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CREB: A Major Mediator of Neuronal Neurotrophin Responses

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Summary

Neurotrophins regulate neuronal survival, differentiation, and synaptic function. To understand how neurotrophins elicit such diverse responses, we elucidated signaling pathways by which brain-derived neurotrophic factor (BDNF) activates gene expression in cultured neurons and hippocampal slices. We found, unexpectedly, that the transcription factor cyclic AMP response element-binding protein (CREB) is an important regulator of BDNF-induced gene expression. Exposure of neurons to BDNF stimulates CREB phosphorylation and activation via at least two signaling pathways: by a calcium/calmodulin-dependent kinase IV (CaMKIV)-regulated pathway that is activated by the release of intracellular calcium and by a Ras-dependent pathway. These findings reveal a previously unrecognized, CaMK-dependent mechanism by which neurotrophins activate CREB and suggest that CREB plays a central role in mediating neurotrophin responses in neurons.

Introduction

The neurotrophins are a family of secreted proteins that potently regulates diverse neuronal responses. Family members include nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin 4/5 (NT4/5). Neurotrophins, released by target neurons, regulate the type and the number of afferent synapses by promoting the survival of discrete neuronal subpopulations (Hyman et al., 1991; Oppenheim et al., 1992; Sendtner et al., 1992; Arenas and Persson, 1994). Neurotrophins also regulate differentiation, influence cell fate choices, and regulate neurite morphology (Snider, 1988; Diamond et al., 1992a, 1992b; Ip et al., 1993a). Neurotrophins alter the synaptic connectivity of the developing cortex (Castren et al., 1992; Maffe et al., 1992; Cabelli et al., 1995; Prakash et al., 1996) and possibly synaptic strength in adult animals (Kang and Schuman, 1995; Korte et al., 1995; Figurov et al., 1996). Clearly, neurotrophins elicit remarkably diverse but specific responses and are central regulators of the structure and function of the nervous system throughout development and adulthood.

How neurotrophins mediate their diverse responses in neurons remains unclear. Some responses, such as rapid changes in synaptic activity, occur quickly and probably result partly from the activation of second messengers or kinases that influence ion channel function, neurotransmitter release, or synaptic structure (Lohof et al., 1993; Levine et al., 1995). Other responses, such as neurotrophin-induced differentiation, occur slowly and depend on new gene expression. NGF induces differentiation of the pheochromocytoma cell line PC12 partly by initiating a cascade of immediate-early genes (IEGs), many of which regulate the expression of particular late response genes (LRGs) (Halegoua et al., 1996). Several of the LRGs encode proteins that comprise the differentiated phenotype, such as voltage-dependent ion channels and the enzymes and receptors necessary to synthesize and respond to certain neurotransmitters (Dichter et al., 1977; Garber et al., 1989; Fanger et al., 1995; Toledo-Aral et al., 1995). Little is known about how neurotrophins regulate gene expression in neurons, but new gene expression is likely to be important for neuronal differentiation during development as well as the long-term adaptive responses of mature neurons.

Work in PC12 cells has revealed mechanisms by which the neurotrophin NGF signals to induce gene expression (Klein et al., 1990; Vetter et al., 1991; D'Arcangelo and Halegoua, 1993; Kaplan and Stephens, 1994; Greene and Kaplan, 1995). NGF binds and dimerizes its cognate receptor, TrkA; dimerization activates the receptor's intrinsic tyrosine kinase. Activated TrkA autophosphorylates several tyrosine residues found within the receptor's cytoplasmic domain. The phosphotyrosines serve as docking sites for adapter proteins and kinases such as phospholipase Cγ (PLCγ), phosphatidylinositol-3′ kinase (PI3K), and the adapter protein SHC (Soltoff et al., 1992; Marsh et al., 1993; Ng and Shooter, 1993; Widmer et al., 1993; Stephens et al., 1994). These molecules trigger kinase cascades that culminate in the phosphorylation and activation of transcription factors that direct gene expression (D'Arcangelo and Halegoua, 1993). For example, SHC triggers the activation of the small GTP-binding protein Ras and the subsequent sequential phosphorylation and activation of the kinases Raf, mitogen- and extracellular-regulated kinase (MEK), and extracellular-regulated kinase (ERK). ERK activates a ternary complex transcription factor (TCF), such as Elk-1 or serum response factor accessory protein (SAP), that forms a complex with a dimer of serum response factor molecules (SRF) to regulate gene expression through the serum response element (SRE). The prevailing view is that NGF and other growth factors induce transcription mainly through the Ras/ERK/pp90 ribosomal S6 kinase (RSK) pathway and the SRE via a TCF such as Elk-1 (Segal and Greenberg, 1996). However, several lines of evidence suggest that other DNA response elements also contribute to neurotrophin responses (D'Arcangelo et al., 1996; Gaiddon et al., 1996; Iwata et al.,

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1996). In PC12 cells, neurotrophins transactivate cyclic AMP response element-binding protein (CREB) by inducing its phosphorylation on a critical residue (Ser-133) that enables CREB to mediate gene expression cooperatively (Bonni et al., 1995). Nevertheless, in neurons, it remains unknown how neurotrophins activate CREB or whether CREB plays a role in neurotrophin-mediated gene expression (Ip et al., 1993b).

To begin to understand how neurotrophins elicit specific neuronal responses, we sought to identify the signaling pathways that mediate BDNF-induced gene expression in neurons (Klein et al., 1991; Marsh et al., 1993; Ip et al., 1993a, 1993b). Surprisingly, we found that the SRE, a common target of receptor tyrosine-kinase signaling, is largely dispensable for BDNF-induced transcription in neurons. Rather, BDNF activates CREB to induce transcription through its cognate-binding site, the Ca^{2+} and cyclic AMP response element (Ca/CRE), independently of other promoter-bound factors. BDNF activates CREB, in part, by stimulating the release of intracellular Ca^{2+} stores that leads to the activation of a CREB kinase, CaMKIV. Exposure to BDNF also activates the Ras/ERK/RSK pathway that culminates in CREB phosphorylation. In addition, BDNF induced CREB (Ser-133) phosphorylation in neurons from hippocampal slices, suggesting that similar pathways could transduce the BDNF signal in the intact nervous system. Taken together, these findings suggest that CREB plays a central role in mediating neurotrophin responses and that neurons have evolved multiple pathways to mediate neurotrophin signaling to CREB. The identification of a Ca^{2+}/CaMKIV-dependent pathway to CREB suggests new mechanisms by which neurotrophins might locally alter synaptic function or rapidly signal from the synapse to the nucleus over long distances.

Results

To characterize mechanisms that link neurotrophin receptors with gene expression, we traced signaling pathways in reverse from a reporter gene, c-*fos*, back to the receptor that initially triggered c-*fos* induction. The c-*fos* gene is a useful reporter because its message levels are low basally, and they increase rapidly and robustly upon neurotrophin stimulation. Notably, neurotrophins induce c-*fos* transcription by pathways that do not require new protein synthesis. Thus, c-*fos* levels represent a sensitive measure of the immediate activity of the pathways that culminate in c-*fos* transcription. In cortical neurons, we have assayed c-*fos* induction by BDNF following specific genetic and pharmacological manipulations of candidate pathway components. This approach has enabled us to deduce sequentially the DNA elements within the c-*fos* promoter that mediate BDNF responses, the transcription factors that bind to these promoter elements, and the kinase cascades activated by BDNF that culminate in the phosphorylation and activation of the critical transcription factors.

Promoter Requirements

We deduced the DNA regulatory elements that mediate BDNF responses in neurons by assaying the transcriptional responsiveness of a series of c-*fos* reporter plasmids under the control of portions of the wild-type c-*fos* promoter. After transfection of these reporter genes into neurons, we analyzed their ability to mediate BDNF-induced transcription by RNase protection assays. The c-*fos* promoter contains several DNA elements that control stimulus-induced gene transcription, including an SRE located approximately 300 nucleotides 5′ of the initiation start site and a Ca/CRE located approximately 60 nucleotides 5′ of the initiation start site. We first tested whether BDNF induced the expression of both the transfected and endogenous c-*fos* in neurons. When neurons were transfected with a c-*fos* reporter plasmid (pF4) that contains a large region (750 bp) of the wild-type promoter, BDNF induced a response from the transfected c-*fos* gene that mimicked the response of the endogenous c-*fos* gene (Figure 1A). Previous work has shown that the SRE is required for growth factor induction of c-*fos* transcription in a variety of cell types (Segal and Greenberg, 1996). To test whether an intact SRE is necessary to mediate BDNF responses in neurons, we tested whether BDNF could induce transcription of pAF222, a reporter that lacks the SRE and only contains 222 nucleotides of the wild-type promoter. Surprisingly, neurons transfected with pAF222 showed strong BDNF responses, suggesting that the presence of an SRE is not necessary to mediate BDNF-induced gene expression (Figure 1A). This result distinguishes neurons from a variety of other cell types in which the SRE is required for growth factors to induce c-*fos* transcription.

In contrast to the results with pAF222, BDNF failed to activate a reporter that only contained 42 nucleotides of the wild-type promoter (pAF42) (Figure 1A). This suggests that a DNA response element(s) between base pairs 42 and 222, such as the Ca/CRE, mediates BDNF-induced transcription. In other cell types, the Ca/CRE does not confer growth factor responses on its own but has been well characterized as a mediator of cAMP and Ca^{2+} responses (Sheng et al., 1991). In PC12 cells, NGF induces c-*fos* transcription by a cooperative mechanism that depends on interactions between CREB and transcription factors bound at other DNA regulatory elements such as the SRE (Bonni et al., 1995). To test whether the Ca/CRE is sufficient to mediate BDNF responses in neurons, we used a reporter gene (pAF42.Ca/CRE) in which a Ca/CRE was inserted immediately upstream of the minimal promoter. The presence of the Ca/CRE restored BDNF-induced transcription to pAF42, suggesting that the Ca/CRE is capable of conferring BDNF responses in neurons. This result also suggests that Ca/CRE-mediated BDNF responses do not require cooperation between proteins bound to the Ca/CRE and proteins bound to other response elements such as the SRE (Figure 1A). Insertion of an SRE immediately 5′ of a minimal promoter was also sufficient to mediate BDNF-induced c-*fos* transcription in neurons, indicating that while the SRE is not essential for a BDNF response, this element can confer a transcriptional response to BDNF (Figure 1A).

