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Ca^{2+} Influx Regulates *BDNF* Transcription by a CREB Family Transcription Factor-Dependent Mechanism

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Summary

CREB is a transcription factor implicated in the control of adaptive neuronal responses. Although one function of CREB in neurons is believed to be the regulation of genes whose products control synaptic function, the targets of CREB that mediate synaptic function have not yet been identified. This report describes experiments demonstrating that CREB or a closely related protein mediates Ca^{2+}-dependent regulation of BDNF, a neurotrophin that modulates synaptic activity. In cortical neurons, Ca^{2+} influx triggers phosphorylation of CREB, which by binding to a critical Ca^{2+} response element (CRE) within the *BDNF* gene activates *BDNF* transcription. Mutation of the *BDNF* CRE or an adjacent novel regulatory element as well as a blockade of CREB function resulted in a dramatic loss of *BDNF* transcription. These findings suggest that a CREB family member acts cooperatively with an additional transcription factor(s) to regulate *BDNF* transcription. We conclude that the *BDNF* gene is a CREB family target whose protein product functions at synapses to control adaptive neuronal responses.

Introduction

Activity-dependent adaptive neuronal responses are believed to be critical for the refinement of neuronal connections during development and for mature brain functions such as learning and memory. Adaptive neuronal responses consist of a variety of long-lasting modifications in neuronal structure and function that can lead to increases or decreases in synaptic strength. At excitatory synapses of the vertebrate CNS, the release of glutamate from the presynaptic terminal triggers postsynaptic responses by binding to specific glutamate receptors on the dendritic spines of postsynaptic neurons. Repetitive stimulation of the synapse can lead to changes in the amount of neurotransmitter that the presynaptic neuron releases or in the responsiveness of the postsynaptic neuron and, depending on the exact nature of the stimulus, can lead to increases or decreases in synaptic strength (Hawkins et al., 1993).

Recently, neurotrophic factors have been shown to modulate synaptic function (Lo, 1995; Thoenen, 1995). One of these factors, the neurotrophin brain-derived neurotrophic factor (BDNF), plays an important role in regulating the survival and differentiation of selective populations of neurons during development (Lindsay et al., 1985; Alderson et al., 1990; Knüsel et al., 1991; Ghosh et al., 1994; Mamounas et al., 1995; Schwartz et al., 1997). BDNF has also been implicated as a mediator of neuronal adaptive responses. In the CNS, BDNF is synthesized predominantly by neurons, and is highly expressed in the hippocampus and cortex (Ernfors et al., 1990; Hofer et al., 1990; Phillips et al., 1990), two brain regions known to be important for learning and memory. The BDNF receptor tyrosine kinase, TrkB, is colocalized with BDNF in the CNS (Kokaia et al., 1993; Cohen-Cory et al., 1996), suggesting that BDNF may act in a paracrine or an autocrine fashion. Consistent with a role of BDNF as a mediator of neuronal adaptive responses, the expression of BDNF is regulated by neuronal activity. Activation of L-type voltage sensitive Ca^{2+} channels (VSCCs) or the N-methyl-D-aspartate (NMDA) subtype of glutamate receptor leads to an enhancement of *BDNF* mRNA levels and stimulates release of the BDNF protein (Zafra et al., 1990, 1991; Ghosh et al., 1994). In addition, stimuli that evoke long-term potentiation (LTP) in hippocampal CA1 pyramidal neurons or dentate granule neurons induce *BDNF* mRNA expression in these same neurons (Patterson et al., 1992; Castrén et al., 1993; Dragunow et al., 1993). Several recent studies have shown that *BDNF* mRNA levels are also induced by physiological stimuli. For example, visual experience regulates the expression of *BDNF* in both the newborn and adult rat visual system, as blocking visual input leads to a rapid downregulation of *BDNF* mRNA in the visual cortex (Castrén et al., 1992).

