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 promoter 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; J ones 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; Hallbock 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 μM 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.
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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 1A). Our deletion analysis suggested, however, that this element was not sufficient to mediate a calcium response since the construct -108BIII-CAT was unable to mediate calcium-dependent transactivation (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

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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) or 5
nifedipine (5 μM, 10 μM) for 10 hr at 5DIV. All KCl stimulations in this and other experiments shown in this paper were performed in the presence of 100 μM APV to inhibit activation of NMDA receptors.

(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 μM, 10 μM) for 10 hr at 5DIV. All KCl stimulations in this and other experiments shown in this paper were performed in the presence of 100 μM APV to inhibit activation of NMDA receptors.
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 (TCACGTCA).

the CRE sequences, and in the third construct the BIII-CRE was mutated in context in a -72BIII-CAT background (-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...
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

The promoter analysis experiments described above and suggest that the complex we see shifted with 72–47 oligonucleotides as well as 56–30 shift a complex not present in shifts 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 contains 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 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
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 transcription 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 (ΔCaM kinase IV/Gr(f); 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.
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 on 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 II and 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 postsynaptic 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 (Gosh 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 min), and dissociated in culture media. The dissociated neurons were plated on 60 mm plates precoated with poly-L-lysine and laminin at a density of 3 × 10^4 cells per plate in either glutamine-free Basal Media Eagle (Sigma) supplemented with glutamine (to 1 mM), N2 (to 1%; GibC0), and fetal bovine serum (5%) or in Neurobasal Media (GibC0) supplemented with glutamine (to 1 mM) and B27 (to 2%; GibC0).

**Plasmids**

-4100BIII-CAT was constructed by subcloning a 4.4 kb HindIII/XbaI fragment containing exon Iii into pBLCAT2 vector. -1500BIII-CAT, -634BIII-CAT, and -108BIII-CAT were similarly constructed from a 1.8 kb EcoRI/XbaI fragment, a 0.9 kb HindIII/XbaI fragment, and an EcoRI/XbaI fragment, respectively. -108BIII-CAT (CRE-1) was constructed by modified inverse PCR mutagenesis (Stratagen), using the following two oligos: 5’-CGCTCTAGATGCACTAGAGTGTCTATTTCG-3’ and 5’-CTAGCTAGCTAGCTATGTATCTCCTTGGAGAATGG-3’. -72BIII-CAT (Δ57-64) and -72BIII-CAT (Δ65-72) were constructed by ligating two separate double-stranded oligonucleotides to a vector made from a BamHI/ XbaI digest of the -108BIII-CAT (CRE-1). The double-stranded oligo was annealed from the following two single-stranded oligos: 5’-GGACAGGAGGACCTATCATGACGCTCT-3’ and 5’-CTAGCTAGCTAGCTATGTATCTCCTTGGAGAATGG-3’. -108BIII-CAT (CRE-1) was prepared by digestion with a modified calcium phosphate transfection procedure, as described previously (Threadgill et al., 1997). Briefly, the culture media was removed and replaced with DMEM and visualized by autoradiography.

**Transfections**

Cells were transfected by a modified calcium phosphate transfection procedure, as described previously (Threadgill et al., 1997). Briefly, the culture media was 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 μg of DNA was used per plate. Typically, this would include 4–5 μg of reporter construct DNA, 2 μg of DNA coding a candidate interacting protein (e.g., CaM Kinase), and carrier DNA. Following a 20–30 min transfection during which a fine sand 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 μCi 14C-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, speedvacuum, 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 14C emissions on TLC plates were quantified by phosphorimagery scans.

**Gel Mobility Shift Assays**

Nuclear extracts were prepared using a rapid technique described previously (Andrews and Fallier, 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 complementary synthetic oligonucleotides and were end labeled using [γ-^32P]ATP and T4 polynucleotide kinase. Binding reactions were performed in a total volume of 30 μl 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 μg/ml RNA, and 10 μg/ml poly dI-dC as nonspecific DNA. Radiolabeled probe (0.5 pmol, 105 cpmp) was incubated with 15 μg 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; αCREB, 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 (1–73) was used for 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 1X 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 × 5 min, room temperature), blocked for...
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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 covered slipped. 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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