The preceding experiments suggest that the Ca/CRE plays a central role in mediating BDNF-induced c-*fos* transcription. However, these conclusions are based on experiments using reporter constructs in which large portions of the c-*fos* promoter have been deleted or the

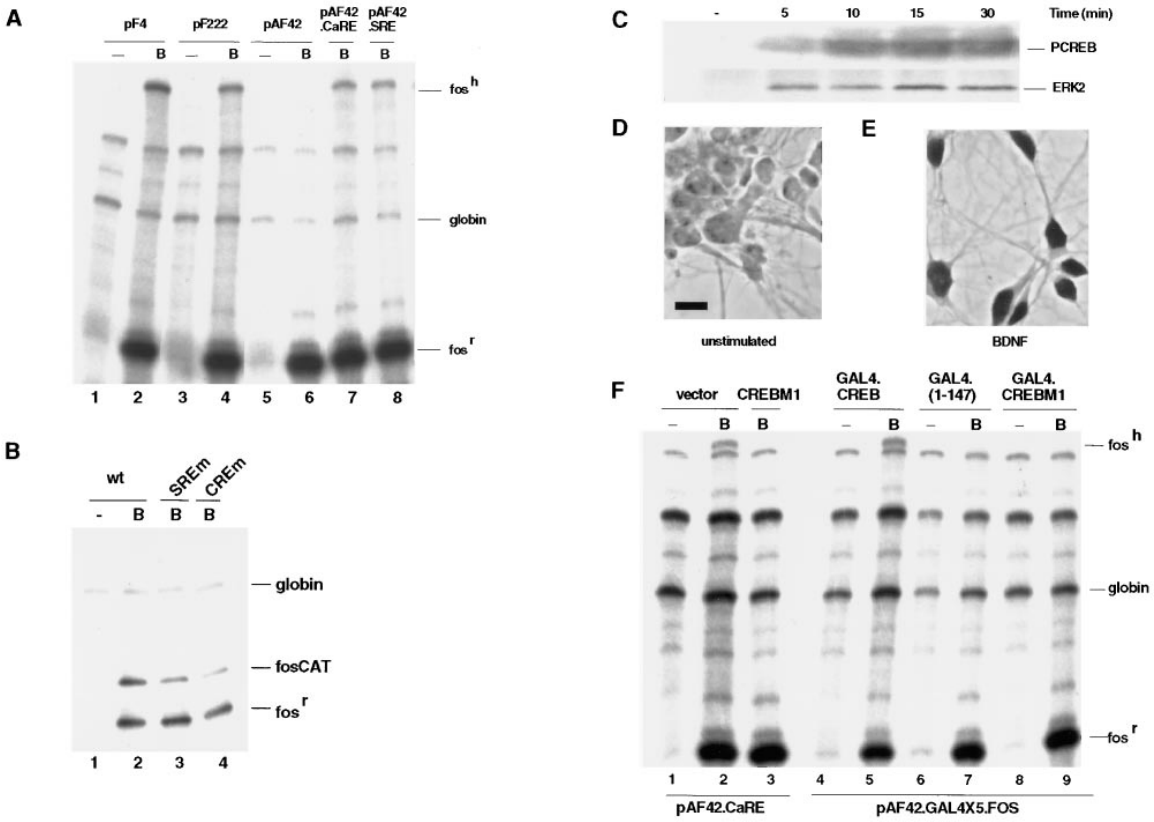

Figure 1. BDNF Induces Gene Transcription in Neurons through the Calcium and Cyclic AMP Response Element (Ca/CRE) via CREB

(A) RNase protection analysis shows BDNF responses from primary cortical neurons transfected with reporter plasmids that contain the human c-*fos* (fos^h) gene under the control of different portions of the wild-type promoter (see text for details). The c-*fos* probe protects the transfected fos^h transcript and a smaller fragment of the endogenous rat c-*fos* (fos^r) transcript. The α-globin gene is cotransfected and probed as an internal control for transfection efficiency and RNA recovery. Neurons were either left untreated (−) or stimulated with BDNF (50 ng/ml) for 1 hr before lysis. BDNF robustly induced fos^h transcription in neurons transfected with a reporter (pF4) that contains the wild-type promoter (B) (lanes 1 and 2) or a reporter (pF222) in which the SRE has been deleted (lanes 3 and 4) but not a reporter (pAF42) in which the Ca/CRE has also been deleted and only 42 bp of the promoter 5′ of the start site remain (lanes 5 and 6). Insertion of either a minimal Ca/CRE sequence (lane 7) or SRE sequence (lane 8) 5′ of the inactive promoter (pAF42.SRE) restores the ability of BDNF to stimulate c-*fos*^{h} transcription. Similar results were obtained from three separate experiments.

(B) The Ca/CRE is an important mediator of BDNF responses in the context of the full promoter. An RNase protection analysis shows neuronal BDNF responses mediated by transfected reporter constructs that express a fusion gene of c-*fos* and choline acetyl transferase gene under the control of mutated or wild-type portions of the c-*fos* promoter (fosCAT, see text and Figure 1A legend for details). Neurons were either left untreated (−) or stimulated with BDNF (50 ng/ml) for 1 hr before lysis. BDNF robustly induced fosCAT transcription from a reporter under the control of a portion of the wild-type promoter (356 bp 5′ of the initiation start site) (B) (lanes 1 and 2: 8.7- $\pm$ 0.2-fold induction). BDNF induced 36% less fosCAT message levels from neurons transfected with SREm.fosCAT, a reporter in which the SRF-binding site has been mutated (lane 3: 5.9- $\pm$ 1-fold induction). CREm.fosCAT, a reporter in which the CREB-binding site has been mutated, mediated 62% less BDNF-induced fosCAT expression than the wild-type promoter (lane 4: 3.9- $\pm$ 1-fold induction; ANOVA, $p < 0.03$).

(C) BNDF rapidly induces phosphorylation of Ser-133 of CREB (PCREB) and tyrosine phosphorylation of ERK2 in cortical neurons. Neurons were stimulated with BDNF (50 ng/ml) for the indicated times, lysed, and subjected to Western analysis either with an antibody that specifically recognizes the Ser-133-phosphorylated form of CREB (anti-PCREB) or anti-phosphotyrosine antibodies (4G10 and PY20). The identity and phosphorylation state of ERK2 was confirmed independently using an antibody specifically developed against the phosphorylated form of ERK (anti-phosphoERK2). These findings have been repeated in four similar experiments.

(D and E) BDNF rapidly induces phosphorylation of Ser-133 of CREB within the nucleus of cortical neurons. Neurons were left unstimulated (D) or stimulated (E) with BDNF (50 ng/ml) for 15 min, fixed, probed with the anti-PCREB antibody, and visualized using diaminobenzidine (DAB) staining. This staining pattern occurred in three separate experiments. Scale bar = 20 μm.

(F) BDNF mediates gene expression through CREB. RNase protection assays show that cotransfection and expression of a dominant-interfering CREB mutant (CREBM1, lane 3) blocks fos^h transcription through the Ca/CRE by BDNF, whereas the empty parent vector does not block BDNF responses (lanes 1 and 2). BDNF-induced gene expression could be reconstituted within neurons by cotransfecting the altered binding site mutant, GAL4CREB, and a fos^h reporter with five GAL4 sites inserted 5′ of the minimal promoter of pAF42 (lanes 4 and 5). By contrast, cotransfection of the GAL4 DNA-binding domain alone without the rest of the CREB moiety or cotransfection with a Ser-133 mutant of GAL4CREB fails to mediate BDNF-induced transcription (lanes 6–9). Similar results were obtained in duplicate experiments.

responsive c-*fos* promoter elements have been moved relative to the initiation start site of transcription. These perturbations could disturb protein–protein interactions at sites besides the SRE or Ca/CRE that normally govern transcription within the c-*fos* promoter. Therefore, we wanted to test whether the Ca/CRE is an important mediator of BDNF-induced c-*fos* transcription by using reporter constructs that contain small mutations in either

the Ca/CRE or SRE within the context of the full promoter. We compared, by RNase protection assay, BDNF responses mediated by neurons transfected with reporters that express a portion of the c-*fos* gene fused to the choline acetyl transferase gene (fosCAT) under the regulation of either wild type or subtly mutated forms of the c-*fos* promoter (Graham and Gilman, 1991; Bonni et al., 1995). Figure 1B shows that BDNF robustly induces fosCAT transcription in neurons transfected with the reporter containing the wild-type promoter (compare lanes 1 and 2). Small in-context mutations in the SRE (CCATATTAGG to ggATATTAcc: SREm.FosCAT) that disrupt SRF binding reduced by one-third the levels of fosCAT induced by BDNF (compare lanes 2 and 3). Disruption of CREB binding by mutating the Ca/CRE (from CACTGCATC to gAgTcgATC: CREm.FosCAT) reduced by half BDNF-induced fosCAT transcription (compare lanes 2 and 4). The fact that small mutations within the Ca/CRE, in the context of the full promoter, significantly reduced BDNF-induced c-*fos* transcription strongly suggests that the Ca/CRE itself and likely proteins bound directly to the Ca/CRE contribute to BDNF responses in neurons. The fact that BDNF induces transcription through the Ca/CRE within c-*fos* is likely to have wide significance, since many neuronal genes contain CREs (Bonni et al., 1995).

We conclude that, in neurons, the Ca/CRE is a major growth factor-responsive DNA element that is at least as potent as the SRE. Consistent with these findings, Gaiddon et al. (1996) have shown that the Ca/CRE also mediates BDNF responses in cerebellar granule cells (Gaiddon et al., 1996). Clearly, BDNF induces transcription in neurons by mechanisms that are somewhat different from those utilized by growth factors in other cell types. The ability of the Ca/CRE to mediate growth factor responses independently of other elements within the c-*fos* promoter may be a property specific to neurons. We have found that the Ca/CRE alone can mediate NGF-induced c-*fos* transcription in TrkA-transfected cortical neurons but not in wild-type PC12 cells or TrkA-transfected nnR5 cells (PC12 cells in which TrkA was mutated) (S. F. and M. E. G., unpublished data). This suggests that intrinsic differences between neurons and PC12 cells rather than stimulus-specific differences between neurotrophins enable CREB, in neurons, to mediate transcription independently of other response elements within the c-*fos* promoter.

CREB: A Major Mediator of Neurotrophin Responses

Having shown that the Ca/CRE is a key neurotrophin response element in neurons and that mutations sufficient to disrupt CREB binding reduce BDNF responses, we set out to define the transcriptional regulators that bind to the Ca/CRE and mediate BDNF-induced c-*fos* transcription. A variety of transcription factors can bind to the Ca/CRE, including CREB, activating transcription factor-1 (ATF-1), and the CCAAT enhancer-binding protein β (C/EBPβ) (Liu et al., 1993; Alberini et al., 1994; Kobayashi and Kawakami, 1995). Gel-shift assays have established that the transcription factor CREB is present in nuclear extracts prepared from neurons and binds to the Ca/CRE (Ginty et al., 1993). In cortical neurons, BDNF robustly induced CREB (Ser-133) phosphorylation within minutes of stimulation, confirming previous findings (Figures 1C–1E) (Bonni et al., 1995).

Having found that CREB is present in the nuclei of cultured neurons and is robustly phosphorylated in response to BDNF, we next sought to determine whether binding of endogenous CREB to the Ca/CRE was required for BDNF-induced pAF42.Ca/CRE-dependent transcription. We tested whether cotransfection and overexpression of a mutant form of CREB (CREBM1) that lacks the essential Ser-133 site necessary for CREB activation would affect BDNF-induced transcription of pAF42.Ca/CRE. CREBM1 has been previously shown to function as a dominant negative, attenuating stimulus-induced CRE-dependent transcription in other cell types (Bonni et al., 1995). We found that BDNF failed to induce Ca/CRE responses in neurons cotransfected with CREBM1 but not with equivalent amounts of the empty vector (Figure 1F). We obtained similar results using a different CREB mutant (KCREB), in which the DNA-binding domain is mutated, but the key Ser-133 site is intact (data not shown) (Walton et al., 1992). The fact that both dominant negative CREBs, functioning by different mechanisms, had similar effects suggests that CREB itself or a closely related family member is required for BDNF-induced Ca/CRE dependent transcription.

To test whether CREB is sufficient to mediate BDNF-induced transcription and whether Ser-133 is necessary for this response, an additional series of experiments were performed. To distinguish transcriptional responses mediated by transfected CREB from responses mediated by endogenous CREB or CREB-related transcription factors, we fused the yeast GAL4 DNA-binding domain to CREB. Upon transfection of the GAL4CREB fusion into neurons, we examined its ability to activate transcription from a pAF42.GAL4X5.FOS reporter in which five tandem GAL4-binding sites were inserted adjacent to the TATA box (Sheng et al., 1991). Proteins in mammalian cells do not normally contain GAL4 DNA-binding domains, so the pAF42.GAL4X5.FOS reporter does not bind any endogenous proteins. Thus, the reporter does not respond to stimuli unless it is cotransfected with a transcriptional regulator such as GAL4CREB that binds to the GAL4 site within the promoter of pAF42.GAL4X5.FOS. We found that BDNF induced transcription from a transfected pAF42.GAL4X5.FOS reporter only if GAL4-CREB was cotransfected and only if the GAL4CREB had a functional Ser-133 site (Figure 1F). Thus, BDNF induces c-*fos* transcription through CREB in a Ser-133-dependent way.

