

Identification of a Signaling Pathway Involved in Calcium Regulation of *BDNF* Expression

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Summary

A signaling pathway by which calcium influx regulates the expression of the major activity-dependent transcript of *BDNF* in cortical neurons has been elucidated. Deletion and mutational analysis of the promoter upstream of exon III reveals that transactivation of the *BDNF* gene involves two elements 5' to the mRNA start site. The first element, located between 72 and 47 bp upstream of the mRNA start site, is a novel calcium response element and is required for calcium-dependent *BDNF* expression in both embryonic and postnatal cortical neurons. The second element, located between 40 and 30 bp upstream of the mRNA start site, matches the consensus sequence of a cAMP response element (*CRE*) and is required for transactivation of the promoter in postnatal but not embryonic neurons. The *CRE*-dependent component of the response appears to be mediated by CREB since it is part of the complex that binds to this *CRE*, and since dominant negative mutants of CREB attenuate transactivation of the promoter. A constitutively active mutant of CaM kinase IV, but not of CaM kinase II, leads to activation of the promoter in the absence of extracellular stimuli, and partially occludes calcium-dependent transactivation. The effects of CaM kinase IV on the promoter require an intact *CRE*. These mechanisms, which implicate CaM kinase IV and CREB in the control of *BDNF* expression, are likely to be centrally involved in activity-dependent plasticity during development.

Introduction

Sensory stimulation can have profound effects on the development of the nervous system. Our understanding of activity-dependent development is largely derived from the pioneering studies of Hubel and Wiesel on the development of ocular dominance columns in the visual cortex (Hubel and Wiesel, 1970). Monocular deprivation during the critical period leads to a permanent shift in ocular dominance in favor of the nondeprived eye. The anatomical basis of this shift is an expansion of lateral geniculate nucleus (LGN) axon terminals receiving input from the nondeprived eye within layer 4 of visual cortex.

The importance of neuronal activity and synaptic stimulation in thalamocortical development has been subsequently confirmed in several major studies (reviewed by Shatz, 1990). For example, it has been shown that pharmacological blockade of afferent input to cortical neurons by intra-ocular injections of tetrodotoxin (TTX, a drug that blocks action potentials by blocking Na⁺ channels) in vivo prevents the segregation of LGN axons into ocular dominance columns (Stryker and Harris, 1986), and synaptic transmission via one of the glutamate receptors (the NMDA receptor) has been shown to be necessary for the shift in ocular dominance caused by monocular deprivation (Kleinschmidt et al., 1987). While it is now generally recognized that synaptic activity can have a profound effect on thalamocortical connectivity, the molecular mechanisms that underlie activity-dependent cortical development have not yet been identified.

Many of the investigations on mechanisms of thalamocortical development have been driven by the hypothesis that thalamic axons compete for growth-promoting factors produced by cortical neurons, and that this competition favors correlated activity between thalamic neurons and their postsynaptic targets. One class of molecules that may serve as target-derived trophic factors are the neurotrophins. Neurotrophins are a family of small secreted proteins related to nerve growth factor (NGF) (Levi-Montalcini, 1987; Crowley et al., 1994) and include brain-derived neurotrophic factor (BDNF) (Barde et al., 1982; Leibrock et al., 1989; Ernfors et al., 1994b; Jones et al., 1994), neurotrophin-3 (NT-3) (Hohn et al., 1990; Ernfors et al., 1994a), and neurotrophin 4/5 (NT-4/5) (Berkemeier et al., 1991; Hallbook et al., 1991; Conover et al., 1995; Liu et al., 1995). The developmental and spatial pattern of expression of the neurotrophins and their receptors (reviewed by Chao, 1992; Barbacid, 1995), along with emerging experimental evidence, suggests that specific neurotrophins are involved in regulating particular aspects of cortical development (Bozzi et al., 1995; McAllister et al., 1995, 1997; reviewed by Bonhoeffer, 1996; Ghosh, 1996; Shieh and Ghosh, 1997). Of particular relevance to the problem of activity-dependent development are recent observations that suggest that BDNF may play an important role in regulating thalamocortical development. Cabelli et al. (1995) have reported that infusion of BDNF or NT-4/5 during the critical period prevents the formation of ocular dominance columns, consistent with the possibility that thalamic axons normally compete for limiting amounts of BDNF or NT-4/5. In addition, Galuske et al. (1996) have shown that intracortical administration of BDNF reverses the activity-dependent shift of ocular dominance columns. More recently, Cabelli et al. (1997) have shown that infusion of TrkB receptor bodies also prevents ocular dominance column formation, and in related experiments we have found that intracortical injections of anti-BDNF or TrkB-IgG inhibits the growth of thalamic axon terminals in the rat barrel cortex (J. S. Roberts, D. Kim, and A. G., unpublished data). These observations suggest that endogenous levels of BDNF play an important role in regulating thalamic innervation of the cortex.

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Electrophysiological studies have provided additional evidence in support of a role for BDNF in synaptic plasticity. It has been reported that BDNF can enhance the efficacy of synaptic transmission in hippocampal and cortical slices (Kang and Schuman, 1995; Figurov et al., 1996; Akaneya et al., 1997) and that treatment with TrkB-IgG or the Trk receptor inhibitor, K252a, prevents the induction of long-term potentiation in both preparations (Akaneya et al., 1997; Kang et al., 1997). Moreover, hippocampal slices from mice with a targeted disruption of *BDNF* show a defect in synaptic plasticity (Korte et al., 1995; Patterson et al., 1996). This defect can be rescued by viral expression of *BDNF* in slices (Korte et al., 1996) or by bath application of BDNF (Patterson et al., 1996), suggesting an acute requirement for BDNF in this process. Interestingly, the defect is seen in both homozygous null and heterozygous animals, indicating that a critical level of BDNF may be required for activity-dependent synaptic modifications. Although the precise role of BDNF in synaptic physiology is not yet fully understood, these studies suggest that BDNF is likely to play an important role in modulating synaptic function.

To explore the possibility that BDNF may be involved in activity-dependent cortical development, we initially examined the role of BDNF in the activity-dependent survival of cortical neurons (Ghosh et al., 1994). We found that the survival of cortical neurons in culture is enhanced by membrane depolarization and requires calcium influx via voltage sensitive calcium channels (VSCCs). We then considered the possibility that calcium influx may lead to increased survival by modulating BDNF levels and found that VSCC activation indeed leads to a robust increase in BDNF expression, that BDNF is a trophic factor for cortical neurons, and that neutralizing antibodies to BDNF block the activity-dependent survival of these cells (Ghosh et al., 1994). Consistent with this observation, several other groups have also reported regulation of BDNF in vivo and in vitro by neuronal activity (Isackson et al., 1991; Castren et al., 1992; Lauterborn et al., 1996; see also Meyer-Franke et al., 1995), suggesting that activity-dependent regulation of BDNF may be broadly involved in mediating adaptive changes in the nervous system. It is therefore of interest to understand the molecular mechanisms by which neuronal activity leads to transcription of the *BDNF* gene.

The *BDNF* gene consists of five exons, with the coding region included entirely within exon V. Each of the first four exons has a putative promoter on its 5' flanking region and a splice donor site on its 3' end. Exon V contains the only splice acceptor site on its 5' end and two alternative polyadenylation sites on its 3' end. Each of the four 5' exons can therefore be alternatively spliced with exon V, and the use of two alternate polyadenylation sites can give rise to eight different *BDNF* transcripts (Timmusk et al., 1993). Experiments using exon-specific probes have indicated that the four 5' exons are differentially responsive to neuronal activity, and that exon III-containing transcripts are most robustly regulated by activity in the hippocampus and cortex (Metsis et al., 1993; Timmusk et al., 1995; Lauterborn et al., 1996). We and others have previously shown that activity-dependent *BDNF* expression is mediated largely by calcium influx via VSCCs (Ghosh et al., 1994). Here, we report

on our findings on the mechanisms by which VSCC activation leads to transactivation of the exon III promoter in cortical neurons.

Results

To begin to understand the mechanisms by which VSCC activation regulates the expression of exon III-containing transcripts, our first goal was to identify regions of the gene that confer calcium responsiveness upon the promoter. Based on the genomic structure of most transcriptionally regulated genes, we suspected that sequences 5' to the exon III mRNA start site should contain such calcium response elements, and we created a construct in which putative exon III promoter and transcript sequences were placed 5' to a chloramphenicol acetyl transferase (CAT) coding sequence. This construct, denoted *-4100BIII-CAT*, begins 4100 bp upstream of the mRNA start site and contains 285 bp of exon III sequence. To determine whether the DNA sequences present in this construct were sufficient to mediate a calcium response, the construct was transfected into embryonic day 18 (E18) cortical cultures at three days in vitro (E18 + 3DIV) using a modified calcium phosphate transfection procedure (Threadgill et al., 1997). Two days later, the cultures were depolarized with 50 mM KCl to activate VSCCs, and the cells were harvested at various times after stimulation to assess reporter activity. (In this and all subsequent experiments, the cells were stimulated in the presence of 100 μ M APV to prevent activation of the NMDA receptors.) As shown in Figure 1A, CAT assays revealed that this reporter was robustly stimulated by VSCC activation. Elevations in CAT activity were first detectable at 4 hr and continued to increase until about 10 hr of stimulation. This kinetics of activation is consistent with our previous studies, which had shown that *BDNF* mRNA peaked between 4 and 6 hr following VSCC activation (Ghosh et al., 1994), and suggested the presence of a calcium response element within the 4100 bp fragment. Transactivation of *-4100BIII-CAT* was attenuated by EGTA and nifedipine (Figure 1B), indicating that transcription of the transgene was regulated by calcium influx via VSCCs, as had previously been described for the endogenous gene.