The functions of BDNF in mature neurons are being intensively investigated, and several studies have shown that BDNF itself can modulate synaptic strength. Studies of the *Xenopus* neuromuscular junction revealed that BDNF enhances the spontaneous release of acetylcholine from presynaptic terminals (Lohof et al., 1993). In addition, the application of BDNF to hippocampal slices produces a dramatic and sustained enhancement of synaptic strength at Schaffer collateral CA1 synapses (Kang and Schuman, 1995). By enhancing the ability of hippocampal synapses to respond to tetanic stimulation, BDNF promotes the induction of long-lasting LTP in young hippocampal slices that normally only show short-term potentiation (Figurov et al., 1996). Conversely, synaptic transmission and LTP are severely impaired in the hippocampus of *BDNF* knockout mice (Korte et al., 1995; Patterson et al., 1996). The synaptic transmission and LTP defects in *BDNF* knockout mice can be largely rescued either by reintroduction of the *BDNF* gene into the cells or by the exogenous application of BDNF (Korte et al., 1996; Patterson et al., 1996). This suggests that BDNF plays a direct role in ongoing basal synaptic transmission and activity-dependent plasticity, above and beyond any role it plays during development. The precise mechanisms by which BDNF modulates synaptic

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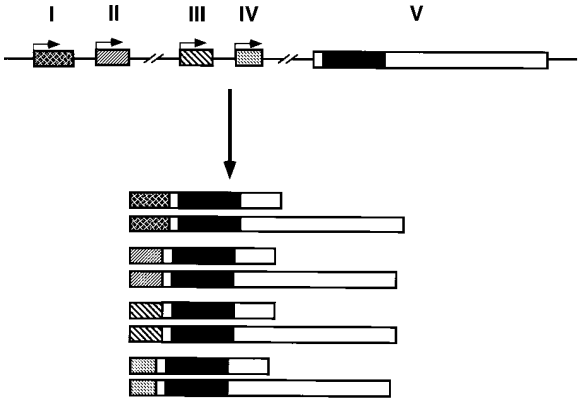

Figure 1. Schematic Representation of the *BDNF* Gene and Mature *BDNF* Transcripts

The *BDNF* coding region is indicated by the closed boxes. The UTRs encoded by *BDNF* exon V are indicated by open boxes. The 5′ UTRs encoded by 5′ exons are indicated by hatched boxes (Timmusk et al., 1993.)

efficacy remain unknown; however, recent studies indicate that BDNF increases both presynaptic firing rate and postsynaptic responses (Lessmann et al., 1994; Levine et al., 1995). In addition to its effects on the mature CNS, BDNF also contributes profoundly to the activity-dependent development and formation of neuronal circuits (Cohen-Cory and Fraser, 1995; Cabelli et al., 1995; McAllister et al., 1995, 1996).

Given BDNF's central role as a mediator of neuronal development and as a regulator of synaptic function, it will be important to characterize the mechanisms by which neuronal activity regulates *BDNF* expression. Despite a wealth of evidence indicating that *BDNF* mRNA levels are enhanced by neuronal activity, the mechanisms by which L-type VSCC stimulation or NMDA receptor activation triggers *BDNF* mRNA upregulation remain poorly understood. While it has been established that Ca^{2+} influx through L-type VSCCs or the NMDA receptor channel is critical for *BDNF* mRNA upregulation (Zafra et al., 1990, 1991; Ghosh et al., 1994), it is still unclear whether the enhancement in *BDNF* mRNA levels is due to a stimulation of *BDNF* transcription, a stabilization of *BDNF* mRNA, or both. Genomic analysis has shown that the rat *BDNF* gene is large, ~40 kb in length, and complex, containing four distinct promoters (Timmusk et al., 1993). During the synthesis of the four primary *BDNF* mRNA transcripts, one of four 5′ exons is spliced to a common 3′ exon that includes the entire *BDNF* coding region (Figure 1). Since each of the four *BDNF* mRNAs can be polyadenylated at either of two sites within the 3′ untranslated region, a total of eight distinct *BDNF* mRNAs are synthesized (Ohara et al., 1992; Timmusk et al., 1993). An important feature of *BDNF* mRNAs is that while there are eight distinct transcripts, each of the *BDNF* messages encodes an identical BDNF protein. The functional significance of the eight distinct *BDNF* mRNAs remains a mystery, but one possibility is that the existence of multiple *BDNF* promoters and transcripts allows *BDNF* expression to be regulated at multiple levels including transcription, stability, subcellular localization, and translatability. The fact that *BDNF* expression may be regulated at several levels could be relevant to BDNF's multiple functions as a modulator of neuronal plasticity and a mediator of neuronal survival and differentiation.

Several studies using exon-specific probes have revealed that Ca^{2+} influx into neurons through L-type voltage-sensitive Ca^{2+} channels (VSCCs) leads to a transient increase in exon I-, II-, and III-containing *BDNF* mRNAs in the hippocampus and cerebral cortex (Metsis et al., 1993; Timmusk et al., 1993), and that a sequence upstream of exon III is capable of mediating the induction of reporter gene expression in transgenic mice (Timmusk et al., 1995). However, the specific sequence elements within the *BDNF* gene that control the *BDNF* response under these circumstances have not been identified.