Since GAL4CREB contains a leucine zipper motif that might enable it to form homo- or heterodimers with endogenous leucine zipper-containing transcription factors, it remained possible that GAL4CREB might affect BDNF-induced transcription through interactions with other endogenous transcription factors (Bonni et al., 1995). Thus, it was possible that GAL4CREB could function partly by recruiting to the pAF42.GAL4X5.FOS promoter endogenous transcription factors that were important for BDNF-induced transcription. To test whether CREB alone is sufficient to mediate BDNF responses, we mutated the leucine zipper of GAL4CREB (GAL4-CREBΔLZ). We found that BDNF remained able to induce

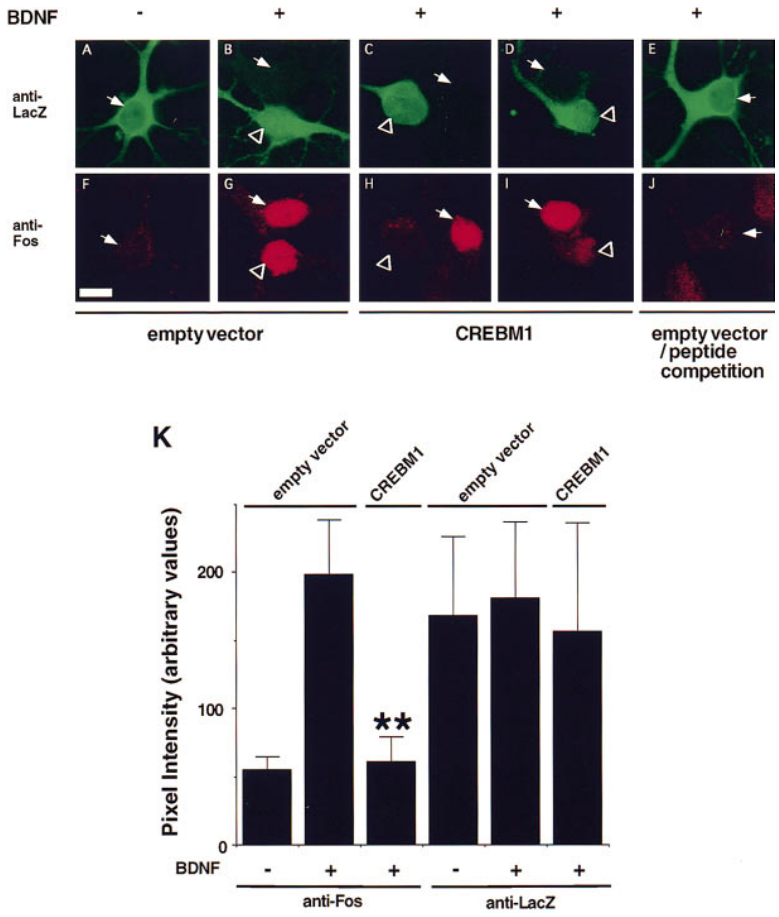

Figure 2. CREB Is a Critical Mediator of BDNF Responses by the Endogenous Gene

Cortical neurons were transfected with an expression plasmid for β-galactosidase (LacZ) and either the empty vector (pRcRSV) or an expression plasmid for dominant-interfering CREB (pRcRSVCREBM1). Two days after transfection, some neurons were left unstimulated and others were stimulated with BDNF (50 ng/ml) for 90 min. After stimulation, neurons were fixed and immunostained against Fos and LacZ (A–J). Transfected neurons typically showed very little Fos immunostaining in the absence of stimulation ([A] and [F], solid white arrow) but in response to BDNF (50 ng/ml), both transfected ([B] and [G], hollow white arrow) and untransfected ([B] and [G], solid white arrow) neurons show striking Fos immunoreactivity. By contrast, neurons transfected with CREBM1 showed minimal BDNF responses ([C], [H] and [D], [I], hollow arrows), although neighboring untransfected neurons showed robust BDNF-induced Fos expression ([C], [H] and [D], [I], solid arrows). These experiments were repeated on over 1000 cells in four different cultures using two different lots of two different c-*fos* antibodies. Fos immunostaining could be largely blocked by preincubation with a 10-fold excess of the peptide against which the antibody was raised (E and J). Scale bar = 20 μm (F). Quantitative confocal microscopy showed that cotransfection of CREBM1 (but not empty vector) significantly reduced (double asterisks = $p < 0.0001$, ANOVA) BDNF-induced Fos levels while not affecting the expression of the transfection marker, LacZ ([K]; see text for details).

transcription from pAF42.GAL4X5.FOS but at reduced levels (GAL4CREB [6.6- ± 2-fold] versus GAL4CREBΔLZ [4.1- ± 2-fold], n = 3, $p < 0.01$ by two-tailed t test). Since CREB mediates responses in the absence of its leucine zipper, we conclude that CREB itself, rather than another dimerization partner, is sufficient to mediate BDNF-induced c-*fos* transcription in neurons. Thus, neurons appear to be a cell type in which CREB is sufficient to mediate a portion of the growth factor response and is required together with other transcription factors for full growth factor-mediated gene expression.

We next wanted to test whether the CREB- and Ca/CRE-dependent pathways revealed by reporter gene assays also mediate BDNF-induced transcription of the endogenous c-*fos* gene. Reporter genes could differ from endogenous genes in ways (e.g., interactions with chromatin) that might alter their respective transcriptional responses. Therefore, we sought to test the role that CREB plays in mediating BDNF responses of the endogenous c-*fos* gene by testing the effect of dominant-interfering CREB expression (CREBM1) on BDNF-induced c-*fos*^{r} expression. Since our method for introducing CREBM1 succeeds in transfecting only a small percentage of neurons, we assayed the level of BDNF-induced c-*fos*^{r} expression in single neurons by immunolabeling with an anti-Fos antibody and quantitatively measuring immunofluorescence by confocal microscopy (Niswender et al., 1995). We transfected neurons with an expression plasmid for the marker, β-galactosidase (LacZ), along with either the expression vector for CREBM1 or the corresponding empty vector and compared BDNF responses by performing Fos and LacZ immunocytochemistry and quantitative confocal microscopy (Figures 2A–2J). We found that BDNF induced Fos expression in untransfected neurons or neurons transfected with the empty vector (3.7- ± 0.9-fold); however, neurons transfected with CREBM1 showed minimal BDNF-induced Fos expression (1.1- ± 0.2-fold) (ANOVA, $p < 0.0001$) (Figure 2K). To assess for the possibility that CREBM1 might be blocking c-*fos* expression by nonspecific mechanisms such as affecting neuronal health, we also quantitated the immunofluorescence of the transfection marker LacZ. We found that there were no statistically significant differences between levels of LacZ within neurons transfected with empty vector or CREBM1 (Figure 2K). Since LacZ is constitutively expressed but is turned over at moderate rates, levels of LacZ likely reflect cumulative expression within hours to days of measurement and might, therefore, be sensitive to nonspecific blockade of gene expression or to the general health of the cell over that period. The fact that CREBM1 specifically blocks BDNF-induced Fos expression without affecting gene expression generally suggests that CREBM1 specifically interferes with the pathways that mediate BDNF-induced expression of endogenous Fos. Together, the reporter gene and Fos

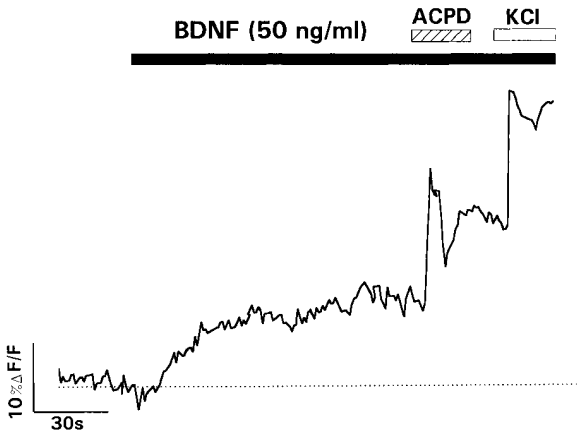

Figure 3. BDNF Induces an Increase in Cytosolic Ca^{2+} in Cortical Neurons

Cortical neurons were bath loaded with the Ca^{2+} indicator fluo-3 and imaged by time-lapse video microscopy. With fluo-3, fluorescence changes are directly proportional to changes in intracellular Ca^{2+}. BDNF induces a slow sustained increase in intracellular Ca^{2+} in cortical neurons, whereas the metabotropic glutamate receptor agonist, (+)-1-aminocyclopentate-*trans*-1,3-dicarboxylic acid (ACPD), or depolarizing concentrations of extracellular potassium (55 mM KCl) induce a rapid Ca^{2+} increase. The figure shows a representative response from three independent experiments in which 97% of cells showed BDNF responses (34 total cells measured).

immunocytochemistry experiments suggest that the Ca/CRE and CREB play critical roles mediating BDNF-induced c-*fos* transcription.

BDNF Activates CREB and Ca/CRE via a Calcium/Calmodulin-Dependent Kinase

Having defined CREB as a mediator of BDNF-regulated gene expression, we sought next to elucidate the pathways by which TrkB receptor activation led to the phosphorylation of CREB and the activation of Ca/CRE-dependent transcription. TrkB, similar to other neurotrophin receptors, activates a variety of intracellular signaling cascades, including the Ras/ERK pathway, the PLCγ pathway, and the PI3K pathway. Activation of PLCγ is known to raise intracellular Ca^{2+} in neurons and PC12 cells by producing inositol 1,4,5-trisphosphate (IP_3), which releases intracellular Ca^{2+} stores (Vetter et al., 1991; Berninger et al., 1993; Baxter et al., 1995). We suspected that BDNF responses might be partly mediated by Ca^{2+}-dependent mechanisms, since several features of BDNF-induced CREB-dependent transcription resemble CREB responses induced by Ca^{2+} in PC12 cells (Sheng et al., 1990; Bonni et al., 1995). In PC12 cells, Ca^{2+} influx through voltage-dependent Ca^{2+} channels induces Ca/CRE-dependent transcription, independently of the SRE. However, it was controversial whether BDNF stimulation leads to Ca^{2+} rises in neurons. One study in hippocampal neurons found that BDNF and NT-3 induced rapid increases in intracellular Ca^{2+} (Gaiddon et al., 1996), whereas another study in cerebellar granule cells found that BDNF did not induce a detectable change in intracellular Ca^{2+} (Berninger et al., 1993).

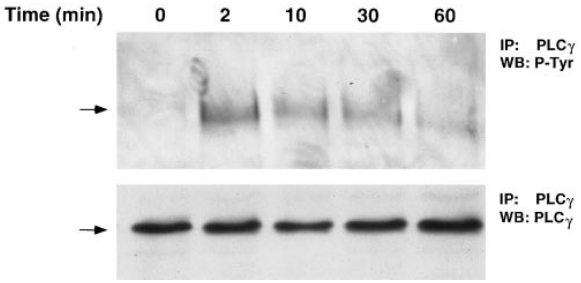

Figure 4. BDNF Induces Rapid Tyrosine Phosphorylation of Phospholipase Cγ

Cortical neurons were stimulated with BDNF (50 ng/ml) for the time points indicated and lysed under nondenaturing conditions. Phospholipase Cγ (PLCγ) was immunoprecipitated and the samples were divided onto two blots. One blot (top panel) was probed with a cocktail of antiphosphotyrosine antibodies. To confirm equal protein loading, the other blot (bottom panel) was probed with anti-PLCγ antibody. Similar results were obtained from two separate experiments.