To further define the DNA sequences in the *BDNF* promoter required for calcium-dependent transcription, a series of deletion constructs were created that progressively removed 5' sequences from the *-4100BIII-CAT* reporter (Figure 2A). These constructs were transfected into E18 cortical cultures at 3DIV, and at 5DIV the cells were stimulated and harvested for CAT assays. The distal deletion constructs, which included 1500, 634, and 108 bp of 5' sequence, were all capable of mediating a robust calcium response comparable to that seen with *-4100BIII-CAT* (data not shown), suggesting the presence of a major calcium response element within the 108 bp promoter fragment. Further analysis of deletion constructs in E18 cultures indicated that whereas constructs that included 108 or 72 bp of upstream sequence could mediate a robust calcium response, constructs that included 47 or 30 bp of upstream sequence were only weakly responsive or nonresponsive to VSCC

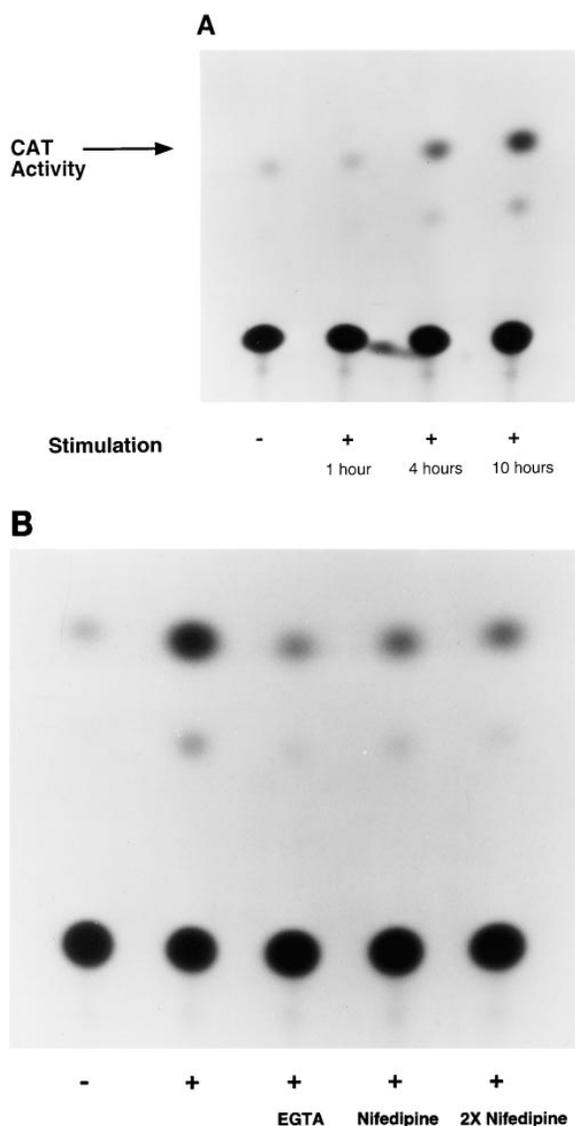


Figure 1. A Reporter Construct that Includes 4100 bp of Sequence Upstream of the Exon III mRNA Start Site Can Mediate Calcium-Dependent Transcription in Response to Depolarization of Cortical Neurons

(A) CAT activity in E18 cortical neurons transfected with the reporter *-4100BIII-CAT* at 3DIV and stimulated for indicated times with 50 mM KCl (to activate VSCCs) at 5DIV.

(B) CAT activity in E18 cortical cultures transfected with the reporter *-4100BIII-CAT* at 3DIV and stimulated with 50 mM KCl in the absence or presence of EGTA (2 mM) or nifedipine (5 μ M, 10 μ M) for 10 hr at 5DIV. All KCl stimulations in this and other experiments shown in this paper were performed in the presence of 100 μ M APV to inhibit activation of NMDA receptors.

activation (Figure 2B). These experiments indicate the presence of a calcium response element within the *BDNF* promoter located between 47 and 72 bp upstream of the mRNA start site. Examination of the sequence in this region (Figure 2D) revealed no known calcium-responsive sequences, suggesting that this 25 bp DNA sequence represents a novel calcium response element required for calcium regulation of *BDNF* exon III expression. We refer to the calcium-responsive element within

this region as calcium-responsive sequence I (*CRS-I*) to distinguish it from the previously described calcium/cAMP response element (*CaRE/CRE*; Sheng et al., 1990).

We were also interested in determining whether the *CRS-I* was involved in regulating *BDNF* expression in postnatal neurons, since distinct roles of *BDNF* during embryonic and postnatal development may require different forms of regulatory control. For example, the regulatory mechanisms important in the embryonic cortex where *BDNF* may function as a trophic factor (Ghosh et al., 1994) may be distinct from those that are important in postnatal neurons where *BDNF* may mediate aspects of cortical plasticity (Cabelli et al., 1995, 1997; Akaneya et al., 1997). To identify regions of the gene that are involved in calcium-dependent expression in postnatal neurons, P1 cortical cultures were transfected with the reporter constructs described above, and once again an element between -72 and -47 was found to be required for transactivation (Figure 2C). These experiments indicate that the *CRS-I* functions as a calcium response element in both embryonic and postnatal cortical neurons.

To further characterize the sequences between -47 and -72 that were involved in mediating a calcium response, we generated reporter constructs that had mutations within this region. As shown in Figure 3A, in E18 cortical cultures mutations between -57 and -64 as well as mutations between -65 and -72 attenuated calcium-dependent transactivation. These results indicate that sequences between -57 and -72 are important for the calcium responsiveness of *CRS-I*.

Previous studies of calcium signaling pathways have indicated that DNA sequences upstream of the mRNA start site can have more than one calcium-responsive element. For example, the *c-fos* promoter, which has long served as a model for calcium-regulated transcriptional activation, contains two calcium response elements—the serum response element (*SRE*) at -300 and the cAMP/calcium response element (*CRE*) at -60 (Sheng et al., 1988; Misra et al., 1994). To determine if the *BDNF* promoter might similarly contain calcium response elements in addition to *CRS-I*, we carried out a sequence analysis of the promoter and found the sequence TCACGTCA, located between -38 and -31, which closely matches the consensus for a *CRE* (TGACGTCA) (Figure 2D). Our deletion analysis suggested, however, that this *CRE*, which we will refer to as *BIII-CRE*, was not sufficient to mediate a calcium response since the construct *-47BIII-CAT*, which included the *BIII-CRE* sequence, was unable to mediate calcium-dependent transactivation.

Although the deletion analysis suggested that the *BIII-CRE* was not sufficient to mediate a calcium response, it did not rule out the possibility that this element might be required for calcium-dependent transactivation in the context of the *BDNF* promoter. To examine this possibility, we created three constructs in which the *BIII-CRE* was mutated in context within the *BDNF* promoter. In one of the constructs, the *BIII-CRE* sequence was mutated (*-108BIII-CAT(CRE-)*) so that it no longer matched the *CRE* consensus sequence necessary for binding the transcription factor CREB (Montminy and Bilezikjian, 1987; Sheng et al., 1990). In the second construct, sequences between -46 and -32 were deleted in a *-108BIII-CAT* background (*-108BIII-CAT[del 46-32]*) to remove

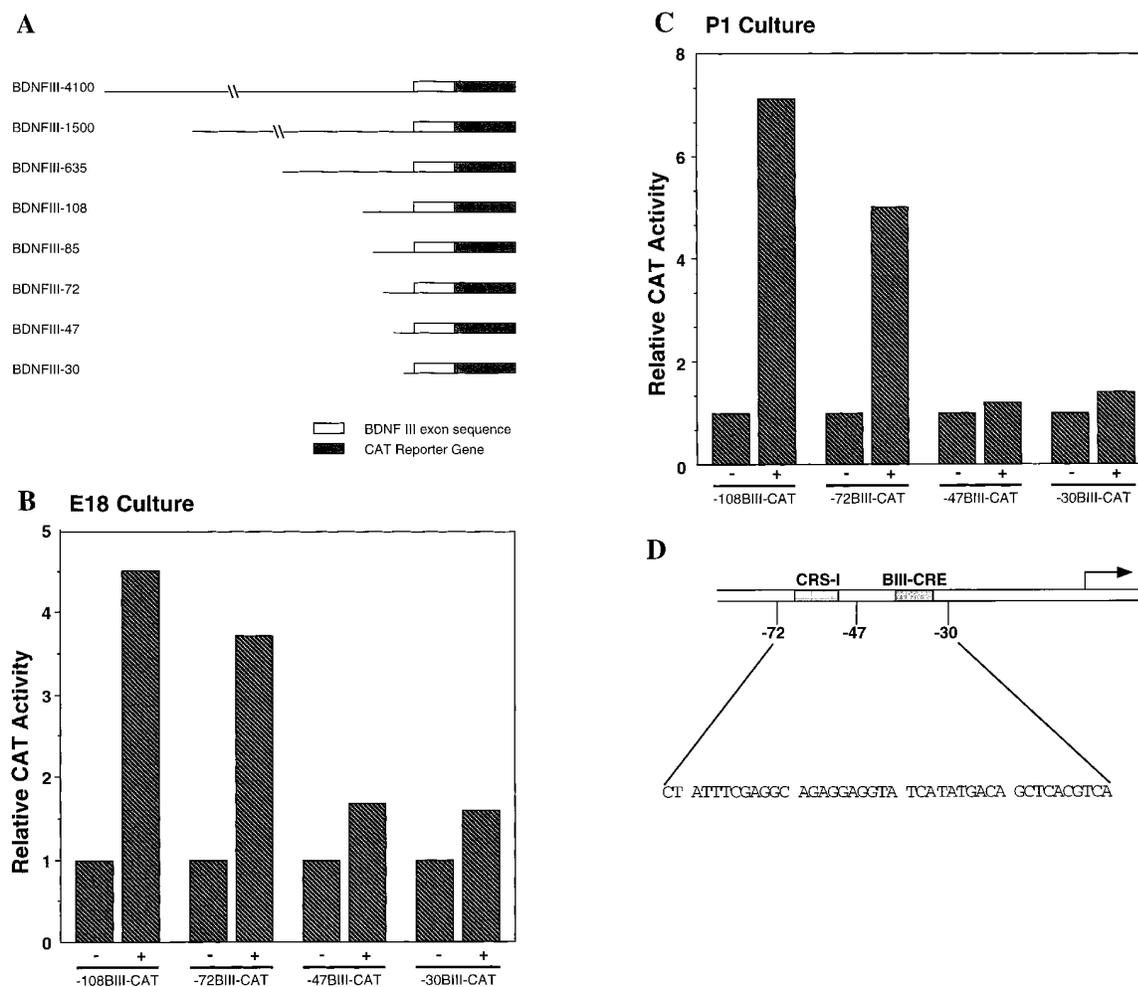


Figure 2. A Calcium Response Element Located between 72 and 47 bp Upstream of the Transcriptional Start Site Is Required for Transactivation of the *BDNF* Promoter in Response to Depolarization

(A) Diagrammatic summary of promoter constructs used to identify the calcium-responsive region within the *BDNF* exon III promoter.

(B) Relative CAT activity in E18 cortical cultures transfected with indicated constructs at 3DIV, left unstimulated (-) or stimulated with 50 mM KCl (+) at 5DIV.

(C) Relative CAT activity in P1 cortical cultures transfected with indicated constructs at 3DIV, left unstimulated (-) or stimulated with 50 mM KCl (+) at 5DIV.

(D) Sequence of the region of the *BDNF* exon III promoter that mediates a calcium response. The region between -72 and -47 does not include any known calcium response element. The region between -47 and -30 includes a *CRE*-like element (*BIII-CRE*) located between -38 and -31 (TACGTC A).

the *CRE* sequences, and in the third construct the *BIII-CRE* was mutated in context in a *-72BIII-CAT(CRE-)*. To evaluate the role of *BIII-CRE* sequences in calcium-dependent transactivation of *BDNF*, these constructs were transfected into E18 and P1 cortical cultures at 3DIV, and the cells were stimulated and harvested at 5DIV. As shown in Figure 3B, in E18 cortical cultures the deletion or the mutation of the *CRE* site attenuated but did not abolish the calcium response, suggesting that in these cells the *BIII-CRE* contributed to the calcium response but was not required for calcium-dependent transactivation of the promoter. To determine whether this *CRE*-independent component of the calcium response was mediated by sequences downstream of -72, we examined the ability of the construct *-72BIII-CAT(CRE-)* to mediate a calcium

response. This construct was also calcium responsive (comparable to *-108BIII-CAT(CRE-)*), indicating that sequences between -72 and the *BIII-CRE* can mediate calcium-dependent transactivation (data not shown). Therefore, in E18 cultures the *BIII-CRE* contributes to but is not absolutely necessary for a calcium response.

