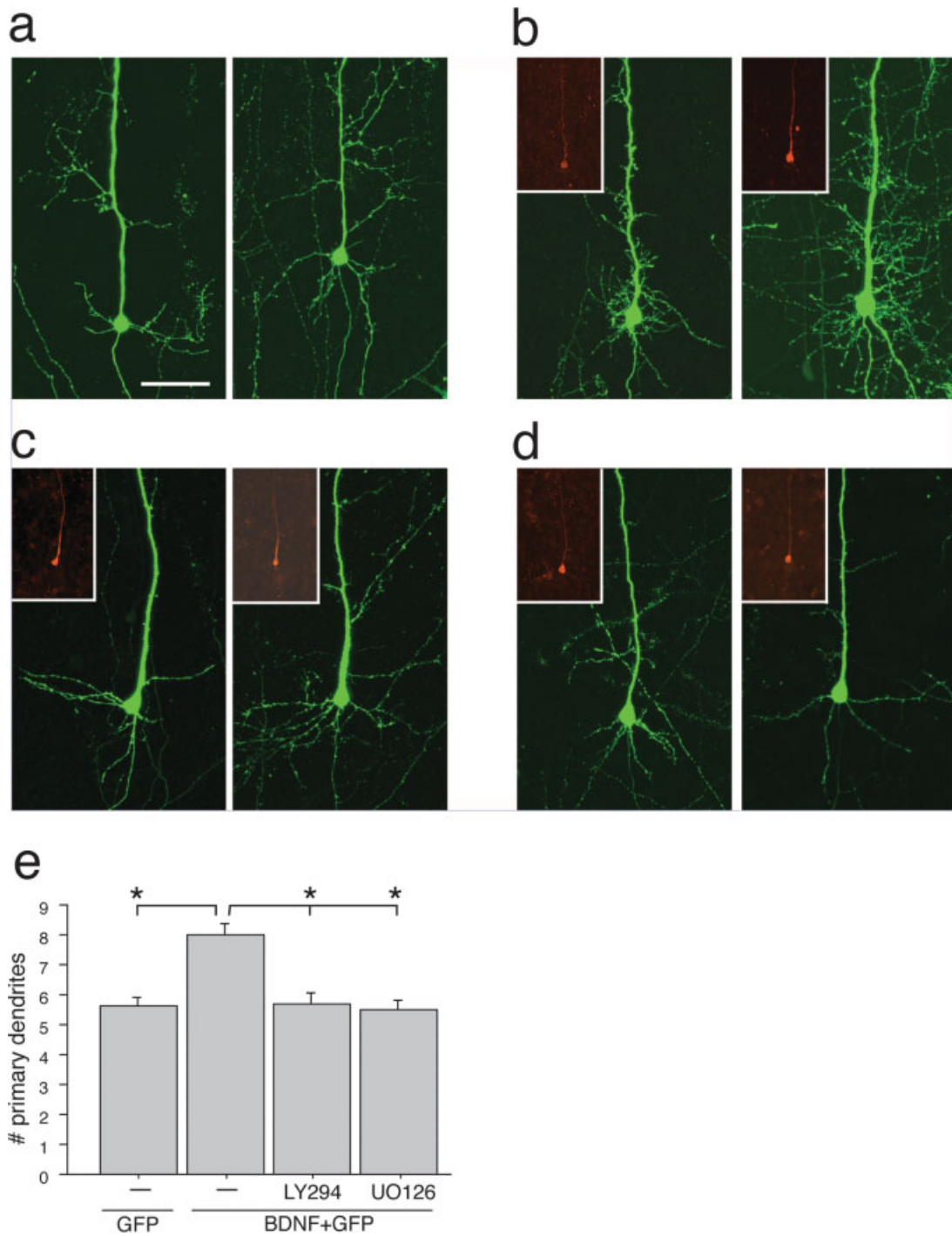


Figure 5 BDNF induces primary dendrite formation in cortical layer 6 pyramidal neurons in a PI3-K and MAPK dependent manner. (a) Examples of layer 6 neurons in rat P7 cortical slices transfected with GFP. These neurons typically contain a single apical dendrite and several primary basal dendrites. (b) Examples of cortical layer 6 neurons transfected with GFP together with an HA-tagged BDNF expression vector. BDNF expression induces the formation of many relatively short unbranched primary dendrites. (c,d) Examples of cortical layer 6 neurons transfected with GFP and BDNF, and treated with inhibitors of PI3-K (LY294002) (c), or MAPK (U0126) (d). (e) Quantification of number of primary dendrites in GFP-transfected cortical layer 6 neurons under indicated conditions. Inserts in (b,c,d) show localization of the transfected BDNF construct based on HA immunofluorescence. Scale bar in (a) = 50 μ m. All neurons are displayed at the same scale.

fection in rat cortical slices led to a dramatic increase in dendritic complexity, as has been previously reported for ferret cortical slices (Horch et al., 1999). Specifically, BDNF-expressing layer 6 pyramidal neurons had a greater number of basal dendritic branches than controls [Fig. 5(b) and (e)]. In addition, BDNF-transfected neurons had more short side branches emerging from the proximal part of the apical dendrite. To determine if the PI3-K and MAPK pathways were involved in mediating BDNF-induced dendrite formation in layer 6 cortical neurons, the inhibitors LY294002 or U0126 were added to the medium 14 h after transfection and slices were fixed 26 h later. Similar to what we observed in dissociated cell cultures, inhibiting either the PI3-K [Fig. 5(c)] or the MAPK [Fig. 5(d)] pathway completely blocked BDNF-induced primary dendrite formation [Fig. 5(e)]. These results confirm that activity of the PI3-K and MAPK pathways mediate the effects of BDNF on dendrite formation.

DISCUSSION

Our experiments indicate that BDNF exerts an important effect on primary dendrite formation and further supports a role for BDNF in cortical dendrite development (McAllister et al., 1996; Horch et al., 1999). The focus of our study was to examine the intracellular signaling mechanisms that mediate the effects of BDNF on dendrite development. We found that BDNF promotes the formation of new primary dendrites within a period of hours. Surprisingly, BDNF-induced initiation of new dendrites within this period does not require protein synthesis. Pharmacological and molecular perturbation experiments indicate that BDNF-induced dendrite formation requires activation of the MAPK and PI3-K signaling pathways, with a minor role for the PLC- γ 1 pathway. These experiments provide the first glimpse of intracellular effectors that regulate dendritic development in response to BDNF stimulation.