We investigated whether Ca^{2+} might mediate BDNF-induced gene expression by testing first if BDNF induces changes in intracellular Ca^{2+} in cortical neurons. Using imaging techniques and the Ca^{2+}-sensitive fluorescent dye fluo-3, we found that BDNF induced a slowly developing but sustained increase in cytosolic Ca^{2+} in a majority of cortical neurons (Figure 3). BDNF induced an increase in intracellular Ca^{2+} in the absence of extracellular Ca^{2+}, suggesting that the source of BDNF-induced Ca^{2+} increase was mainly via the release of intracellular Ca^{2+} stores (data not shown). One mechanism by which BDNF could lead to the release of intracellular Ca^{2+} is through the production of IP_3 due to PLCγ activation. Therefore, we asked whether BDNF induces PLCγ phosphorylation in cortical neurons with activation kinetics that were consistent with a role for PLCγ in CREB phosphorylation (Vetter et al., 1991). PLCγ was immunoprecipitated from untreated or BDNF-treated cortical neurons and its state of tyrosine phosphorylation determined by Western blotting with antibodies that specifically recognize phosphotyrosine residues. We found that BDNF robustly induces tyrosine phosphorylation of PLCγ in cortical neurons within 5 min of stimulation and that the levels of tyrosine phosphorylation remain increased for up to an hour following stimulation (Figure 4). Thus, PLCγ becomes activated early enough for it potentially to play a role in mediating CREB phosphorylation.

We next examined whether PLCγ activation can trigger Ca/CRE-dependent c-*fos* transcription. We sought a way to activate PLCγ selectively, without activating other signaling cascades that are linked to growth factor receptors. To test the effects of selectively activating PLCγ, we began by transfecting cortical neurons with a growth factor receptor, the platelet-derived growth factor receptor (PDGFR). The PDGFR couples to PLCγ but is not normally expressed at high levels in cortical neurons (Valius and Kazlauskas, 1993; Kim et al., 1997). Neurons transfected with the PDGFR should become responsive to PDGF. By mutating the PDGFR at various residues and testing the ability of the receptor mutants to confer responses in transfected cells, it should be

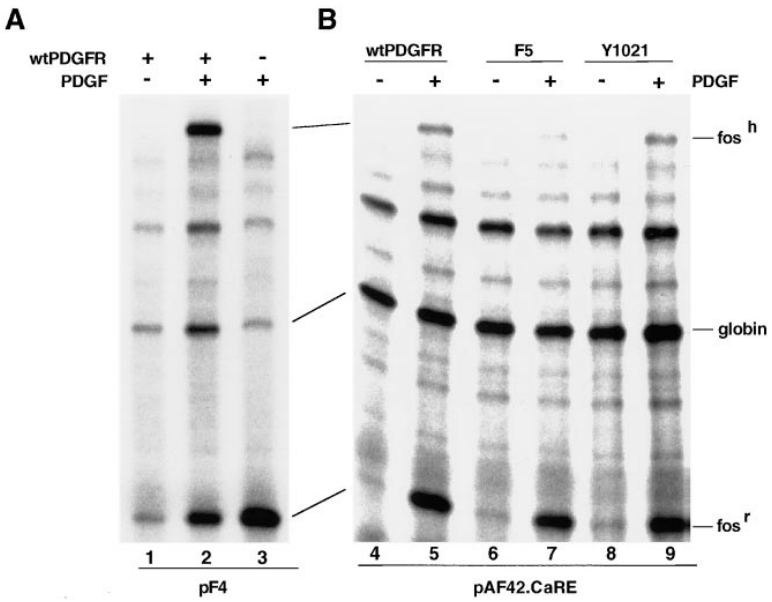

Figure 5. Activation of Phospholipase Cγ Triggers Ca/CRE-Dependent Transcription

(A) RNase protection shows that transfection with the PDGFR confers PDGF-dependent fos^h responses to neurons. Transcriptional responses mediated by the transfected c-*fos*^{h} reporter absolutely required the presence of a cotransfected PDGF receptor (lanes 1–3). PDGF did induce detectable c-*fos*^{r} gene responses in cultures that were not transfected by the PDGFR (lane 1) that, by immunocytochemical analysis, appeared to arise mainly from the small number of contaminating glia present in these cultures (data not shown; see text for details). Thus, fos^h responses can be used to assay the signaling activity of transfected PDGFRs in neurons.

(B) RNase protection shows that neurons transfected with the wild-type PDGF (wtPDGFR) receptor (5 μg) are also able to activate Ca/CRE-dependent transcription in response to PDGF treatment. Neurons transfected with a mutant PDGFR (F5) that lacks the tyrosine residues that are necessary for the PDGFR to signal to PLCγ, RasGAP, p85/PI3K, or SHP1PTP no longer show significant PDGF responses through the Ca/CRE. If the binding site for PLCγ is restored to F5, the resultant PDGFR (Y1021), when transfected into neurons, can mediate PDGF-induced Ca/CRE-dependent transcription that is quantitatively similar to responses mediated by the wild-type PDGFR. Similar results were obtained in two separate experiments.

possible to determine whether PLCγ activation can trigger Ca/CRE-dependent transcription.

We first tested whether transfection of the wild-type PDGFR (wtPDGFR) confers PDGF responses to neurons by assaying the receptor and PDGF dependence of c-*fos* expression using RNase protection assay and immunocytochemistry. We tested for PDGF-induced fos^h transcription from a reporter under the control of the wild-type promoter (pF4) and found responses only in cultures transfected with the PDGFR (Figure 5A). Although PDGF treatment of cortical neurons failed to induce detectable pF4 transcription in the absence of cotransfected PDGFR, PDGF did induce the endogenous c-*fos*^{r} in cells that were not transfected with the PDGFR. This response appears to be mediated mainly by endogenous PDGFR expression within the small number of contaminating glia present in these cultures. Immunostaining experiments showed that PDGF induced Fos expression within most of the glial cells, although glia represented only a small fraction of the cells in these cultures (data not shown). PDGF also induced Fos in neurons transfected with PDGF receptors but not untransfected neurons (data not shown). Importantly, our methods have been optimized to transfect almost exclusively neurons rather than glia. Consequently, PDGF-induced c-*fos*^{h} transcription by transfected reporter genes appears to be mediated almost exclusively by transfected PDGF receptors in neurons. Thus, it should be possible to deduce the signaling consequences of specific mutations in the PDGFR by comparing responses mediated by a cotransfected c-*fos*^{h} reporter.

To test whether the activation of PLCγ can trigger Ca/CRE-dependent transcription, we next compared PDGF responses in cells transfected with the wtPDGFR to responses in cells transfected with mutant PDGF receptors in which the PLCγ-binding site is mutated or selectively restored (F5 or Y1021). We first confirmed by immunocytochemistry that transfected wild-type, F5, and Y1021 PDGFRs are comparably expressed, to be certain that obvious expression differences would not contribute significantly to differences in PDGF-induced transcription (data not shown). To compare signaling by wild-type and mutant PDGFRs, we used RNase protection assays and found that the Ca/CRE is sufficient to mediate PDGF-dependent c-*fos* transcription in neurons cotransfected with wild-type PDGF receptors (Figure 5B). However, PDGF only weakly activated pAF42.Ca/CRE in cultures transfected with F5, the PDGFR mutant that lacks phosphotyrosine-binding sites for PLCγ, RasGAP, SHP1PTP, and p85/PI3K. Thus, one or more of the signaling pathways affected by mutating the PDGFR mediates PDGF-induced Ca/CRE-dependent transcription. When the PLCγ-binding site was restored to F5 by adding back tyrosine 1021, the resultant receptor (Y1021) was capable of mediating levels of Ca/CRE-dependent transcription that were similar to the wtPDGFR. Since PLCγ is the only protein known to bind to this site, these results suggest that activation of the PLCγ-dependent signaling pathway is sufficient to mediate growth factor-dependent transcription through the Ca/CRE.

One likely mechanism by which PLCγ activation and cytosolic Ca^{2+} increases trigger Ca/CRE-dependent transcription is through activation of CaMKs. To begin to test whether BDNF induces CREB phosphorylation and transcription through CaMKs, we examined the effects of the CaMK antagonist KN62 (Tokumitsu et al., 1990; Enslen et al., 1994). We found that KN62 attenuated BDNF-induced CREB phosphorylation and Ca/CRE-dependent transcription (Figures 6A and 6B). KN62 failed to block forskolin-induced CREB phosphorylation, which occurs by a Ca^{2+}-independent mechanism, suggesting KN62 is acting specifically to block Ca^{2+}-dependent BDNF responses. We also found that the CaMK

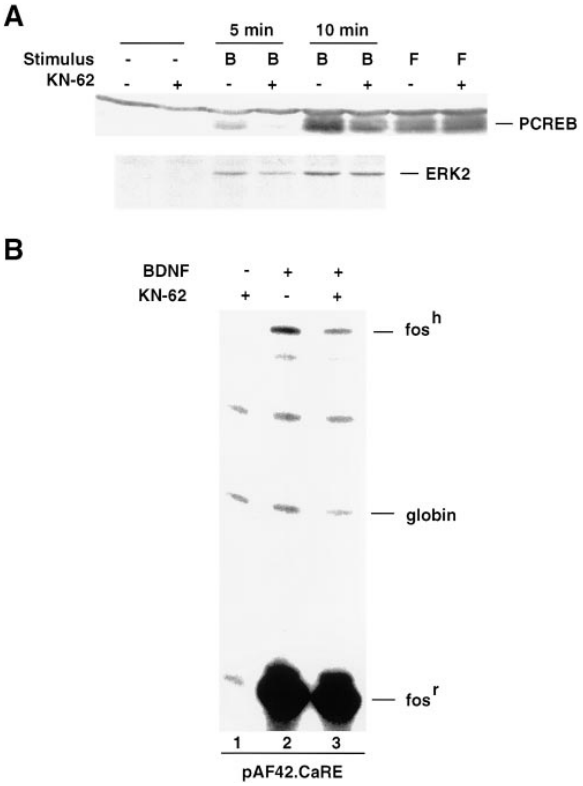

Figure 6. The CaMK Antagonist KN62 Inhibits BDNF-Induced CREB Phosphorylation and Ca/CRE-Dependent Transcription

(A) Western blot showing lysates from cortical neurons stimulated with BDNF (50 ng/ml) for the indicated time or with forskolin (10 μM) for 10 min in the presence (+) or absence (−) of KN62 (10 μM). After stimulation, neurons were lysed in SDS lysis buffer, proteins separated by SDS-PAGE, and transferred to nitrocellulose. Blots were probed with anti-PCREB antibody to detect Ser-133 phosphorylated CREB or with antiphosphotyrosine antibodies to detect phosphorylated ERK. Similar results have been found in eight separate experiments.