To determine if *BIII-CRE* functions in a similar way in neonatal neurons, we transfected the various *CRE* mutant reporters into P1 cortical cultures. The effects of *BIII-CRE* mutations in P1 cultures were notably different from what we had seen in E18 cultures. In these cultures, as shown in Figure 3C, mutations of the *BIII-CRE* completely prevented calcium-dependent transactivation of the reporter, indicating that *BIII-CRE* sequences were absolutely necessary for a calcium response in this age. These experiments strongly suggest that the *BIII-CRE*

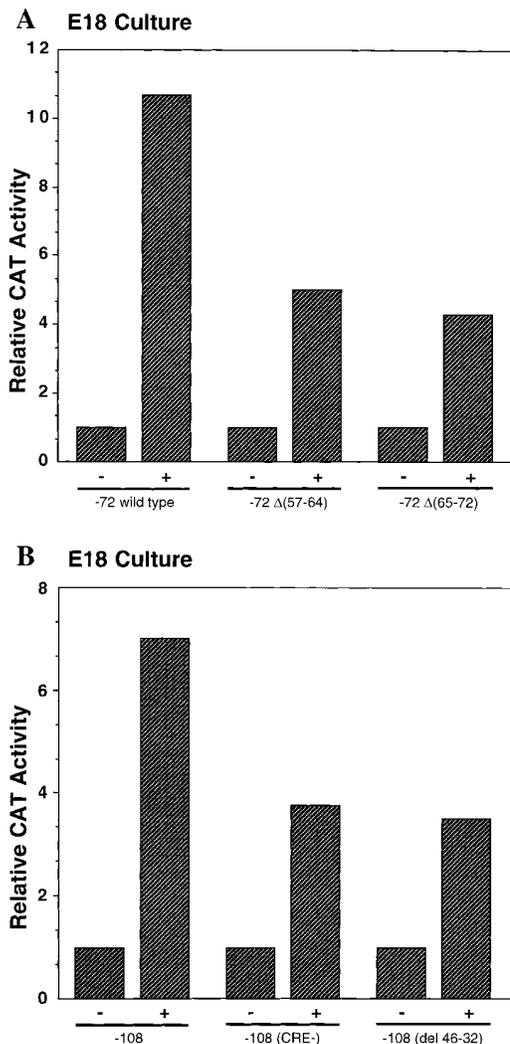


Figure 3. Mutational Analysis of the *BDNF* Promoter

E18 and P1 cortical cultures were transfected with constructs indicated at 3DIV and stimulated with 50 mM KCl at 5DIV.

(A) Relative CAT activity in E18 cortical cultures transfected with *-72BIII-CAT* or with constructs that have in-context mutations between -57 and -72, as indicated.

(B) Relative CAT activity in E18 cultures transfected with *-108BIII-CAT* or with constructs that have mutations that disrupt *BIII-CRE*.

(C) Relative CAT activity in P1 cortical cultures transfected with *-108BIII-CAT* or with constructs that have mutations that disrupt *BIII-CRE*.

functions as a developmental stage-specific calcium response element within the *BDNF* promoter, and that it is a critical mediator of calcium responses in postnatal neurons.

The promoter analysis experiments described above suggested the involvement of two elements in calcium regulation of *BDNF* expression. We were therefore interested in characterizing the proteins that bind to the *BDNF* promoter to see if we could find evidence for the formation of two separate protein complexes in the proximal region of the *BDNF* promoter. For these experiments, we made nuclear protein extracts from E18 and P1 cells in culture, and used the extracts in gel mobility shift experiments with three oligonucleotides that spanned the sequence between -72 and -20. As shown in Figure 4A, shifts with E18 extracts clearly indicated the existence of two protein complexes at the promoter. Importantly, the 72-47 oligonucleotide and the 47-20 oligonucleotide shifted distinct complexes, indicating that one complex forms between -72 and -47 and a second complex forms between -47 and -20. Gel shifts with P1 extracts were qualitatively similar to those with E18 extracts with one noteworthy difference. The complex that can clearly be

seen in 72-47 shifts with E18 extracts (indicated with an arrow) is barely apparent in shifts with P1 extracts (Figure 4A). A failure of this complex to effectively form in P1 cells may account for the absence of a *BIII-CRE*-independent pathway for transactivation in P1 cultures. The results of these gel shift experiments are consistent with the promoter analysis experiments described earlier and suggest that the complex we see shifted with 72-47 binds to *CRS-I*. The oligonucleotides 47-20 as well as 56-30 shift a complex not present in shifts with 72-47, and we infer that this complex should include protein(s) that bind to *BIII-CRE*.

To determine whether the *BIII-CRE* sequence could in fact bind to CREB, we carried out a series of gel shift experiments with a recombinant CREB peptide (which includes the DNA-binding domain [CREB1 bZIP; Santa Cruz]). As shown in Figure 4B, the recombinant CREB peptide could shift oligonucleotides containing a consensus *CRE*, 56-30, and 47-20; the peptide, however, failed to shift oligonucleotides containing 72-47 and 72-40, indicating that CREB can bind to a sequence between -40 and -30. Moreover, an oligonucleotide that contain a mutated *CRE*, 47-20 (CRE-), could not be

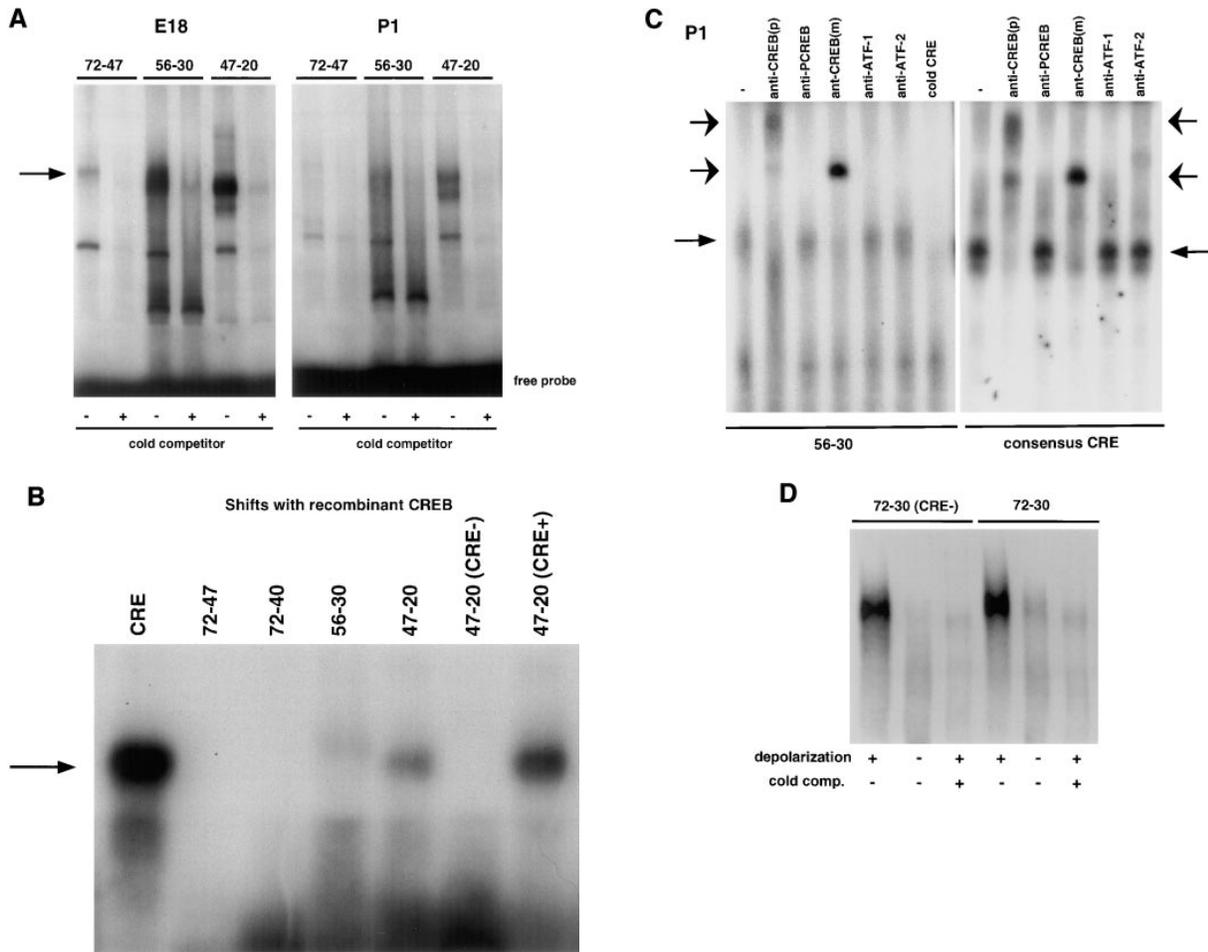


Figure 4. Analysis of Protein Complexes at the *BDNF* Promoter

(A) At least two different protein complexes bind to the *BDNF* promoter between 72 and 30 bp upstream of the mRNA start site. Nuclear protein extracts from E18 and P1 cultures were used to shift indicated ³²P-labeled double-stranded oligonucleotides in the absence (-) or presence (+) of 100-fold excess unlabeled (cold) competitor as described in the Experimental Procedures. Note that the complex in the 72–47 lane (indicated by an arrow) moves at a different mobility than the major complex in the 56–30 and 47–20 lanes. Note also that the complex at 72–47 is more abundant in E18 extracts compared to P1 extracts. The lower shifted band represents a nonspecific DNA–protein interaction since it is present in all lanes and is competed by DNA sequences unrelated to the *BDNF* promoter (data not shown).

(B) A recombinant CREB peptide can bind to the *BDNF* promoter in a CRE-dependent manner. A recombinant peptide containing the DNA-binding domain of CREB (see Experimental Procedures) was used in gel shift experiments with indicated oligonucleotides. CRE: consensus CRE from the *c-jun* promoter; 72–47, 72–40, 56–30, 47–20: fragments of the *BDNF* promoter; 47–20(CRE-): 47–20 oligonucleotide with a mutation in *BIII-CRE* to alter it from consensus CREB-binding sequence; and 47–20(CRE+): 47–20 oligonucleotide with a base substitution so that the *BIII-CRE* (TCACGTCA) is changed to a perfect CRE (TGACGTCA). The arrow indicates the location of the shifted complex.