Our results are consistent with previous findings showing marked effects of BDNF on dendritic development. BDNF appears to be more important for primary dendrite formation than for dendrite extension. BDNF stimulation induces the rapid formation of highly dynamic primary basal dendrites (Horch et al., 1999). This increase in proximal basal dendrites is at the expense of more distal dendritic branches. A similar phenotype is observed following overexpression of the BDNF receptor *trkB* in pyramidal neurons (Yacoubian and Lo, 2000). These neurons also displayed an increase in proximal primary dendrites,

while more distal branches are lost. Interestingly, overexpression of a dominant negative truncated form of BDNF had an opposite effect, leading to fewer primary dendrites, and longer distal branches. Our observations suggest that BDNF can induce these changes rapidly, and independent of new protein synthesis. Many of these effects can be explained based on an effect of BDNF on cytoskeletal stability.

How do the PI3-K and MAPK pathways mediate the morphological changes associated with BDNF-induced dendrite remodeling? The fact that BDNF can induce dendrite formation independent of new protein synthesis suggests that these pathways regulate dendrite development by directly regulating cytoskeletal components. In support of that possibility, PI3-K activation has been implicated in regulating actin cytoskeleton dynamics through regulation of Rho family GTPases, and Rho GTPases have a central role in mediating dendritic growth and remodeling in developing neurons (Threadgill et al., 1997; Redmond and Ghosh, 2001; Luo, 2000). These proteins are activated by guanosine exchange factors (GEFs) that switch Rho family GTPases from an inactive GDP-bound state to an active GTP-bound state (reviewed in Kjoller and Hall, 1999). The activity of many GEFs can be modulated by PI3-K activation, providing a possible link between the PI3-K pathway and dendrite formation through Rho family GTPases (Han et al., 1998; Ma et al., 1998; Nimmual et al., 1998). Indeed, it was recently reported that the Rho GTPase member Rac1 is activated in the PC12 cell line in response to neurotrophin signaling in a PI3-K dependent manner (Yasui et al., 2001), and we have found that BDNF induces Rac1 activation in cortical neurons (P. A. D. and A. G., unpublished observation). We have also found that transfection of Rho-GAP, a protein that suppresses the activity of Rho family GTPases, inhibits the ability of BDNF to induce new dendrites, suggesting that Rho family GTPases are important effectors of the dendritic effects of BDNF.