(B) RNase protection assay showing that KN62 (10 μM) partially inhibits BDNF-induced fos^h transcription through the Ca/CRE in cortical neurons. KN62 reduced levels of fos^h transcription (after normalization to the cotransfected α-globin) by an average of 32% ± 6% ($n = 4$, $p < 0.05$, two-tailed t test). KN62 also reduces the induction of endogenous c-*fos* (fos^r) by approximately 25% (20- ± 4-fold induction of c-*fos*^{r} in the absence of KN62 versus 15 ± 7 in the presence of KN62 [$n = 4$, $p < 0.05$, two-tailed t test]).

antagonist KN93, but not the inactive congener KN92, blocked BDNF-induced CREB phosphorylation, further suggesting that KN62 is acting specifically (data not shown). KN62 has no consistent effect on BDNF-induced ERK phosphorylation, suggesting that it also does not interfere with Ras activation. The fact that KN62 does not interfere with ERK phosphorylation is important because PLCγ is known to activate Ras and, via Ras, to activate ERKs. Previously, it has been shown that growth factors can stimulate CREB phosphorylation via the Ras/ERK pathway through RSK. Since KN62 treatment does not effectively block BDNF induction of the Ras/ERK pathway, we conclude that one way that BDNF induces CREB phosphorylation in neurons is through a Ras-independent pathway that involves CaMKs.

Since KN62 might have effects on cortical neurons in addition to inhibiting CaMKs, the possibility that CaMKs might mediate BDNF-induced CREB phosphorylation was further investigated in several other ways. For a particular CaMK to be a good candidate to mediate BDNF-induced CREB phosphorylation, it must be present in the nucleus where CREB is, and it must become activated with kinetics that precede CREB phosphorylation. Of the three CaMKs that are known to function as CREB kinases, CaMKIV and certain isoforms of CaMKII have been found in the nucleus of neurons. Western blotting revealed that CaMKIV is highly expressed in cortical cultures although apparently not in PC12 cells (Figure 7A). Using either an N or a C terminus anti-CaMKIV antibody, a protein doublet of 65–67 kDa was detected in extracts of cortical neurons (Figure 7A) that comigrated with recombinant CaMKIV that was overexpressed in COS cells (data not shown). Immunostaining of cortical neurons confirmed that CaMKIV is localized to the soma and nuclei of neurons in these cultures, consistent with previous observations (Figure 7B) (Nakamura et al., 1995). We tested whether BDNF activates CaMKIV in cortical neurons by first assaying whether BDNF induces CaMKIV phosphorylation. CaMKIV phosphorylation was examined by immunoprecipitating CaMKIV from ^{32}P-labeled neurons and then assessing its level of phosphorylation by phosphorimaging. We found that BDNF induces CaMKIV phosphorylation within 1–5 min of stimulation, consistent with a possible role for this kinase in BDNF-induced CREB phosphorylation (Figure 7C). In order to determine whether BDNF-induced phosphorylation of CaMKIV resulted in an increase in CaMKIV activity, an immune complex kinase assay was performed. BDNF induced CaMKIV activity as assayed by phosphorylation of the synthetic substrate syntide-2 (Figure 7D) in a manner that could be largely blocked by the addition of KN62 (data not shown). While membrane depolarization and BDNF treatment also induced CaMKII autophosphorylation in neurons, BDNF induction of CaMKII phosphorylation occurred slowly and at time points too delayed to be consistent with a major role mediating rapid BDNF-induced CREB phosphorylation (Figure 7E; compare with Figure 1C). Others have recently found evidence that BDNF activates CaMKII in the hippocampal slice by a pathway that depends on the activation of PLCγ and the release of intracellular Ca^{2+} and that peak activity occurs hours after stimulation (Blanquet and Lamour, 1997).

Having shown that BDNF induced cytosolic Ca^{2+} increases and CaMKIV activation, we next considered whether CaMKIV might mediate BDNF-induced Ca/CRE-dependent transcription (Matthews et al., 1994; Sun et al., 1994). To test whether CaMKIV is necessary for BDNF responses, we overexpressed a kinase-inactive mutant of CaMKIV and found that we could inhibit a portion of BDNF-induced Ca/CRE-dependent transcription (Figure 8B). The fact that the mutant CaMKIV did not block BDNF-induced SRE-dependent transcription suggests that the mutant CaMKIV specifically interferes with the pathway by which BDNF activates the Ca/CRE (Figure 8B). We have also found that the mutant CaMKIV does not interfere with forskolin-induced Ca/

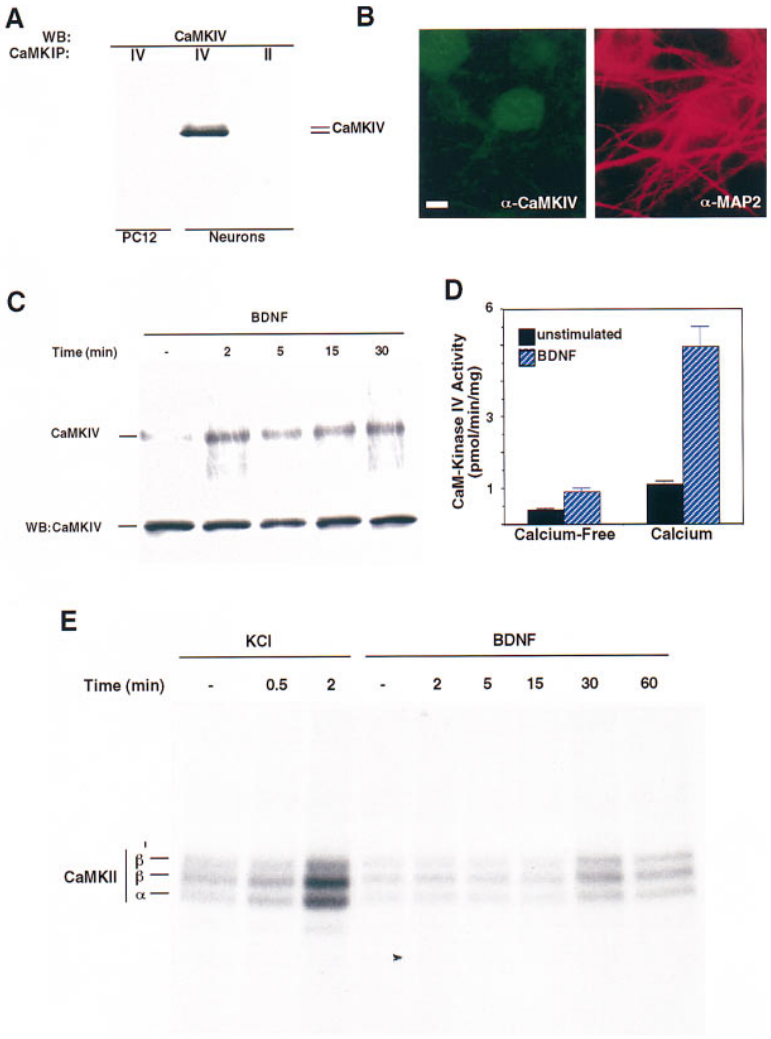

Figure 7. BDNF Activates Ca^{2+}/Calmodulin-Dependent Kinases

(A) Western blot analysis shows that CaMKIV is abundant in neurons. CaMKIV and CaMKII were immunoprecipitated from neurons and PC12 cells and blotted for CaMKIV. The blot shows that CaMKIV is abundant in neurons but not PC12 cells and establishes that the CaMKII antibodies do not immunoprecipitate CaMKIV.

(B) CaMKIV is found in the nucleus and in the cytosol of the cell soma (α-CaMKIV). By contrast, the microtubule-associated protein MAP-2 is found extensively in neurites as well as the cell soma (α-MAP-2). Scale bar = 10 μm.

(C) BDNF induces CaMKIV phosphorylation in neurons. Neurons were metabolically labeled in vivo with ^{32}P and stimulated for the indicated times with BDNF (50 ng/ml), and CaMKIV was immunoprecipitated and subjected to gel electrophoresis. To confirm that approximately equal amounts of CaMKIV were immunoprecipitated, the immunoprecipitate was divided and a portion was analyzed by Western analysis. BDNF induces a 2-fold increase in CaMKIV phosphorylation as shown here by the increased intensity of CaMKIV by autoradiography with no change in the amount of CaMKIV by Western analysis. Similar results were obtained in three independent experiments.

(D) BDNF induces an increase in CaMKIV activity in neurons. Neurons were stimulated for 15 min with BDNF (50 ng/ml), and CaMKIV was immunoprecipitated. CaMKIV activity was measured by ^{32}P incorporation into syntide-2. On average, BDNF induced a 4- to 5-fold increase in CaMKIV activity (n = 3).

(E) BDNF induces CaMKII phosphorylation in neurons with delayed kinetics compared to depolarization. Neurons were metabolically labeled in vivo with ^{32}P and stimulated for the indicated times with BDNF (50 ng/ml) or KCl (55 mM). The α and β isoforms of CaMKII were immunoprecipitated and subjected to gel electrophoresis. To confirm that we recovered approximately equal amounts of CaMKII, the immunoprecipitate was divided and a portion was analyzed by Western analysis (data not shown). BDNF induces an increase in CaMKII phosphorylation that is delayed relative to the effects of depolarization and relative to kinetics of BDNF-induced CREB phosphorylation (see Figure 1C). The experiment was repeated twice with similar results.

CRE-dependent transcription, consistent with the idea that it specifically blocks Ca^{2+}-dependent signaling to the Ca/CRE (data not shown). To test whether CaMKIV is sufficient, we transfected neurons with a constitutively active form of CaMKIV and found that this was sufficient to induce transcription through the Ca/CRE (Figure 8A). Taken together, these results suggest that BDNF activates CREB phosphorylation and Ca/CRE-dependent transcription partly through a Ras/ERK-independent pathway that involves the release of intracellularly stored Ca^{2+} and CaMKIV activation.

BDNF Activates CREB by the Ras/ERK/RSK Pathway

Although the preceding experiments indicate that BDNF induces CREB phosphorylation through TrkB by a pathway that involves PLCγ activation, intracellular Ca^{2+} release, and CaMKIV activation, several observations suggest that additional mechanisms may exist by which BDNF triggers CREB Ser-133 phosphorylation. In addition to its ability to stimulate intracellular Ca^{2+} rises, activated PLCγ can induce Ras (Stephens et al., 1994). TrkB is also capable of activating Ras via the adapter protein SHC and the guanine nucleotide exchange factor GRB2/SOS (Zirrgiebel et al., 1995). Finally, KN62 only partially blocks CREB phosphorylation. Therefore, we considered whether the Ras/ERK pathway might also mediate BNDF-induced CREB phosphorylation. In PC12 cells, NGF activates CREB phosphorylation through sequential activation of Ras, Raf, MEK, ERK, and RSK (Xing et al., 1996). However, it is unknown whether similar pathways are important in neurons and whether their activation contributes to CREB phosphorylation and Ca/CRE-dependent transcription.

To test for the involvement of the Ras/ERK/RSK pathway in BDNF signaling, we performed three sets of experiments. We tested whether BDNF activates components of the Ras/ERK/RSK pathway, whether activation

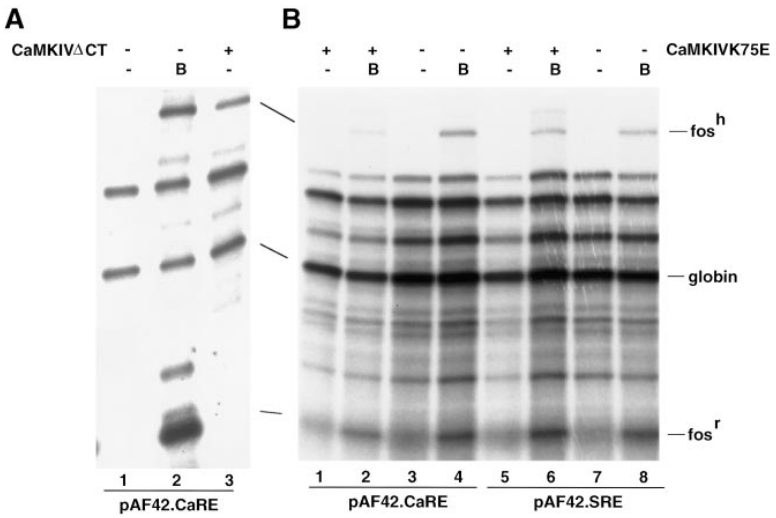

Figure 8. CaMKIV Mediates BDNF-Induced Ca/CRE-Dependent Transcription

(A) CaMKIV activates Ca/CRE-dependent transcription. RNase protection assay shows that cotransfection of a constitutively active form of CaMKIV (5 μg; CaMKIVΔCT) is sufficient to induce transcription through the Ca/CRE (pAF42.CaRE) to levels significantly greater than control but less than those achieved by stimulation with BDNF. Similar results were obtained in three separate experiments.