(C) CREB is part of the complex that binds to *BIII-CRE*. Antibodies to CREB, but not to ATF-1 or ATF-2, can supershift the protein complex from P1 cultures, which binds to the 56–30 oligonucleotide. Similar results were obtained with extracts from E18 cultures (data not shown). Anti-CREB(p): polyclonal antibody to CREB; anti-PCREB: polyclonal antibody to phosphorylated CREB (serine 133); anti-CREB(m): monoclonal antibody to CREB; and anti-ATF-1, anti-ATF-2: monoclonal antibodies to ATF-1 and ATF-2.

(D) A protein complex that binds to the 72–30 fragment of the *BDNF* promoter independent of the *BIII-CRE* is regulated by stimulation (depolarization). Nuclear proteins from unstimulated E18 cultures (-) or cultures stimulated with 50 mM KCl for 4 hr (+) were used to shift ³²P-labeled oligonucleotides in the absence (comp. -) or presence (comp. +) of 100-fold excess cold competitor. Note that the shifted complex is present only in stimulated cultures.

shifted by the recombinant CREB, indicating that CREB binds to the *BIII-CRE* sequence within the *BDNF* promoter (Figure 4B).

To examine more directly whether the *BDNF* promoter-binding activity in nuclear extracts contains CREB or a CREB family protein, we carried out a series of gel shift experiments with the 56–30 oligonucleotide in the presence of antibodies to CREB and to CREB family

members ATF-1 and ATF-2. As shown in Figure 4C, addition of two different CREB antibodies led to supershifted complexes (top two arrows), indicating that the complex contains CREB. The shifted complexes did not show a supershift with antibodies to ATF-1 and ATF-2, arguing in favor of the presence of CREB and not another CREB family member in the complex. This conclusion is further reinforced by the fact that the pattern of shifts

and supershifts seen with the 56–30 oligonucleotide was virtually identical to the pattern of shifts seen with a consensus *CRE* oligonucleotide (derived from the *c-jun* promoter), and that excess of this cold consensus *CRE* could compete the complex that shifts 56–30 (Figure 4C). Similar results were obtained with nuclear extracts from both E18 and P1 cultures. (An antibody to phospho-CREB was unable to supershift either the 56–30 oligonucleotide or a consensus *CRE*; this failure was most likely due to dephosphorylation of CREB during the nuclear protein isolation procedure, since in the absence of stimulus CREB gets dephosphorylated quite rapidly [S.-C. H. and A. G., unpublished data]).

It is known that CREB can bind to the *CRE* in the absence of stimulus. To determine whether the binding of other cellular proteins to the *BDNF* promoter was also stimulus independent, we carried out shifts with the fragment 72–30 with extracts from stimulated and unstimulated E18 cortical neurons. As shown in Figure 4D, extracts from stimulated but not unstimulated neurons caused a strong shift of this DNA fragment. Moreover, the formation of this complex did not require an intact *CRE*, since mutations within *BIII-CRE* did not affect formation of this complex. These experiments indicate that the formation of a major protein–DNA complex at the *BDNF* promoter is calcium dependent, and it is reasonable to speculate that the calcium-dependent formation of this complex might play an important role in calcium-dependent transactivation of the reporter.

Although the gel shift experiments suggest that CREB is part of a complex that forms at the *BDNF* promoter, they do not indicate whether CREB is functionally required for transactivation of the promoter. In the next series of experiments, we therefore examined the effects of expressing a dominant negative mutant of CREB (KCREB; Walton et al., 1992) on transactivation of the *-108BIII-CAT* reporter. Strikingly, the results of this experiment were very similar to the effects of *BIII-CRE* mutations. Whereas KCREB completely abolished calcium-dependent transactivation in P1 cultures, it attenuated but did not abolish transactivation in E18 cultures (Figure 5). These experiments indicate that CREB-dependent transcription is important for transactivation of the *BDNF* promoter in both E18 and P1 cultures, and they also support the existence of a CREB-independent transactivating mechanism that can function in E18 cultures.

In our final series of experiments, we investigated the mechanism by which the calcium signal might be propagated to the nucleus to regulate *BDNF* expression. The calcium/calmodulin-dependent protein kinases, CaM kinase II and CaM kinase IV, have been shown to mediate several calcium-dependent biochemical and physiological changes in neurons. Importantly, both CaM kinase II and CaM kinase IV can phosphorylate the transcription factor CREB and have been implicated in calcium regulation of the *c-fos* gene (Sheng et al., 1991; Matthews et al., 1994; Sun et al., 1994). To determine whether CaM kinase II or CaM kinase IV might be involved in calcium regulation of *BDNF* expression, we first examined the distribution of these two kinases in cortical cultures. As shown in Figure 6, immunofluorescence using monoclonal antibodies indicated that both CaM kinase II and

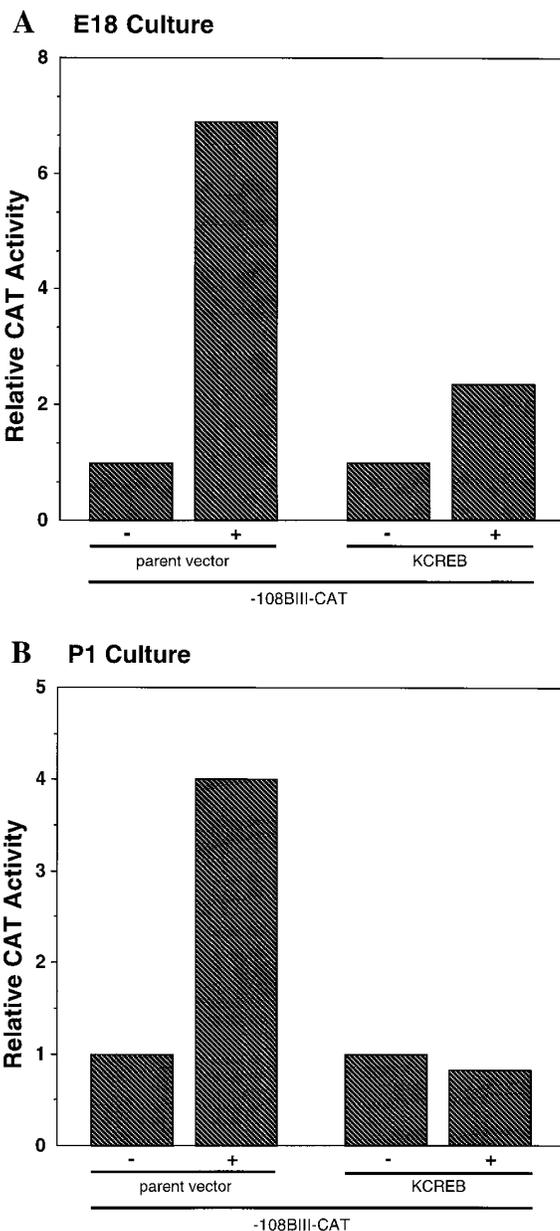


Figure 5. Expression of a Dominant Negative CREB Attenuates Transactivation of the *BDNF* Promoter

(A and B) Relative CAT activity in E18 (A) and P1 (B) cultures transfected with *-108BIII-CAT* together with a control vector or dominant negative CREB (KCREB). Cells were transfected at 3DIV and stimulated with 50 mM KCl at 5DIV.

IV are expressed in cortical cultures. Consistent with the distribution reported in hippocampal cultures (Bito et al., 1996), in cortical neurons CaM kinase II is present predominantly in the cytoplasm, and CaM kinase IV is present primarily in the nucleus (Figure 6), suggesting that one or both of these kinases might be involved in propagating the calcium signal to the nucleus.

To evaluate more directly the role of CaM kinase in *BDNF* expression, we next examined the effects of transfecting wild type and constitutively active mutants

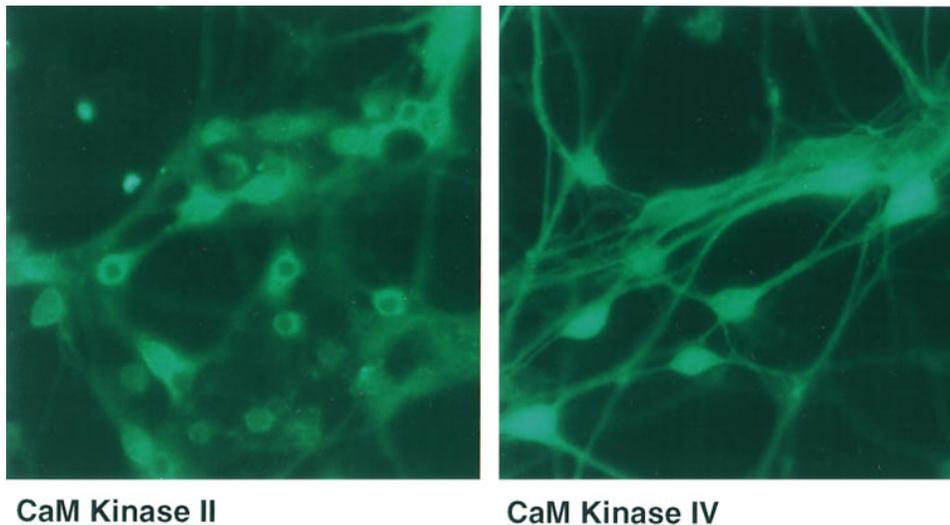


Figure 6. Distribution of CaM Kinase II and CaM Kinase IV in E18 Cortical Neurons in Culture Revealed by Immunofluorescence Using Monoclonal Antibodies to CaM Kinase II and CaM Kinase IV

Note that CaM kinase II, but not CaM kinase IV, is largely excluded from the nucleus.

of CaM kinase II and IV on BDNF transcription. The constitutively active mutants used in this experiment were deletion mutations in which the autoinhibitory domain of the kinase had been removed (Sun et al., 1994). In this series of experiments, wild type and constitutively active mutants of CaM kinase II or IV were cotransfected with *-634BIII-CAT* (data not shown) or *-108BIII-CAT* (Figure 7). As shown for E18 (Figure 7A) and P1 (Figure 7D) cultures, calcium-dependent transactivation was preserved in cells expressing wild-type CaM kinase II, although the level of transactivation was somewhat attenuated in P1 cultures (cf. Figure 2). Expression of constitutively active CaM kinase II, however, almost completely inhibited transactivation of the promoter in both E18 and P1 cultures (Figures 7A and 7D). These experiments suggest that activation of CaM kinase II is unlikely to be directly involved in transactivation of the *BDNF* promoter (see Discussion).

The effects of expressing CaM kinase IV constructs were quite different from those of expressing CaM Kinase II. Although the expression of wild-type CaM kinase IV did not have a marked effect on the induction of the reporter constructs, in both E18 and P1 cultures expression of constitutively active CaM kinase IV was sufficient for transactivation of the *BDNF* promoter even in the absence of stimulus (Figures 7B and 7E). In addition, expression of constitutively active CaM kinase IV largely occluded calcium-dependent transactivation of

the promoter (compare – and + lanes in cells transfected with CaM kinase IV ca), which is consistent with CaM kinase IV being part of the normal signaling pathway by which calcium influx leads to BDNF expression. Finally, to determine whether CaM kinase IV modulates transactivation via *CRS-I*, *BIII-CRE*, or both, we examined the effects of expressing wild-type and constitutively active CaM Kinase IV on transactivation via the *-108BIII-CAT(CRE-)* reporter. As shown in Figure 7C, constitutively active CaM kinase IV was not sufficient to activate transcript of this mutated reporter construct, indicating that CaM kinase IV acts via the *BIII-CRE* to regulate transcription.