In this study, the molecular mechanisms by which neuronal activity controls *BDNF* expression were characterized using primary embryonic cortical neurons exposed to elevated levels of KCl to stimulate an influx of Ca^{2+} into neurons through L-type VSCCs. We find that Ca^{2+} influx through L-type VSCCs stimulates transcription from *BDNF* promoter III via a CREB family–dependent mechanism. Together with the previous studies demonstrating the importance of BDNF in synaptic function, these findings suggest that BDNF is a critical target of the CREB family of transcription factors and that this target may mediate the CREB family's effects on synaptic function.

Results

Ca^{2+} Influx through L-type VSCCs Activates *BDNF* Transcription

Previous studies have shown that membrane depolarization-triggered Ca^{2+} influx through L-type VSCCs induces an increase in *BDNF* mRNA expression in cultured neurons (Zafra et al., 1990; Ghosh et al., 1994). This increase in *BDNF* mRNA could be the result of increased transcription initiation, or increased *BDNF* mRNA stability, or both. To determine if membrane depolarization stimulates *BDNF* transcription, nuclear run-on analyses were performed using nuclei isolated from cultured embryonic day 17 (E17) or E18 cortical neurons that were left untreated or exposed to KCl for varying periods of time. After incubation of the nuclei with [^{32}P]UTP and unlabeled nucleotide triphosphates to label mRNAs as they elongate in vitro, the ^{32}P-labeled mRNA was purified and hybridized to plasmid DNAs (*BDNF*, c-*fos*, *nur/77*, and *GAPDH*) that had been spotted onto nitrocellulose filters. As shown in Figure 2, in unstimulated cortical neurons very few transcripts were present that hybridized to the *BDNF* cDNA, indicating that the level of *BDNF* transcription prior to membrane depolarization was low. A clear increase in *BDNF* transcription was detected within 30 min of membrane depolarization. *BDNF* transcription peaked within 60 min of the initial stimulus and remained at a moderate level for at least 3 hr after stimulation. In contrast, membrane depolarization induced the transcription of two immediate-early genes (IEGs), c-*fos* and *nur/77*, with more rapid kinetics. Transcription of the *GAPDH* gene was unaffected when cortical neurons were exposed to elevated levels of KCl and

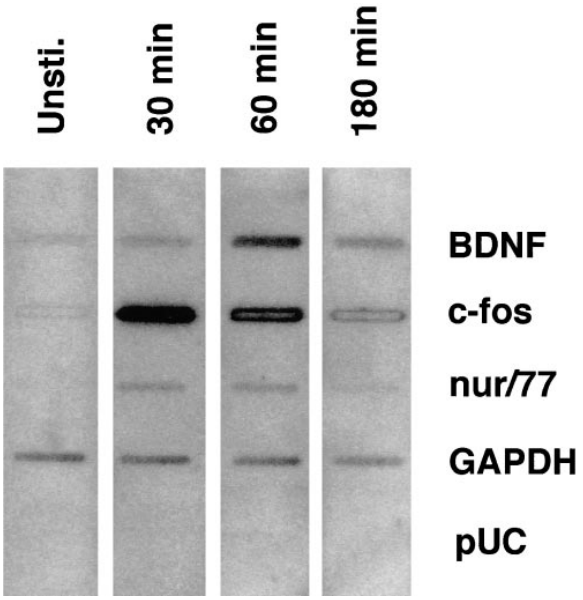

Figure 2. Membrane Depolarization Induces Transcriptional Activation of the *BDNF* Gene in Cultured Cortical Neurons

Nuclei were isolated from cortical neurons that were left unstimulated or stimulated for the indicated times with 50 mM KCl. Nascent RNA transcripts were labeled in an in vitro nuclear run-on assay. Equal amounts of ^{32}P-labeled RNA isolated from cells stimulated for varying periods of time were hybridized to filters that were spotted with DNA probes as indicated. The radioactivity is visualized by PhosophorImager analysis.

therefore *GAPDH* was used as a control for variations in sample handling. Taken together, these findings indicate that the effects of membrane depolarization on the level of *BDNF* mRNA reflect, at least in part, an effect of membrane depolarization on *BDNF* transcription.