(B) CaMKIV is required for BDNF to activate fully Ca/CRE-dependent transcription. RNase protection assay shows that cotransfection of a kinase-inactive CaMKIV (10 μg; CaMKIVK75E) inhibits BDNF-induced transcription through the Ca/CRE (pAF42.CaRE) but not the SRE (pAF42.SRE). Cotransfection of the empty parent vector (pSG5) had no effect. Similar results were obtained in two other separate experiments.

of these pathway components is necessary to mediate BDNF signaling, and whether activation of these components was sufficient to activate CREB and Ca/CRE-dependent transcription. To test whether BDNF activates the Ras/ERK/RSK pathway, we examined the effects of BDNF on Ras, ERK, and RSK. We found by GTP loading assay that BDNF stimulation consistently increased GTP binding to Ras compared with unstimulated neurons, confirming others' results that suggest that BDNF activates Ras in primary cortical neurons (Ng and Shooter, 1993; Zirrgiebel et al., 1995) (Figure 9A). We also found that BDNF activated ERK2 and RSK2 as shown by an increase in ERK2 phosphorylation and a retardation in the gel migration of RSK2 (Figures 10 and 11A). To test directly whether BDNF increases RSK2 activity, we performed immune complex kinase assays and found that BDNF induces an 8- to 15-fold increase in RSK2 activity against the synthetic substrate CREBtide (Figure 11B).

Having shown that BDNF activates components of the Ras/ERK/RSK pathway, we tested whether this pathway was necessary for BDNF responses by interfering with Ras, MEK, or RSK signaling. We found that neurons cotransfected with a dominant-interfering form of Ras (RasN17) showed a dose-dependent decrease in BDNF-induced Ca/CRE-dependent transcription (Feig and Cooper, 1988) (Figure 9B). We also found that dominant-interfering Ras attenuated BDNF-induced transcription in neurons cotransfected with GAL4CREB and pAF42.GAL4X5.FOS, suggesting that the effects of BDNF on reporter gene transcription are mediated by CREB (data not shown). The MEK inhibitor PD098059 blocked BDNF-induced ERK2 phosphorylation, RSK2 gel retardation shift, and RSK2 activation (Dudley et al., 1995) (Figures

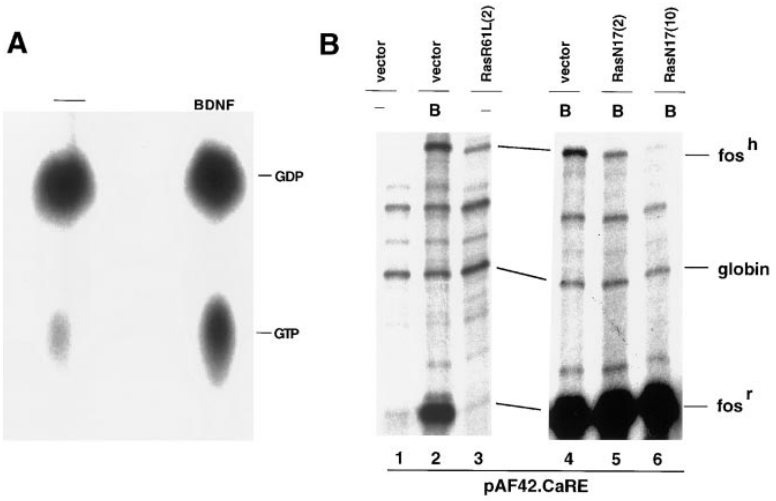

Figure 9. BDNF Activates Ras in Cortical Neurons, and Ras Mediates BDNF-Induced Ca/CRE-Dependent Transcription

(A) BDNF activates Ras in cortical neurons. Neurons were metabolically labeled in vivo with ^{32}P and stimulated for 2 min with BDNF (50 ng/ml). Ras was immunoprecipitated and the bound guanine nucleotides eluted on thin layer chromatography plates. BDNF induced a 2.1- ± 0.4-fold increase in the amount of GTP relative to GDP bound to Ras, suggesting that BDNF activates Ras ($n = 8$, $p < 0.003$, two-tailed t test).

(B) Ras mediates BDNF-induced Ca/CRE-dependent transcription. RNase protection analysis shows that cotransfection of an expression vector for a constitutively active form of Ras (RasR61L, 2 μg) was sufficient to activate Ca/CRE-dependent transcription in neurons. Constitutively active Ras increased fos^h transcription by an average of 2.1- ± 0.3-fold ($n = 3$). Additionally, cotransfection of a dominant-interfering form of Ras (RasN17, 2 = 2 μg; 10 = 10 μg) attenuated BDNF-induced Ca/CRE-dependent transcription in a dose-dependent way. BDNF induced fos^h by 4.6- ± 0.6-fold in the presence of the empty vector (pMT3), which fell to 3.5- ± 0.4-fold in neurons cotransfected with 2 μg of RasN17, and which further fell to 2.7- ± 0.2-fold ($p < 0.05$, ANOVA) in neurons transfected with 10 μg of RasN17.

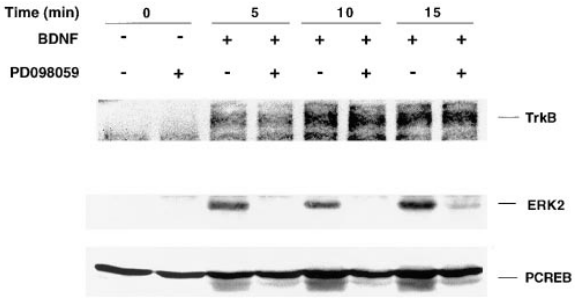

Figure 10. BDNF Activates CREB Phosphorylation through a MEK-Dependent Pathway

Cortical neurons were stimulated with BDNF (50 ng/ml) for the indicated times in the presence or absence of the MEK antagonist PD098059 (20 μM). Neurons were lysed in boiling SDS sample buffer immediately after stimulation and analyzed by Western blotting using anti-phosphoCREB (PCREB) antibodies or anti-phosphotyrosine (shown for ERK2 and TrkB) or anti-phosphoERK antibodies (not shown). PD098059 inhibited BDNF-induced ERK- and CREB-phosphorylation without interfering with TrkB phosphorylation.

10, 11A, and 11B). PD098059 also partially attenuated BDNF-induced CREB phosphorylation, suggesting that the Ras/ERK/RSK pathway is a major pathway by which BDNF induces CREB phosphorylation. PD098059 did not interfere with BDNF-induced TrkB phosphorylation, consistent with the idea that PD098059 is a specific MEK inhibitor (Figure 10). Currently, in experiments where we have pharmacologically or genetically activated the Ras/ERK/RSK or CaMK pathways independently, we have found no evidence for significant cross talk between the two as measured by changes in CREB phosphorylation (data not shown). To test further the involvement of RSK2, we cotransfected neurons with a kinase-inactivated form of RSK2 and found that it inhibited BDNF-induced Ca/CRE-dependent transcription (Figure 11C). However, the kinase-inactive RSK2 is less effective than the dominant negative Ras at blocking BDNF-induced Ca/CRE-dependent transcription, suggesting the possibility that Ras-dependent, RSK2-independent pathways exist that regulate Ca/CRE-dependent transcription. Ras may induce CREB Ser-133 phosphorylation independently of RSK2 by activating other RSK family members that are capable of phosphorylating CREB. Notably, BDNF also increases the CREB kinase activity in cortical neurons of two other RSK2-related molecules, RSK1 and RSK3, in a MEK-dependent way (S. F. T., S. F., and M. E. G., unpublished data).

These results suggest that BDNF activates components of the Ras/ERK/RSK pathway and that the Ras/ERK/RSK pathway must be intact for BDNF to induce fully CREB phosphorylation and Ca/CRE-dependent transcription. We next sought to test whether activation of components of the Ras/ERK/RSK pathway is sufficient to mediate Ca/CRE-dependent transcription. We found that cotransfection of a constitutively active form of Ras (RasR61L) was sufficient to induce Ca/CRE-dependent transcription (Kremer et al., 1991) (Figure 9B). Overexpression of wild-type RSK2 in neurons also enhanced BDNF-induced Ca/CRE-dependent transcription (Figure 11C). These results suggest that the Ras/ERK/RSK pathway is present in neurons and is critical for BDNF to activate fully Ca/CRE and CREB-dependent transcription. These results also suggest that neurons have evolved multiple signaling pathways by which neurotrophins activate CREB and Ca/CRE-dependent transcription, including activation of the two CREB kinases RSK and CaMKIV.

BDNF Induces CREB Phosphorylation in Acute Hippocampal Slice

Experiments shown above implicate CREB as a central mediator of BDNF responses in cortical cultures and define pathways that mediate BDNF-induced CREB phosphorylation. As these experiments used dissociated cultures of neurons, it was unclear whether the

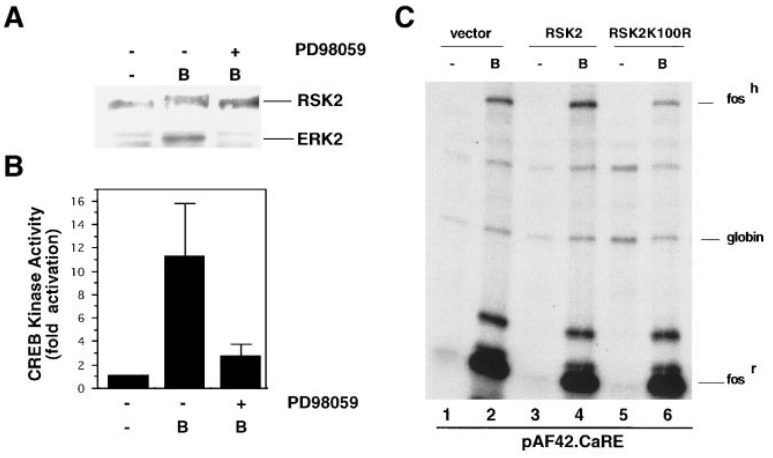

Figure 11. BDNF Activates CREB through a RSK-Dependent Pathway

(A) BDNF activates RSK2. Western analysis reveals that BDNF (50 ng/ml for 15 min) stimulation of cortical neurons retards the mobility of RSK2 by gel electrophoresis, suggesting that BDNF induces RSK2 phosphorylation. The MEK antagonist PD098059 (20 μM) blocks the BDNF-induced shift in RSK2 mobility, suggesting that BDNF activates RSK2 through a MEK-dependent pathway. Immediately after stimulation, neurons were lysed in boiling SDS buffer, and RSK2 was resolved by gel electrophoresis (10% gel) and Western blotting with a RSK2-specific antibody (UBI).