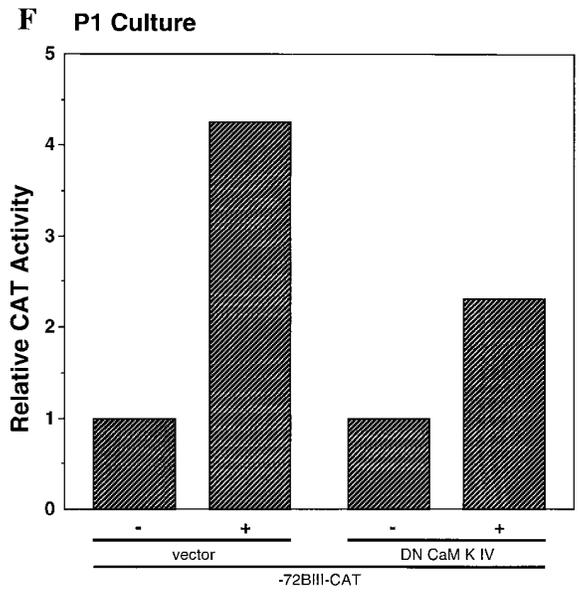
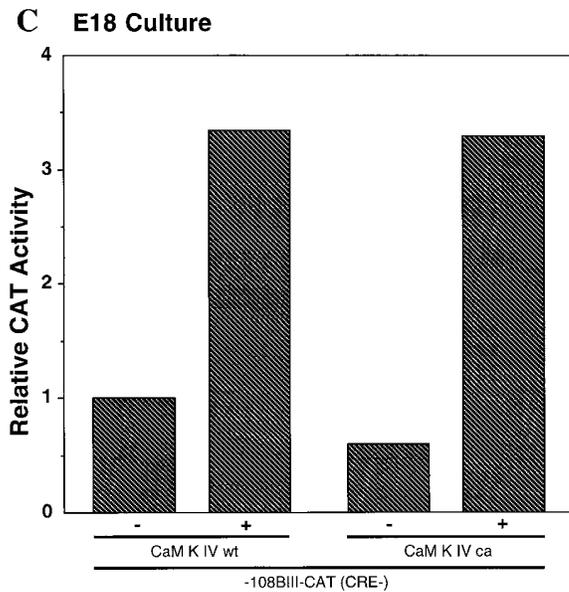
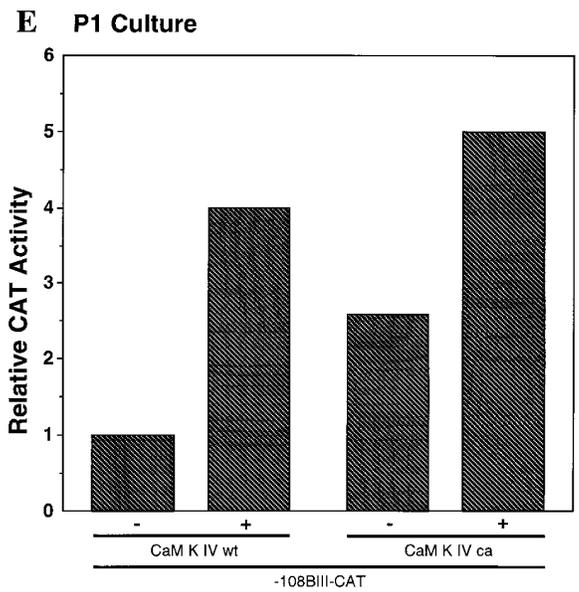
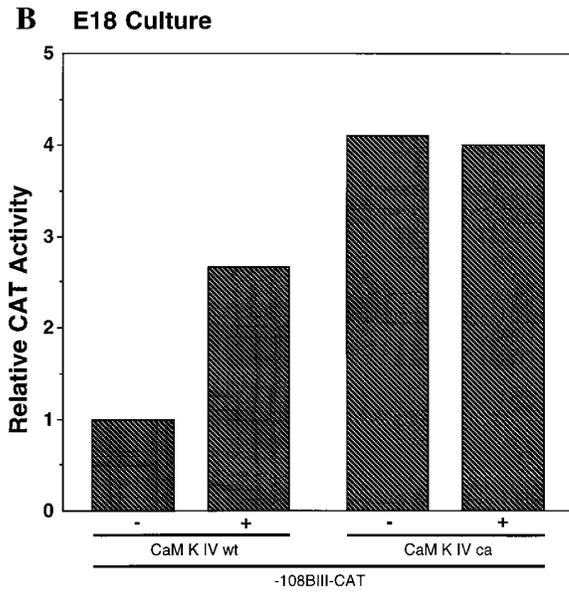
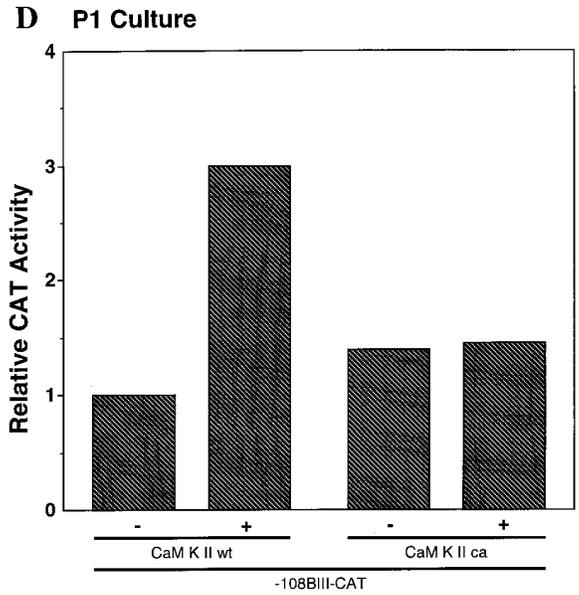
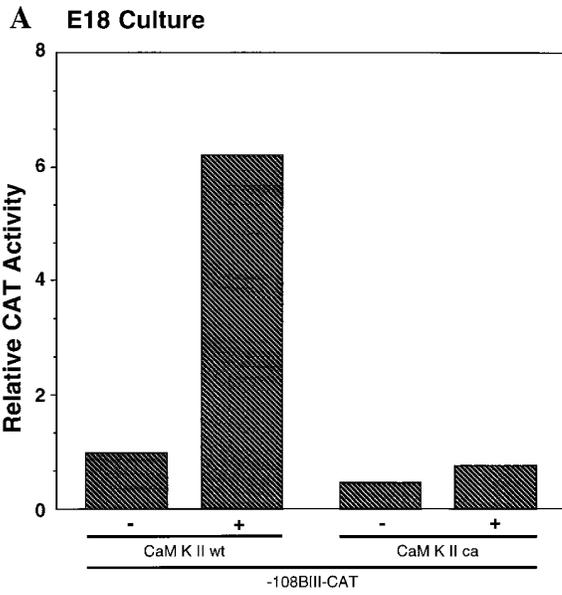
Finally, to determine whether CaM kinase IV activity was necessary for calcium-dependent transactivation of *BDNF*, we examined the effects of inhibiting CaM kinase IV using an inactive CaM kinase IV construct (Δ CaM kinase IV/*Gr(i)*; Ho et al., 1996). Overexpression of this construct has been shown to exhibit a dominant negative effect on endogenous CaM kinase IV (Finkbeiner et al., 1997). As shown in Figure 7F, expression of dominant negative CaM kinase IV markedly attenuated calcium-dependent transactivation of the reporter *-72BIII-CAT*. This observation, taken together with the occlusion effect of constitutively active CaM kinase IV described above, strongly suggests that calcium-dependent activation of the *BDNF* gene is mediated via activation of CaM kinase IV.

Figure 7. Effects of Perturbing CaM Kinase Activity on Transactivation of the *BDNF* Promoter

(A–C) Relative CAT activity in E18 cultures transfected with indicated *BDNF* reporter constructs together with wild-type (wt) or constitutively active (ca) CaM kinase II or CaM kinase IV at 3DIV and stimulated with 50 mM KCl at 5DIV.

(D and E) Relative CAT activity in P1 cultures transfected with indicated *BDNF* reporter constructs together with wild-type (wt) or constitutively active (ca) CaM kinase II or CaM kinase IV at 3DIV and stimulated with 50 mM KCl at 5DIV. Note that the effect of CaM kinase IV on transactivation (B) is abolished when the *CRE* is mutated in the reporter (C), indicating that CaM kinase IV acts via the *CRE* to mediate transactivation.

(F) Relative CAT activity in P1 cultures transfected with *-72BIII-CAT* together with a control vector or dominant negative mutant of CaM kinase IV at 3DIV and stimulated with 50 mM KCl at 5DIV.



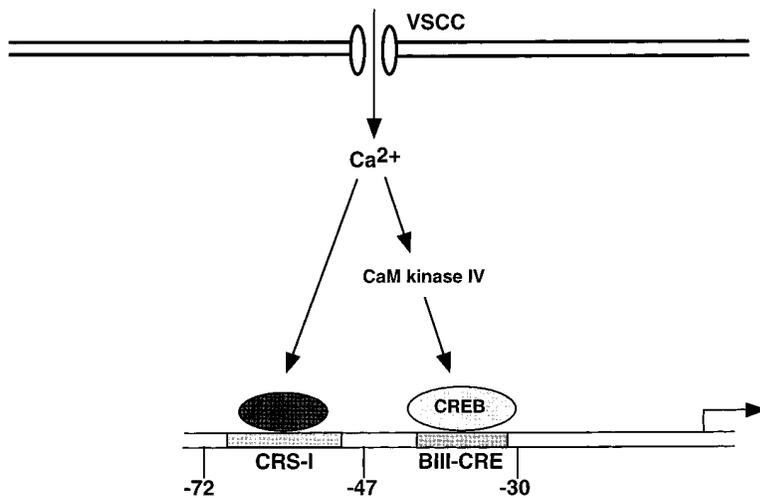


Figure 8. Diagrammatic Representation of the Proposed Mechanism by which Calcium Influx Regulates *BDNF* Expression

Transactivation of the *BDNF* promoter is mediated by two elements, *CRS-I* and *BIII-CRE*. The *BIII-CRE*-dependent component of transactivation is mediated by CREB and can be regulated by CaM kinase IV.