Compared to the relationship between PI3-K activation and cytoskeletal control, less is known about the mechanisms by which MAP kinase may contribute to dendrite formation. It is noteworthy, however, that the MAPK pathway has been implicated in regulating microtubule dynamics, which is likely to be important for dendrite formation (Reszka et al., 1997; reviewed in Gundersen and Cook, 1999). Activated Erk1 and 2 associate with microtubules (Morishima-Kawashima and Kosik, 1996; Reszka et al., 1995), and can phosphorylate several microtubule-associated proteins (Hoshi et al., 1992). Indeed, it was recently reported that NGF supported dendrite development of sensory neurons through its activation of the MAP

kinase pathway and subsequent phosphorylation of MAP2 (Vaillant et al., 2002). Thus, dual activation of the PI3-K and MAPK pathways may be necessary to regulate actin and microtubule dynamics, respectively.

Our experiments also show that activation of the PI3-K pathway alone, but not the MAP kinase pathway, is sufficient to induce primary dendrite formation. This is surprising given the observation that BDNF-induced dendrite formation requires both the PI3-kinase and MAP pathways. One possible explanation is that MAP kinase plays a permissive role in dendrite development, and that basal levels of MAPK activity might cooperate with induced activation of PI3-kinase to drive dendrite formation.

The MAPK and PI3-K pathways have previously been implicated in neurotrophin-mediated survival and axon outgrowth (Datta et al., 1999; Atwal et al., 2000; Kuruvilla et al., 2000; Markus et al., 2002), and here we show that they play an important role in dendritogenesis. Given that these kinases appear to regulate multiple cellular events, it is worth considering how the same signaling molecules might mediate such diverse functions. One possibility is that these kinases regulate survival, axon outgrowth, and dendrite formation via distinct secondary effectors. In support of this proposition, it is noteworthy that MAPK activation mediates neurotrophin-dependent survival via phosphorylation of the transcription factor CREB (Xing et al., 1996; Riccio et al., 1999), whereas the dendritogenic effects of BDNF are protein synthesis-independent. If indeed the primary signaling targets of neurotrophins regulate distinct cellular responses via activation of distinct secondary effectors, one wonders how the same signaling molecules lead to activation of distinct secondary effectors. One possibility is that the effectors for the different cellular responses may be present in spatially restricted domains. Neurons are highly polarized cells, and it is becoming clear that proteins that function in the same signaling cascades colocalize at specialized cellular sites (Teruel and Meyer, 2000). Therefore, the signaling components localized in the axon or dendrite might be different from those localized close to the cell body. Even if activation of the Trk receptors led to activation of core signaling components such as PI3-K and MAPK throughout the neuron, signaling specificity could be achieved by restricted localization of downstream effectors close to the activated receptor. In future studies, it will be of interest to identify the downstream effectors of PI3-K and MAPK that mediate BDNF-induced dendrite formation, and to determine if they are indeed distinct from those that mediate differentiation and survival.

We thank Julian Downward for providing us with the constitutively active p110 construct, Alexander Gray for his gift of the PH-Akt-GFP fusion construct, and Dong Xia Li for providing us with the constitutively active MEK construct. BDNF was a gift from AMGEN Inc. We thank Vivian Fenstermaker for help with the slice transfection experiments, Inga Gurevich for help with dissociated cultures and preparing figures, members of the Ghosh Lab for suggestions and comments, and LeeAnna Ghosh for help in preparation of the manuscript.

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