(B) RSK2 is a MEK-dependent CREB kinase in neurons. BDNF stimulation induces an 11- ± 5-fold increase in RSK2-mediated CREB kinase activity (n = 3) in cortical neurons. BDNF-induced, RSK2-dependent CREB kinase activity is also blocked by PD098059. After stimulation, RSK2 was immunoprecipitated from neurons, and CREB kinase activity was measured as the amount of ^{32}P incorporation into Ser-133 of CREBtide by in vitro kinase assay.

(C) BDNF activates Ca/CRE-dependent transcription partly through a RSK-dependent pathway. RNase protection shows that overexpression of wild-type RSK2 (pMT2RSK2, 5 μg) enhanced BDNF-induced Ca/CRE-dependent transcription compared with the empty parent vector (pMT2, 5 μg), whereas overexpression of kinase-inactive RSK2 (pMT2RSK2K100R, 5 μg) partially attenuated BDNF-induced Ca/CRE-dependent transcription. Similar results were obtained from three independent experiments.

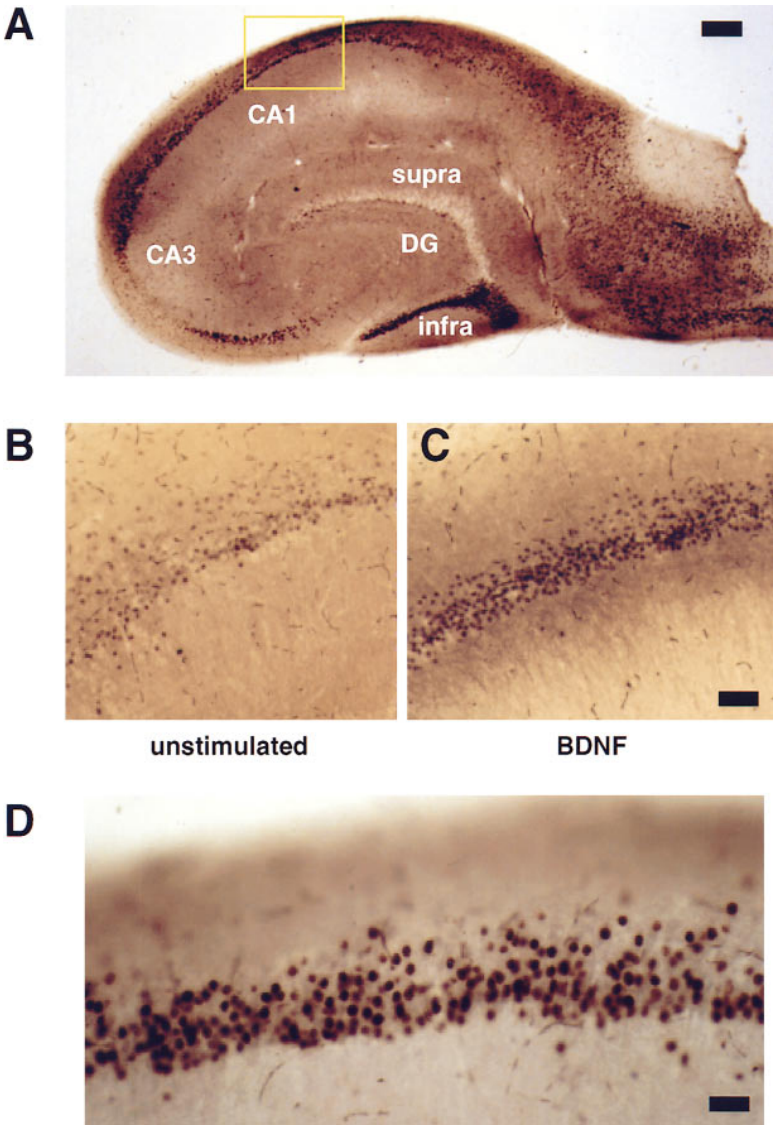

Figure 12. BDNF Induces CREB Phosphorylation in Acute Hippocampal Slice

(A) A hippocampal slice, stimulated by BDNF, illustrates intense nuclear staining in subiculum, areas CA1, CA2, and CA3c, and the infrapyramidal blade of the dentate gyrus. The absence of nuclear staining in the suprapyramidal blade of the dentate gyrus was consistent across most experiments, while the absence of staining in the CA3a-b region was not consistent across slices. Responses to BDNF stimulation were further assessed in the CA1 region of hippocampal slices (yellow rectangle). Scale bar = 230 μm.

(B and C) BDNF induced a substantial increase in anti-PCREB immunoreactivity in the nucleus of CA1 hippocampal neurons when unstimulated slices (n = 4 slices, [B]) were compared to BDNF-stimulated slices (n = 5 slices, [C]). Scale bar = 80 μm.

(D) A high magnification of CA1 hippocampal neurons illustrating the intense anti-PCREB staining in BDNF-treated slices is confined to the nuclei. Scale bar = 40 μm.

preceding results would be relevant under conditions where the physiologic functions and many of the anatomic relationships of neurons are preserved. Therefore, we sought a way to test whether a pathway from BDNF to CREB might also be present in neurons from a preparation that more closely resembled the intact brain. We tested by immunostaining the effects of BDNF application on CREB phosphorylation in acute hippocampal slices. Since BDNF application to hippocampal slices induces rapid increases in synaptic activity, and neurotransmitters are known from previous experiments to induce CREB phosphorylation, synaptic activity was pharmacologically blocked for each experiment (see Experimental Procedures). We confirmed the efficacy of synaptic blockade by performing extracellular field recordings. To test whether a pathway between BDNF and CREB exists in the adult, we exposed slices to BDNF or PBS control in a static bath for 3–5 hr to ensure that BDNF permeated the slice. We subsequently fixed and immunostained slices with an antibody that specifically recognizes Ser-133 phosphorylated CREB (Figure 12A). Analysis of neuronal cell bodies in the CA1 region showed that BDNF induced a substantial increase in the number of Ser-133 CREB-stained nuclei (n = 4–5) (Figures 12B and 12C). Taken together, these results demonstrate that BDNF signaling pathways that trigger CREB Ser-133 phosphorylation function in an intact slice from a mature animal and raise the possibility that these signaling paths may operate in the intact brain as well.

Discussion

Neurotrophins regulate a wide array of diverse processes in neurons during development through adulthood. To identify the mechanisms by which neurotrophins exert their effects, we have elucidated the intracellular signaling pathways that the neurotrophin BDNF activates to mediate gene expression in cortical neurons. In contrast to the prevailing view that growth factors require TCFs and SRF to mediate IEG expression, we find that in neurons the transcription factor CREB is sufficient to mediate BDNF-induced gene expression. In neurons,

BDNF activates CREB-dependent gene expression by at least two pathways: one that depends on the release of intracellular Ca^{2+} and the activation of CaMKIV and another that depends on activation of the Ras/ERK/RSK pathway. These findings suggest that CREB regulates growth factor responses differently in neurons than in several other cell types.

Clues as to how CREB might mediate diverse cellular responses are provided by the finding that CREB regulation and function is different in neurons than in several other cell types. In undifferentiated PC12 cells, CREB must interact with other proteins bound at the promoter to contribute to NGF-induced c-*fos* transcription. In well-differentiated neurons, we find that CREB can function independently of other promoter-bound transcription factors such as SRF and TCFs. This would allow neurotrophins to induce, in mature neurons, an array of genes that contains CREB-binding sites within their regulatory regions regardless of the presence of other regulatory elements. It is interesting to note that the results we obtained by assaying BDNF-induced c-*fos* expression in transient gene reporter assays were qualitatively similar to those we obtained by assaying BDNF-induced expression of endogenous Fos (compare Figure 1 and Figure 2). However, we found quantitatively that disruption of CREB or Ca/CRE function nearly completely blocked BDNF-induced expression of endogenous Fos but only partly blocked BDNF-induced c-*fos* expression from a transiently transfected FosCAT reporter that contained an intact SRE and an in-context Ca/CRE mutation. The quantitative difference between results obtained from transiently transfected reporter genes and endogenous genes could simply reflect differences in the assays. However, the results could also suggest that while CREB can function on its own to mediate BDNF responses in mature neurons, transcription factors bound to other growth factor-responsive elements may require interactions with CREB in order to mediate c-*fos* transcription (Figure 2; also see Robertson et al., 1995). In mature neurons, neurotrophin induction of the expression of CREB-regulated genes may be a mechanism by which the neurotrophin effects long lasting changes in synaptic function. There is considerable evidence that, in mature neurons, the long lasting effects of neuronal activity on synaptic function are partly mediated by CREB (Frank and Greenberg, 1994). Since, in response to either neurotrophins or neurotransmitters, CREB mediates transcriptional responses independently of other promoter-bound factors, it is likely that the effects of these different extracellular stimuli on synaptic function may involve the activation of a similar set of CREB-regulated genes. It remains to be determined whether CREB's ability to function by distinct mechanisms in different cell types explains how CREB might mediate diverse cellular responses.

We have found that BDNF stimulates CREB Ser-133 phosphorylation through two distinct intracellular signaling pathways in neurons, a CaMKIV- and a Ras-dependent pathway. Neurons may have evolved multiple mechanisms for catalyzing CREB Ser-133 phosphorylation to enable BDNF to regulate specific neuronal functions and to broaden the number of genes that are responsive to BDNF. BDNF stimulation of CaMKIV may lead to the activation of other members of the leucine zipper family of transcription factors that typically respond to Ca^{2+} but not to activation of the Ras/ERK pathway (Foulkes et al., 1991; Ginty et al., 1993; Liu et al., 1993; Bonni et al., 1995; Ellis et al., 1995). CREB can dimerize with a number of related transcription factors, including ATF-1 and C/EBPβ. Thus, by activating different CREB partners, BDNF could determine the dimers that form, which, in turn, may regulate both the type and level of gene that is expressed. Whether the CaMKIV or Ras/ERK/RSK pathways might differentially regulate CREB heterodimer formation is not yet clear.

BDNF activation of the CaMKIV pathway suggests mechanisms by which BDNF might rapidly signal to the nucleus over long distances within neurons. By activating PLCγ and the local production of IP_3, BDNF might transmit a long distance signal by triggering a regenerative Ca^{2+} wave that propagates to the nucleus (Cornell-Bell et al., 1990; Spitzer, 1994; Spacek and Harris, 1997). Since Ca^{2+} waves travel roughly 20 μm/s, much faster than long-range protein diffusion or active transport, Ca^{2+}-dependent signals might be expected to arrive in the nucleus from the tip of a neurite minutes to hours before signals sent by the Ras pathway. The possible coexistence of slow and fast pathways to the nucleus also suggests a novel way that a neuron could localize the source of a stimulus in space and produce distinct and appropriate transcriptional responses. If BDNF stimulation at one point on a neurite activates two signaling pathways (e.g., CaMKIV and Ras/ERK/RSK pathways) that send signals at different but constant velocities, the difference between the time it takes each signal to reach the nucleus will be directly proportional to the distance that the signals traveled. Thus, the precise pattern of the resultant IEG and LRG responses might depend on the temporal overlap of the signals as they arrive at the nucleus from the two pathways, on whether the promoters of the target genes contain CREs and/or SREs, and on whether or not the genes are responsive to the CaMKIV and/or Ras/ERK/RSK signaling pathways.