Discussion

In this study, we have examined the mechanism by which calcium influx via voltage-sensitive calcium channels regulates the expression of *BDNF* in cortical neurons. We have focused on analysis of the promoter of exon III, which is the major activity-regulated exon of the *BDNF* gene. Our deletion analysis as well as pharmacological and molecular perturbation experiments suggest that calcium-dependent transcription of the *BDNF* gene is mediated by elements located within 72 bp upstream of the exon III mRNA start site. This segment of the promoter contains two elements that contribute to the calcium response. The first element, denoted *CRS-I*, is located between -72 and -47 and is required for induction of exon III in both embryonic and postnatal cortical cultures. The second element is located between -40 and -30 bp upstream of the mRNA start site, contains a *CRE*-like sequence (TCACGTCA), and is required for calcium-dependent transactivation of *BDNF* in postnatal but not embryonic cultures. Based on this analysis, we conclude that calcium-dependent *BDNF* gene expression is mediated by distinct mechanisms in embryonic and postnatal cortical cultures. In embryonic cultures, *CRS-I* can function in a *BIII-CRE*-independent manner to mediate calcium-dependent transactivation. In postnatal cultures, two elements, *CRS-I* and *BIII-CRE*, cooperate to mediate a calcium response.

One of the major findings of this study is the identification of a novel calcium response sequence, *CRS-I*, which is involved in calcium-dependent expression of *BDNF*. Based on deletion analysis, this element is located between -72 and -47 and is required for calcium-dependent transactivation in both embryonic and postnatal neurons. Initial mutational analysis of the region between -72 and -47 indicates that sequences between -57 and -72 contribute substantially to the responsiveness of the *CRS-I* element. Although we do not know much about the transcription complex that forms at *CRS-I*, we can infer certain properties of this element based on mutational analysis of the promoter and various gel shift assays. Importantly, this element can function in a *CRE*-independent manner to mediate a calcium response in

embryonic neurons, suggesting that *CRS-I* is both necessary and sufficient for calcium-dependent transactivation. Gel mobility shift experiments indicate sequences between -47 and -72 bind to a complex that is more abundant in nuclear extracts of E18 cultures than in extracts of P1 cultures. Importantly, we find that the *CRE*-independent complex that forms at the *BDNF* promoter is regulated by activity. It will be of interest to determine if this reflects activity-dependent DNA binding of an existing protein complex or the activity-dependent synthesis of proteins that are involved in regulating *BDNF* transcription. It is likely that this complex contains the transcription factor that mediates transactivation via *CRS-I*. The signaling mechanisms involved in mediating transactivation via *CRS-I* have not yet been identified. Our experiments, however, indicate that unlike transactivation via the *BIII-CRE*, CaM kinase IV is neither sufficient nor necessary for transactivation via *CRS-I*.

Our experiments indicate that together with *CRS-I*, the *BIII-CRE* contributes significantly to calcium-dependent transactivation. In embryonic cultures, in-context mutations of the *BIII-CRE* attenuate calcium-dependent transactivation, and in postnatal cultures the same mutations completely abolish the calcium response. Therefore, the *BIII-CRE* contributes to calcium-dependent transactivation at all ages and is absolutely required in postnatal cultures. Several lines of evidence suggest that the *BIII-CRE* component of the calcium-dependent transactivation is mediated by CREB. First, the *BIII-CRE* (TCACGTCA) closely resembles a perfect *CRE* (TGACGTCA), suggesting that the *BIII-CRE* might function as a binding site for CREB. Second, recombinant CREB binds to oligonucleotides derived from the *BDNF* promoter in a *BIII-CRE*-dependent manner. Third, gel mobility shifts with a *BDNF* promoter fragment (56–30) are competed with cold consensus *CRE* oligonucleotides, indicating that the complex at the *BDNF* promoter contains a *CRE*-binding protein. Fourth, two different antibodies to CREB (a polyclonal and a monoclonal), but not antibodies to ATF-1 or ATF-2, supershift a complex formed at the *BDNF* promoter, indicating that CREB is part of the complex. Fifth, expression of K-CREB, a dominant negative mutant of CREB, markedly attenuates calcium-dependent transactivation of the promoter.

These pieces of evidence taken together strongly suggest that the *BIII-CRE* contributes to calcium regulation of *BDNF* and that this component of the calcium response is mediated by CREB.

An interesting aspect of the way the *BDNF* promoter seems to be regulated is the functional interaction between the *CRS-I* and *BIII-CRE* elements. Whereas *CRS-I* can function independently of *BIII-CRE* in embryonic cultures, it requires the presence of *BIII-CRE* in the promoter to mediate a calcium response in postnatal cultures. This cooperation between the *CRS-I* and *BIII-CRE* is reminiscent of growth factor regulation of the *c-fos* promoter. In that case, the *c-fos CRE* by itself cannot mediate a growth factor response, much as the *BIII-CRE* by itself cannot mediate calcium-dependent activation of the *BDNF* promoter. In the context of the full promoter, however, the *c-fos CRE* cooperates with the serum response element (SRE) to mediate transactivation (Ginty et al., 1994; Bonni et al., 1995). In an analogous manner, we propose that *BIII-CRE* cooperates with *CRS-I* to mediate calcium-dependent transactivation of the *BDNF* promoter in postnatal neurons.

One of the most striking observations of this study is the developmental stage-specific use of the *BIII-CRE* sequences. Whereas mutations of this site do not prevent calcium-dependent transactivation of the promoter in E18 cultures, the same mutations completely block calcium-dependent transcription in postnatal cultures. This is a noteworthy finding, since it suggests that the *BIII-CRE* can act as a gate that can regulate calcium-dependent transcription at particular developmental stages. The fact that a stringent dependence on the *BIII-CRE* should emerge in postnatal cultures is particularly interesting, since the cortex begins to receive environmental stimuli during postnatal development and undergoes a period of activity-dependent plasticity. Based on recent evidence that BDNF may be involved in mediating aspects of activity-dependent plasticity (reviewed by Bonhoeffer, 1996; Shieh and Ghosh, 1997), we would suggest that transcriptional gating in postnatal cultures via the *BIII-CRE* provides a mechanism for the production of BDNF only in response to stimuli that can lead to long-term adaptive changes. For example, only those presynaptic stimuli that lead to firing of the postsynaptic neurons may be effective in activating the signaling pathway that controls *BIII-CRE*-dependent transcription.

Analysis of the signaling pathways involved in regulation of BDNF expression fits well with this model. It has been previously reported that CREB can be phosphorylated by CaM kinases at serine 133 (Gonzalez and Montminy, 1989; Sheng et al., 1991; Matthews et al., 1994; Sun et al., 1994), a site critical for CREB-dependent transcription. Immunofluorescence experiments indicate that both CaM kinase II and CaM kinase IV are expressed in the cortical neurons in culture. We find that constitutively active mutants of CaM kinase IV can drive transactivation of the promoter in the absence of extracellular stimuli, but that constitutively active mutants of CaM kinase II have an opposite effect and inhibit calcium-dependent transactivation. The observation that expression of a constitutively active mutant CaM kinase II inhibits the calcium-dependent expression of *BDNF* was surprising, since activation of CaM kinase II is positively

correlated with synaptic strengthening in cellular models of plasticity (Malinow et al., 1989; Silva et al., 1992). It should be noted, however, that the ability of activated CaM kinase II to inhibit transactivation is similar to its reported inhibition of CREB-dependent transcription in PC12 cells (Sun et al., 1994). That study indicated that constitutively active mutants of CaM kinase II led to phosphorylation of CREB at serine 142 in addition to serine 133, and this second phosphorylation appeared to inhibit the CREB-mediated transcription. Unlike wild-type CaM kinase II, the constitutively active mutants of CaM kinase II can translocate to the nucleus and therefore can directly phosphorylate CREB at an inhibitory site. The effect of constitutively active CaM kinase II on transactivation may therefore be related to its ability to translocate to the nucleus. In coexpression experiments, we have found that constitutively active CaM kinase II inhibits constitutively active CaM kinase IV-induced transactivation of the *BDNF* promoter, suggesting that the effects of CaM kinase II in this context may be mediated by a similar phosphorylation event.

Our observations are most consistent with the interpretation that CaM kinase IV mediates calcium activation of the *BDNF* promoter, since we find that expression of a constitutively active mutant of CaM kinase IV is sufficient for transactivation of the promoter in the absence of extracellular stimulation, since expression of a constitutively active mutant of CaM kinase IV largely occludes calcium-dependent transactivation of the *BDNF* promoter, and since inactive, dominant negative mutants of CaM kinase IV largely inhibit calcium-dependent transactivation. The effect of CaM kinase IV on the promoter requires the presence of *BIII-CRE*, suggesting that the CaM kinase IV effect is mediated by CREB. Such an interpretation would be consistent with previous reports (Sun et al., 1994), which indicate that CREB is directly phosphorylated by CaM kinase IV at serine 133. We therefore propose that the *BIII-CRE* component of transactivation is mediated by CaM kinase IV, which acts by phosphorylating CREB at serine 133. We should also note that since *CRE* mutations abolish CaM kinase IV effects on the promoter, but do not abolish calcium-dependent transactivation of the promoter in embryonic cultures, the *BIII-CRE*-independent component of transactivation (via *CRS-I*) is likely to involve another pathway.

Given the evidence that BDNF may be involved in synaptic strengthening, the observation that CaM kinase IV may regulate transactivation of the *BDNF* gene is interesting, since it suggests a mechanism that may be involved in activity-dependent plasticity in postnatal neurons. Hebbian models of plasticity require correlated activity of the pre- and postsynaptic neurons for strengthening of connections. If a synaptic input is weak and does not lead to a firing of the postsynaptic neuron, it may not lead to the activation of VSCC at the soma and the consequent activation of CaM kinase IV in the nucleus. Such a stimulation would therefore not lead to *BDNF* expression, and the synapse would not undergo long-term strengthening or stabilization. In contrast, strong stimuli that cause the postsynaptic cell to fire action potentials would lead to the activation of CaM kinase IV and phosphorylation of CREB, which in turn

would lead to transactivation of the *BDNF* gene. BDNF thus produced could contribute to long-term synaptic strengthening and perhaps to the growth of pre- or post-synaptic processes. According to this model, the CaM kinase IV-BDNF pathway could turn out to be a central component of activity-dependent plasticity in the developing cortex, a prediction that is testable at various levels.

Experimental Procedures

Primary Cell Cultures

Cortical neurons from E18 Long-Evans rat embryos or P1 Long-Evans rat pups were cultured as previously described (Ghosh and Greenberg, 1995) with the following minor modifications. The cortex was dissected in ice-cold HBSS (6.5 g/L glucose), digested in 10 U/ml Papain in dissociation media (2×20 mins), and dissociated in culture media. The dissociated neurons were plated on 60 mm plates precoated with poly-D-lysine and laminin at a density of 3×10^6 cells per plate in either glutamine-free Basal Media Eagle (Sigma) supplemented with glutamine (to 1 mM), N2 (to 1%; GIBCO), and fetal bovine serum (5%) or in Neurobasal Media (GIBCO) supplemented with glutamine (to 1 mM) and B27 (to 2%; GIBCO).

Plasmids

-100BIII-CAT was constructed by subcloning a 4.4 kbp HindIII/XbaI fragment containing exon III into pBLCAT2 vector. -1500BIII-CAT, -634BIII-CAT, and -108BIII-CAT were similarly constructed from a 1.8 kbp EcoRV/XbaI fragment, a 0.9 kbp HincII/XbaI fragment, and a EcoRI/XbaI fragment, respectively. -108BIII-CAT(CRE-) was constructed by modified inverse PCR mutagenesis (Stratagene), using the following two oligos: 5'-CAGTCTAGAAGGAGGCAGCGTGGAGCCTC-3' and 5'-CAGTCTAGAGCTGCATATGATGATCCTC-3', which places an XbaI restriction site in the place of BIII-CRE. After PCR amplification, the fragment was gel purified, cut with XbaI, gel purified, and self ligated. -72BIII-CAT(CRE-) was constructed by ligating a double-stranded oligonucleotide to a vector made from a BamHI/XbaI digest of the -108BIII-CAT(CRE-). The double-stranded oligo was annealed from the following two single-stranded oligos: 5'-GATCCCTATTTTCGAGGCAGAGGAGGTATCATATGACAGCTCT-3' and 5'-CTAGAGAGCTGCATATGATGATCCTCCTCGAAATAGG-3'. -72BIII-CAT Δ (57-64) and -72BIII-CAT Δ (65-72) were constructed by ligating two separate double-stranded oligonucleotides to a vector made from a BamHI/NdeI digest of the -108BIII-CAT. The double-stranded oligos were annealed from the following single-stranded oligos: 5'-GATCCCTATTTTCGAGATCTTGGATATCA-3' and 5'-TATGATACCTCAAGATCTCGAAATAGG-3' for -72BIII-CAT Δ (57-64) and 5'-GATCCAGATCCGTAGGAGGAGGATCA-3' and 5'-TATGATACCTCCTCTGCCTAAGATCTGG-3' for -72BIII-CAT Δ (65-72). -47BIII-CAT was constructed from -108BIII-CAT by digestion with NdeI (located at -47) and BamHI (located in the 5' polylinker sequence), blunt ended, gel purified, and religated. -30BIII-CAT was constructed by digestion of -108BIII-CAT(CRE-) with XbaI (located at -34) and BamHI, blunt ended, gel purified, and religated. -85BIII-CAT and -72BIII-CAT were created by PCR subcloning with the primers 5'-CAGGATCCCGTGCACCTAGAGTGTCTATTTTCG-3' (for -85BIII-CAT) and 5'-CAGGATCCCTATTTTCGAGGAGGAGG-3' (for -72BIII-CAT) at one end and the primer 5'-CAGCTCGAGGTCGACGGTATCG-3'. After gel purification, PCR product was digested with BamHI/XhoI, gel purified again, and ligated with prepared vector. Vector was prepared by digesting -108BIII-CAT with BamHI (located in the 5' polylinker sequence) and XhoI (located at +300).

Transfections

Cells were transfected by a modified calcium phosphate transfection procedure, as described previously (Threadgill et al., 1997). Briefly, the culture media was removed and replaced with DMEM at least 1 hr prior to transfection. The calcium phosphate/DNA precipitate was formed in HEPES buffered saline (pH 7.07) and added dropwise to the DMEM. A total of 9–10 μ g of DNA was used per plate. Typically, this would include of 4–5 μ g of reporter construct

DNA, 2 μ g of DNA coding a candidate interacting protein (e.g., CaM Kinase), and carrier DNA. Following a 20–30 min transfection during which a fine sandy precipitate covered the cells, the cultures were washed in DMEM and returned to the original culture media. The efficiency was typically between 1% and 5%, and there was no apparent toxicity to the cells. In all cases, the DNA was purified by two rounds of CsCl banding.

CAT Assays

Cells were harvested immediately after stimulation in isotonic TNE (10 mM Tris [pH 7.8], 150 mM NaCl, 1 mM EDTA). Cells were spun down gently and subjected to three cycles of freeze-thaw lysis. Lysis supernatant was incubated with 0.5 μ Ci 14 C-labeled chloramphenicol (Amersham) and 0.8 mmol acetyl CoA (Boehringer Mannheim) at pH 7.8 and 37°C for 1 hr. Reaction mixtures were extracted with ethyl acetate, speedvacuumed, resuspended in chloroform, spotted on thin-layer chromatography (TLC) plates (J. T. Baker), and separated by ascending chromatography for 2 hr (95% chloroform, 5% methanol). Data from any single histogram are quantified from experiments performed on cells that were simultaneously cultured, transfected, and assayed to minimize variability due to subtle differences in experimental procedure. Normalization was calculated relative to an unstimulated control with the same reporter construct. Shown are representative examples of experiments that were performed two or more times. For measurements of relative CAT activity, levels of 14 C emissions on TLC plates were quantified by phosphorimager scans.

Gel Mobility Shift Assays

Nuclear extracts were prepared using a rapid technique described previously (Andrews and Faller, 1991) with the following modifications. Cultured P1 and E18 cells were stimulated with 50 mM KCl at 2DIV–5DIV as described earlier and scraped into 1.5 ml cold phosphate-buffered saline (PBS). The cells were pelleted by centrifugation for 2 min at 4°C and then resuspended in 0.5 ml cold lysis buffer (10 mM HEPES-KOH [pH 7.9], 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT, and 0.2 mM PMSF). A Dounce homogenizer was used to generate a lysate containing whole nuclei, which were then collected by a brief 2 min centrifugation. The pellets were resuspended in 0.05–0.1 ml cold extraction buffer (20 mM HEPES-KOH [pH 7.9], 25% glycerol, 400 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT, and 0.2 mM PMSF plus additional protease inhibitors) and mixed continuously for 2 hr at 4°C to extract nuclear proteins. Cellular debris was removed by centrifugation for 2 min at 4°C and the supernatant was stored frozen at -80°C.

DNA probes were generated by annealing complimentary synthetic oligonucleotides and were end labeled using [γ -³²P]dATP and T4 polynucleotide kinase. Binding reactions were performed in a total volume of 30 μ l containing 5 mM HEPES, 6.5% glycerol, 0.5 mM EDTA, 1 mM DTT, 1 mM MgCl₂, 100 mM NaCl, 0.5 mM spermidine, 50 μ g/ml tRNA, and 10 μ g/ml poly dI-dC as nonspecific DNA. Radiolabeled probe (0.5 pmol, 10⁵ cpm) was incubated with 15 μ g nuclear extract at room temperature for 60 min. Specificity of the protein-DNA complexes was determined by preincubating the extracts with an excess of homologous unlabelled DNA (10 pmol) for 30 min. Similarly, competition assays were performed by preincubating with an excess of unlabeled competitor DNA. Super-shift antibodies (CREB-1, ATF-1, ATF-2, Santa Cruz Biotechnology; α CREB, P-CREB, Dr. David Ginty) were added to the reactions following the initial incubation and allowed another 60 min at room temperature to bind. A polypeptide containing the DNA-binding domain of CREB (1 μ g CREB-1 bZIP, Santa Cruz) and an oligonucleotide containing the CRE consensus binding site (Promega) were used as controls for these binding studies. Samples were loaded directly onto a 4% nondenaturing gel (80:1 poly-acrylamide:bis-acrylamide) prerun in 1 \times TG buffer (50 mM Tris, 380 mM glycine, and 2 mM EDTA [pH 8.5]). Electrophoresis was performed at 35 mA for 3–4 hr to resolve protein-DNA complexes from free DNA, and then gels were fixed in 20% methanol–20% acetic acid, dried onto Whatman 3MM paper, and visualized by autoradiography.

Immunofluorescence

Cultures were fixed with 4% paraformaldehyde in PBS (37°C) for 15 min, washed with PBS (2 \times 5 min, room temperature), blocked for

2 hr with 3% bovine serum albumin (BSA) and 0.3% Triton X-100 in PBS (room temperature), and incubated in the primary antibody (diluted in the blocking solution) overnight at 4°C. After primary antibody incubation, cells were washed in PBS (3 × 5 min), incubated in fluorescein-conjugated goat anti-mouse secondary antibody for 1 hr, washed in PBS (3 × 5 min), and coverslipped. The following antibodies were used: mouse anti-CaM kinase IIa (Boehringer Mannheim; 1:200) and mouse anti-CaM kinase IV (Transduction Laboratories; 1:200).