The finding that CREB mediates BDNF responses is of particular interest, given that BDNF and CREB have been shown to regulate synaptic transmission (Kang and Schuman, 1995). BDNF most likely strengthens synaptic transmission through both gene expression-dependent and gene expression-independent mechanisms. BDNF-induced CREB phosphorylation and activation may regulate gene expression-dependent synaptic changes. In Aplysia californica, CREB has been shown to regulate synaptic plasticity in part by regulating genes that mediate changes in synaptic structure or the formation of new synaptic connections (Bartsch et al., 1995; Martin and Kandel, 1996). In mammals, the application of a neurotrophin to neurons in vitro and in vivo results in increases in axonal sprouting and dendritic length and arbor (Snider, 1988; Diamond et al., 1992b; Schnell et al., 1994). Neurotrophins are known to mediate neurite outgrowth through both Ras- and PLCγ-dependent pathways that could involve CREB (Bar-Sagi and Feramisco, 1985; Hagag et al., 1986; Domenico et al., 1989; Vaillancourt et al., 1995).

BDNF could also influence synaptic transmission by mechanisms that do not depend on new gene expression but do involve the Ras/ERK/RSK pathway and/or

the CaMKIV-signaling pathway. In hippocampal slices, BDNF can increase synaptic responses quickly by mechanisms that are partly independent of new gene expression (Kang and Schuman, 1996). Electrophysiological analyses suggest that neurotrophins rapidly enhance synaptic responses in part by presynaptic mechanisms, presumably by increasing the amount of neurotransmitter that is released (Lohof et al., 1993; Kang and Schuman, 1995; Levine et al., 1995; Stoop and Poo, 1995). BDNF might effect presynaptic changes through TrkB by activating signaling pathways characterized in this study. For example, BDNF might affect synaptic changes by stimulating PLCγ and by producing IP_3 or by activating the Ras/ERK/RSK pathway. PLCγ activation and IP_3 production could modulate presynaptic function by increasing neurotransmitter release through increases in presynaptic Ca^{2+} levels, by affecting CaMK- and PKC-dependent changes in ion channel function, and/or by inducing cytoskeletal changes that might increase synaptic responses. Therefore, BDNF, via the PLCγ/Ca^{2+}/CaMKIV and Ras/ERK/RSK pathways, might affect rapid changes in synaptic strength through local effects at the synapse and might consolidate synaptic changes through new CREB-dependent gene expression.

Experimental Procedures

Materials

Plasmids pF4 and pF222 were gifts from Dr. R. Treisman (Treisman, 1985); pRcRSV, pRcRSV-KCREB were gifts from Dr. R. Goodman (Walton et al., 1992); wild-type, Y1021, and F5 PDGF receptor expression vectors were gifts of Dr. A. Kaslauskas (Valius and Kazlauskas, 1993); pSG5CaMKIVΔCT and CaMKIV pSG5CaMKIVK75E were gifts of Dr. T. Chatila (Miranti et al., 1995); the expression vectors pMT3RasR61L and pMT3RasN17 were gifts from Dr. L. Feig (Feig and Cooper, 1988). The following constructs have been described previously: pSVα1 (Shyu et al., 1989); pRcRSVCREBM1 (Bonni et al., 1995); pON260 (Cherrington and Mocarski, 1989); pAF42 and pAF42.Ca/CRE (Sheng et al., 1990); pAF42.SRE (Rivera et al., 1990); GAL4CREB, GAL4CREBΔLZ, GAL4CREBM1 (Sheng et al., 1991); pMT2RSK2 and pMT2RSK2K100R (Pende et al., 1994).

Cell Culture, Stimulation, and Labeling

Cortical neurons from postnatal day 0 (P0) rats were cultured according to previously published procedures and grown in a media based on basal medium eagle (BME) (Xia et al., 1996). For Western blotting, immunoprecipitation, or immune complex kinase assay experiments, cells were used between 2 days in vitro (DIV) and 7 DIV; for transfection and RNase protection analysis, cells were stimulated and lysed on 6 DIV. For Western blotting and immunoprecipitation experiments, 2 hr prior to stimulation, growth media was replaced by an equal amount of BME that contained pharmacological antagonists designed to attenuate neuronal electrical activity including tetrodotoxin (TTX, 1 μM), amino-5-phosphonovaleric acid (APV, 100 μM), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 40 μM), and nimodipine (not used when depolarization was the stimulus, 5 μM). Other inhibitors such as KN62 (10 μM, Calbiochem) or PD098059 (20 μM, NEB) were added 30 min before stimulation. BDNF (50 ng/ml) or human recombinant PDGF-$\beta\beta$ (UBI, 50 ng/ml) was added directly to the media in the dish, mixed thoroughly, and allowed to incubate for the indicated times. In vivo ^{32}P labeling of neurons and PC12 culture was performed as previously described (Rosen et al., 1994).

Transfection, RNA Isolation, and RNase Protection

Primary cortical neurons were transfected using a calcium phosphate method described previously (Xia et al., 1996). Typically, reporter plasmids and expression vectors for transcription factors were transfected at 1 μg and 700 ng per 60 mm dish. Expression vectors for other molecules such as constitutively active or dominant-interfering molecules were used at indicated levels. RNA was isolated and RNase protections for c-*fos*^{h} were performed as described (Chomczynski and Sacchi, 1987; Xia et al., 1996). RNase protections for fosCAT were performed as described (Bonni et al., 1995). For quantitative comparisons, RNase protection results were analyzed using a Phosphorimager.

Immunoprecipitation, Western Immunoblotting, and Protein Kinase Assays

Immunoblotting for CREB and ERK2 was performed using previously published methods (Bonni et al., 1995) using the following sources of antibody: anti-PCREB (Ginty et al., 1993); anti-phosphoERK2 (NEB); antiphosphotyrosine antibodies (4G10 [UBI] and PY20 [ICN]); and anti-CaMKIV polyclonal (UBI) and monoclonal (Signal Transduction Laboratories). Immunoprecipitations of PLCγ were performed using an HNTG buffer and protocol previously described (Bonni et al., 1993).

CaMKIV was immunoprecipitated by two methods. To detect CaMKIV phosphorylation from in vivo labeled neurons, BDNF stimulation was terminated by the addition of 200 μl of a modified boiling SDS buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.5% sodium dodecyl sulfate). Lysates were boiled, cooled to 4°C, and diluted with 800 μl of 1.25$\times$ RIPA buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 2.5 mM EDTA, 1.25 mM phenylmethylsulfonylfluoride, 1.25 μg/ml aprotinin, 1.25 μg/ml leupeptin, 1.25 μg/ml pepstatin, 1.25 mM Na_3VO_4, 1.25 mM NaF). Immunoprecipitation kinase assays were performed using previously published methods (Park and Soderling, 1995). In both assays, 5 μg of anti-CaMKIV antibody (Signal Transduction Laboratories) and 40 μl of a 50% slurry of a 1:1 mixture of protein G-Sepharose (Sigma) and protein A-Sepharose (Pharmacia) were added to volumes of lysate that contained equal amounts of protein. Ras loading assays were performed on in vivo labeled neurons using methods described previously (Rosen et al., 1994). RSK2 immunoprecipitations, RSK2 Western analyses, and RSK2-dependent CREB kinase activity measurements were performed as described previously except that neurons were used instead of PC12 cells (Xing et al., 1996).

Immunocytochemistry and Immunohistochemistry

Our general method of immunocytochemistry has been previously published (Xia et al., 1996). Additional antibodies used for immunocytochemistry not described for immunoblotting include anti-MAP-2 (gift of Dr. Valee), anti-LacZ (Promega), and anti-c-Fos (Ab-2 and Ab-5 from Oncogene Science). Primary antibody binding was variously detected with Cy-2 (1:300), Texas red or a biotinylated secondary antibody (1:150; Vector), and fluorescein-conjugated streptavidin (1:25; Vector) or Cy-3 conjugated streptavidin (1:500; Jackson Labs).

Imaging and Electrophysiology

Cortical neurons were seeded on coated glass coverslips (22 $\times$ 22) and used between 2–7 DIV. On the day of an experiment, neurons were washed in BME containing TTX, APV, CNQX, and nimodipine and were bath loaded with fluo-3 (5 μM) and inhibitors for 30 min. The glass coverslip was mounted in a superfusion chamber (Warner Instrument) and placed on the stage of a Leitz Fluovert FU epifluorescence microscope. Cells were illuminated with a mercury arc lamp through neutral density filters (Omega Optical) and a long pass filter suitable for fluorescein optics. Images were collected with a silicon intensified tube (SIT) camera, digitized using Image-1 software (Universal Imaging Corp.) and an 8-bit Matrox frame grabber, and stored on a computer hard drive. Pixel intensities were sampled over the soma of each neuron, and the relative changes in intracellular Ca^{2+} were calculated as percent $\Delta F/F$. To calculate percent $\Delta F/F$, an average of baseline fluorescence values is subtracted from each fluorescence value of the record, and the result is divided by the same average baseline fluorescence value. Quantitative confocal scanning laser microscopy (CSLM) of Fos immunostaining was performed using a Noran Oz CSLM. A Z series of ten images, 1 μm apart, was captured for each transfected neuron using a 100$\times$ Nikon objective. A plane midway through the vertical axis of the nucleus

was identified, and the average pixel intensity of a 3 μm^2 area in the center of the nucleus was sampled.

For electrophysiology, hippocampal slices were prepared from male rats of the Long-Evans strain approximately 30–45 days old (63–82 g) according to previously published methods (Harris and Teyler, 1984; Kremer et al., 1991). Half of the slices was received into ice cold media (116.4 mM NaCl, 5.4 mM KCl, 3.2 mM $CaCl_2$, 1.6 mM $MgSO_4$, 26.2 mM $NaHCO_3$, 1.0 mM NaH_2PO_4, 10 mM D-glucose), and the other half was received into ice cold media that also contained the activity antagonists TTX (1 μM), CNQX (40 μM), nimodipine (5 μM), and APV (100 μM). Slices were transferred to interface chambers and maintained in a humidified atmosphere at 30–31°C (Harris and Teyler, 1984). BDNF (50 ng/ml) was included in the media and applied directly to the surface of the slice for half of the wells containing activity antagonists. An equal amount of media was included in the bath and applied directly to the surface of slices in the other wells. All slices equilibrated for at least 1 hr before physiological recordings.

Slices without activity antagonists were tested for health and/or the capacity to induce LTP at the Schaffer collateral-CA1 synapses. Slices with activity antagonists were tested physiologically to ensure that no synaptic responses could be generated. Two concentric bipolar stimulating electrodes (ultrasmall, 50 μm pole separation, Fred Haer Co., Brunswick, ME) were positioned 600–800 μm apart in the middle of stratum radiatum surrounding a single extracellular recording electrode (glass micropipette filled with 0.12 M NaCl). Alternating stimuli were delivered at one per 15 s at an intensity that evoked field excitatory postsynaptic potentials (EPSPs) slopes of approximately 1 mV/ms. Increasing stimulus intensities were used to generate input–output (I–O) curves, and the half-maximal responses were monitored for 20–40 min for each independent pathway prior to induction of LTP (two trains of 100 Hz stimulation for 1 s separated by a 20 s interval, repeated three times). All responses were digitized and analyzed online using the Scope software (RC Electronics Inc., Santa Barbara, CA). Extracellular recordings in CA1 in response to stimulation along the Schaffer collateral pathway showed that the slices exhibited normal field EPSPs and could undergo long-term potentiation in response to tetanization (data not shown). However, in the presence of synaptic activity antagonists, slices showed no EPSPs, suggesting that the pharmacological antagonists were effective. After 2–4 hr, slices were rapidly fixed in 4% paraformaldehyde, 0.2% glutaraldehyde in 0.1 M PBS using a microwave-enhanced protocol (Jensen and Harris, 1989). Slices were then transferred to PBS containing 1 mM NaF until thin (50 μm) sections were cut using a vibratome. Immunostaining with the anti-PCREB antibody on these free-floating sections was performed as described previously (Ginty et al., 1993).

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