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References

- Akaneya, Y., Tsumoto, T., Kinoshita, S., and Hatanaka, H. (1997). Brain-derived neurotrophic factor enhances long-term potentiation in rat visual cortex. *J. Neurosci.* **7**, 6707–6716.
- Andrews, N.C., and Faller, D.V. (1991). A rapid micropreparation technique for extraction of DNA-binding proteins from limiting numbers of mammalian cells. *Nucleic Acids Res.* **19**, 2499.
- Barbacid, M. (1995). Neurotrophic factors and their receptors. *Curr. Opin. Cell Biol.* **7**, 148–155.
- Barde, Y.A., Edgar, D., and Thoenen, H. (1982). Purification of a new neurotrophic factor from mammalian brain. *EMBO J.* **1**, 549–553.
- Berkemeier, L.R., Winslow, J.W., Kaplan, D.R., Nicolics, K., Goeddel, D.V., and Rosenthal, A. (1991). Neurotrophin-5: a novel neurotrophic factor that activates trk and trkB. *Neuron* **7**, 857–866.
- Bito, H., Deisseroth, K., and Tsien, R.W. (1996). CREB phosphorylation and dephosphorylation: a Ca²⁺- and stimulus duration-dependent switch for hippocampal gene expression. *Cell* **87**, 1203–1214.
- Bonhoeffer, T. (1996). Neurotrophins and activity-dependent development of the neocortex. *Curr. Opin. Neurobiol.* **6**, 119–126.
- Bonni, A., Ginty, D.D., Dudek, H., and Greenberg, M.E. (1995). Serine 133-phosphorylated CREB induces transcription via a cooperative mechanism that may confer specificity to neurotrophin signals. *Mol. Cell. Neurosci.* **6**, 168–183.
- Bozzi, Y., Pizzorusso, T., Cremisi, F., Rossi, F.M., Barsacchi, G., Maffei, L. (1995). Monocular deprivation decreases the expression of messenger RNA for brain-derived neurotrophic factor in the rat visual cortex. *Neuroscience* **69**, 1133–1144.
- Cabelli, R.J., Hohn, A., and Shatz, C.J. (1995). Inhibition of ocular dominance column formation by infusion of NT-4/5 or BDNF. *Science* **267**, 1662–1666.
- Cabelli, R.J., Shelton, D.L., Segal, R.A., and Shatz, C.J. (1997). Blockade of endogenous ligands of TrkB inhibits formation of ocular dominance columns. *Neuron* **19**, 63–76.
- Castren, E., Zafra, F., Thoenen, H., Lindholm, D. (1992). Light regulates expression of brain-derived neurotrophic factor mRNA in rat visual cortex. *Proc. Natl. Acad. Sci. USA* **89**, 9444–9448.
- Chao, M.V. (1992). Neurotrophin receptors: a window into neuronal differentiation. *Neuron* **9**, 583–593.
- Conover, J.C., Erickson, J.T., Katz, D.M., Bianchi, L.M., Poueymirou, W.T., McClain, J., Pan, L., Helgren, M., Ip, N.Y., Boland, P., et al. (1995). Neuronal deficits, not involving motor neurons, in mice lacking BDNF and/or NT4. *Nature* **375**, 235–238.
- Crowley, C., Spenser, S.D., Nishimura, M.C., Chen, K.S., Pitts-Meek, S., Armanini, M.P., Ling, L.H., McMahon, S.B., Shelton, D.L., Levinson, A.D., and Phillips, H.S. (1994). Mice lacking nerve growth factor display perinatal loss of sensory and sympathetic neurons yet develop basal forebrain cholinergic neurons. *Cell* **76**, 1001–1012.
- Ernfors, P., Lee, K.F., Kucera, J., and Jaenisch, R. (1994a). Lack of neurotrophin-3 leads to deficiencies in the peripheral nervous system and loss of limb proprioceptive afferents. *Cell* **77**, 503–512.
- Ernfors, P., Lee, K.F., and Jaenisch, R. (1994b). Mice lacking brain-derived neurotrophic factor develop with sensory deficits. *Nature* **368**, 147–150.
- Figurov, A., Pozzo-Miller, L.D., Olafsson, P., Wang, T., and Lu, B. (1996). Regulation of synaptic responses to high-frequency stimulation and LTP by neurotrophins in the hippocampus. *Nature* **381**, 706–709.
- Finkbeiner, S., Tavazoie, S.F., Maloratsky, A., Jacobs, K.M., Harris, K.M., and Greenberg, M.E. (1997). CREB: a major mediator of neuronal neurotrophin responses. *Neuron* **19**, 1031–1047.
- Galuske, R.A.W., Kim, D.-S., Castren, E., Thoenen, H., and Singer, W. (1996). Brain-derived neurotrophic factor reverses experience-dependent synaptic modifications in kitten visual cortex. *Eur. J. Neurosci.* **8**, 1554–1559.
- Ghosh, A. (1996). Cortical development: with an eye on neurotrophins. *Curr. Biol.* **6**, 130–133.
- Ghosh, A., and Greenberg, M.E. (1995). Distinct roles for bFGF and NT-3 in the regulation of cortical neurogenesis. *Neuron* **15**, 89–103.
- Ghosh, A., Carnahan, J., and Greenberg, M.E. (1994). Requirement for BDNF in activity-dependent survival of cortical neurons. *Science* **263**, 1618–1623.
- Ginty, D.D., Bonni, A., and Greenberg, M.E. (1994). Nerve growth factor activates a Ras-dependent protein kinase that stimulates *c-fos* transcription via phosphorylation of CREB. *Cell* **77**, 713–725.
- Gonzalez, G.A., and Montminy, M.R. (1989). Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133. *Cell* **59**, 675–680.
- Hallbook, F., Ibanez, C.F., and Persson, H. (1991). Evolutionary studies of the nerve growth factor family reveal a novel member abundantly expressed in *Xenopus* ovary. *Neuron* **6**, 845–858.
- Ho, N., Cullberg, M., and Chatila, T. (1996). Activation Protein 1-dependent transcriptional activation of Interleukin 2 gene by Ca²⁺/calmodulin kinase type IV/Gr. *J. Exp. Med.* **184**, 101–112.
- Hohn, A., Leibrock, J., Bailey, K., and Barde, Y.A. (1990). Identification and characterization of a novel member of the nerve growth factor/brain-derived neurotrophic factor family. *Nature* **344**, 339–341.
- Hubel, D.H., and Wiesel, T.N. (1970). The period of susceptibility to the physiological effects of unilateral eye closure in kittens. *J. Physiol.* **206**, 419–436.
- Isackson, P.J., Huntsman, M.M., Murray, K.D., and Gall, C.M. (1991). BDNF mRNA expression is increased in adult rat forebrain after limbic seizures: temporal patterns of induction distinct from NGF. *Neuron* **6**, 937–948.
- Jones, K.R., Farinas, I., Backus, C., and Reichardt, L.F. (1994). Targeted disruption of the BDNF gene perturbs brain and sensory neuron development but not motor neuron development. *Cell* **76**, 989–999.
- Kang, H., and Schuman, E.M. (1995). Long-lasting neurotrophin-induced enhancement of synaptic transmission in adult hippocampus. *Science* **267**, 1658–1662.
- Kang, H., Welcher, A.A., Shelton, D., and Schuman, E.M. (1997). Neurotrophins and time: different roles for TrkB signaling in hippocampal long-term potentiation. *Neuron* **19**, 653–664.
- Kleinschmidt, A., Bear, M.F., and Singer, W. (1987). Blockade of NMDA receptors disrupts experience-dependent plasticity of kitten striate cortex. *Science* **238**, 355–358.
- Korte, M., Carroll, P., Wolf, E., Brem, G., Thoenen, H., and Bonhoeffer, T. (1995). Hippocampal long-term potentiation is impaired in mice lacking brain-derived neurotrophic factor. *Proc. Natl. Acad. Sci. USA* **92**, 8856–8860.
- Korte, M., Griesbeck, O., Gravel, C., Carroll, P., Staiger, V., Thoenen, H., and Bonhoeffer, T. (1996). Hippocampal long-term potentiation is impaired in mice lacking brain-derived neurotrophic factor. *Proc. Natl. Acad. Sci. USA* **92**, 8856–8860.

- Lauterborn, J.C., Rivera, S., Stinis, C.T., Hayers, V.Y., Isackson, P.J., and Gall, C.M. (1996). Differential effects of protein synthesis inhibition on the activity-dependent expression of BDNF transcripts: evidence for immediate-early gene responses from specific promoters. *J. Neurosci.* **16**, 7428–7436.
- Leibrock, J., Lottspeich, F., Hohn, A., Hofer, M., Hengerer, B., Masiakowski, P., Thoenen, H., and Barde, Y.A. (1989). Molecular cloning and expression of brain-derived neurotrophic factor. *Nature* **341**, 149–152.
- Levi-Montalcini, R. (1987). The nerve growth factor 35 years later. *Science* **127**, 1154–1162.
- Liu, X., Ernfors, P., Wu, H., and Jaenisch, R. (1995). Sensory but not motor neuron deficits in mice lacking NT4 and BDNF. *Nature* **375**, 238–241.
- Malinow, R., Schulman, H., and Tsien, R.W. (1989). Inhibition of postsynaptic PKC or CaMKII blocks induction but not expression of LTP. *Science* **245**, 862–866.
- Matthews, R.P., Guthrie, C.R., Wailes, L.M., Zhao, X., Means, A.R., and McKnight, G.S. (1994). Calcium/calmodulin-dependent protein kinase types II and IV differentially regulate CREB-dependent gene expression. *Mol. Cell. Biol.* **14**, 6107–6116.
- McAllister, A.K., Lo, D.C., and Katz, L.C. (1995). Neurotrophins regulate dendritic growth in developing visual cortex. *Neuron* **15**, 791–803.
- McAllister, A.K., Katz, L.C., and Lo, D.C. (1997). Opposing roles for endogenous BDNF and NT-3 in regulating cortical dendritic growth. *Neuron* **18**, 767–778.
- Metsis, M., Timmusk, T., Arenas, E., and Persson, H. (1993). Differential usage of multiple brain-derived neurotrophic factor promoters in the rat brain following neuronal activation. *Proc. Natl. Acad. Sci. USA* **90**, 8802–8806.
- Meyer-Franke, A., Kaplan, M.R., Pfrieder, F.W., and Barres, B.A. (1995). Characterization of the signaling interactions that promote the survival and growth of developing retinal ganglion cells in culture. *Neuron* **15**, 805–819.
- Misra, R.P., Bonni, A., Miranti, C.K., Rivera, V.M., Sheng, M., and Greenberg, M.E. (1994). L-type voltage-sensitive calcium channel activation stimulates gene expression by a serum response factor-dependent pathway. *J. Biol. Chem.* **269**, 25483–25493.
- Montminy, M.R., and Bilezikjian, L.M. (1987). Cyclic binding of a nuclear protein to the cyclic-AMP response element of the somatostatin gene. *Nature* **328**, 175–178.
- Patterson, S.L., Abel, T., Deuel, T.A.S., Martin, K.C., Rose, J.C., and Kandel, E.R. (1996). Recombinant BDNF rescues deficits in basal synaptic transmission and hippocampal LTP in BDNF knockout mice. *Neuron* **16**, 1137–1145.
- Shatz, C.J. (1990). Impulse activity and the patterning of connections during CNS development. *Neuron* **5**, 745–756.
- Sheng, M., Dougan, S.T., McFadden, G., Greenberg, M.E. (1988). Calcium and growth factor pathways of c-fos transcriptional activation require distinct upstream regulatory sequences. *Mol. Cell. Biol.* **8**, 2787–2796.
- Sheng, M., McFadden, G., and Greenberg, M.E. (1990). Membrane depolarization and calcium induce c-fos transcription via phosphorylation of transcription factor CREB. *Neuron* **4**, 561–582.
- Sheng, M., Thompson, M.A., and Greenberg, M.E. (1991). CREB: a Ca^{2+} -regulated transcription factor phosphorylated by calmodulin-dependent kinases. *Science* **252**, 1427–1430.
- Shieh, P.B., and Ghosh, A. (1997). Neurotrophins: new roles for a seasoned cast. *Curr. Biol.* **7**, R627–R630.
- Silva, A.J., Stevens, C.F., Tonegawa, S., Wang, Y. (1992). Deficient hippocampal long-term potentiation in alpha-calcium-calmodulin kinase II mutant mice. *Science* **257**, 201–206.
- Stryker, M.P., and Harris, W.A. (1986). Binocular impulse blockade prevents the formation of ocular dominance columns in cat visual cortex. *J. Neurosci.* **6**, 2117–2133.
- Sun, P., Enslin, H., Myung, P.S., and Maurer, R.A. (1994). Differential activation of CREB by Ca^{2+} /calmodulin-dependent kinases type II and type IV involves phosphorylation of a site that negatively regulates activity. *Genes Dev.* **8**, 2526–2539.
- Threadgill, R., Bobb, K., and Ghosh, A. (1997). Regulation of dendritic growth and remodeling by Rho, Rac, and Cdc42. *Neuron* **19**, 625–634.
- Timmusk, T., Palm, K., Metsis, M., Reintam, T., Paalme, V., Saarma, M., and Persson, H. (1993). Multiple promoters direct tissue-specific expression of the rat BDNF gene. *Neuron* **10**, 475–489.
- Timmusk, T., Lendahl, U., Funakoshi, H., Arenas, E., Persson, H., and Metsis, M. (1995). Identification of brain-derived neurotrophic factor promoter regions mediating tissue-specific, axotomy-, and neuronal activity-induced expression in transgenic mice. *J. Cell Biol.* **128**, 185–199.
- Walton, K.M., Rehfuss, R.P., Chrivia, J.C., Lochner, J.E., and Goodman, R.H. (1992). A dominant repressor of cyclic adenosine 3',5'-monophosphate (cAMP)-regulated enhancer-binding protein activity inhibits the cAMP-mediated induction of the somatostatin promoter in vivo. *Mol. Endocrinol.* **6**, 647–655.