BDNF mRNA that Contains Exon III Is the Major Ca^{2+}-Inducible Transcript in Cortical Neurons

Given the finding that Ca^{2+} influx through L-type VSCCs induces *BDNF* transcription, experiments were carried out to determine which of the four *BDNF* promoters is capable of mediating a Ca^{2+} response. As described above, the rat *BDNF* gene consists of four distinct 5′ exons each driven by a specific promoter and each spliced to a common 3′ exon that encodes the BDNF protein. Since each of the four primary *BDNF* transcripts can be polyadenylated at one of two sites, a total of eight *BDNF* transcripts are generated. In principle, the eight transcripts can be distinguished by Northern blotting using 5′exon–specific probes, since each of the four 5′ exon probes should detect a short and a long *BDNF* transcript. By identifying the specific *BDNF* mRNAs induced upon Ca^{2+} influx through L-type VSCCs, it should be possible to identify which of the four *BDNF* promoters is Ca^{2+} responsive, since the Ca^{2+}-responsive promoter(s) would be expected to be located just 5′ of the initiation site of *BDNF* mRNA synthesis.

By Northern blotting, we were able to detect the 4.2 kb and the 1.6 kb exon III–containing *BDNF* mRNAs and we found that the expression of both of these transcripts is induced upon exposure of cortical neurons to elevated levels of KCl. KCl induction of *BDNF* mRNA requires Ca^{2+} influx through L-type VSCCs. It has been previously found that KCl induction of *BDNF* mRNA is completely blocked by chelation of extracellular Ca^{2+} or blockage of L-type VSCCs with nifedipine (Ghosh et al., 1994). As shown in Figure 3A, exon III–containing *BDNF* transcripts were induced within 60 min of membrane depolarization, and the induction was more apparent when cortical neurons were depolarized for 3–6 hr. These findings suggest that the *BDNF* promoter adjacent to exon III (*BDNF* promoter III) is a Ca^{2+}-responsive promoter, and subsequent studies (see below) focused on identifying the key regulatory element(s) within *BDNF* promoter III that control the Ca^{2+} response. In contrast to the exon III–containing transcripts, we were unable to detect by Northern blotting exon I-, II-, or IV-containing *BDNF* transcripts either before or after membrane depolarization. Given that the ^{32}P-labeled 5′ exon probes used to detect each of these *BDNF* transcripts were comparable in size and specific activity, this suggests that the level of expression of the exon I-, II-, and IV-containing *BDNF* mRNAs is considerably lower than that of the exon III–containing mRNAs and may be below the level of detection by Northern analysis.

To investigate further whether the other exon I-, II-, and IV-containing *BDNF* transcripts were responsive to membrane depolarization, a quantitative reverse transcription–coupled polymerase chain reaction (RT-PCR) assay was developed to measure the level of each *BDNF* mRNA. For this purpose, 5′ end primers were designed that are specific for *BDNF* exons I, II, III, or IV, and a 3′ end primer was generated that is complementary to sequences in exon V, the exon that is common to all eight *BDNF* transcripts. Using these PCR primers in the RT-PCR assay with mRNA prepared from membrane-depolarized cortical neurons, it was possible to amplify the exon I-, II-, III-, and IV-containing *BDNF* transcripts so that each could be distinguished by gel electrophoresis based on their difference in molecular weight. For the PCR reaction, the 3′ end primer was labeled with ^{32}P so the amount of each PCR product generated could be quantitated based on the amount of radioactivity incorporated. By adjusting the reaction parameters, it was possible to obtain conditions in which each PCR reaction has comparable amplification efficiency and the amount of PCR product correlated linearly with the template input. The quantitative PCR analysis revealed that while each of the *BDNF* transcripts is at least weakly inducible by membrane depolarization, the exon III *BDNF* transcripts were by far the most responsive to Ca^{2+} influx, accounting for more than 80% of total *BDNF* mRNA (Figures 3B and 3C). For each group of transcripts, the RT-PCR assay could not distinguish the small and large *BDNF* transcripts that differ with respect to their site of polyadenylation, although by Northern analysis both the small and the large exon III–containing *BDNF* mRNAs were found to be responsive to Ca^{2+} influx (Figure 3A). Together with the nuclear run-on transcription experiments, the PCR analysis indicates that the membrane depolarization induction of the exon III–containing *BDNF* mRNAs is regulated at the transcriptional level and is probably driven by Ca^{2+}-responsive element(s) within

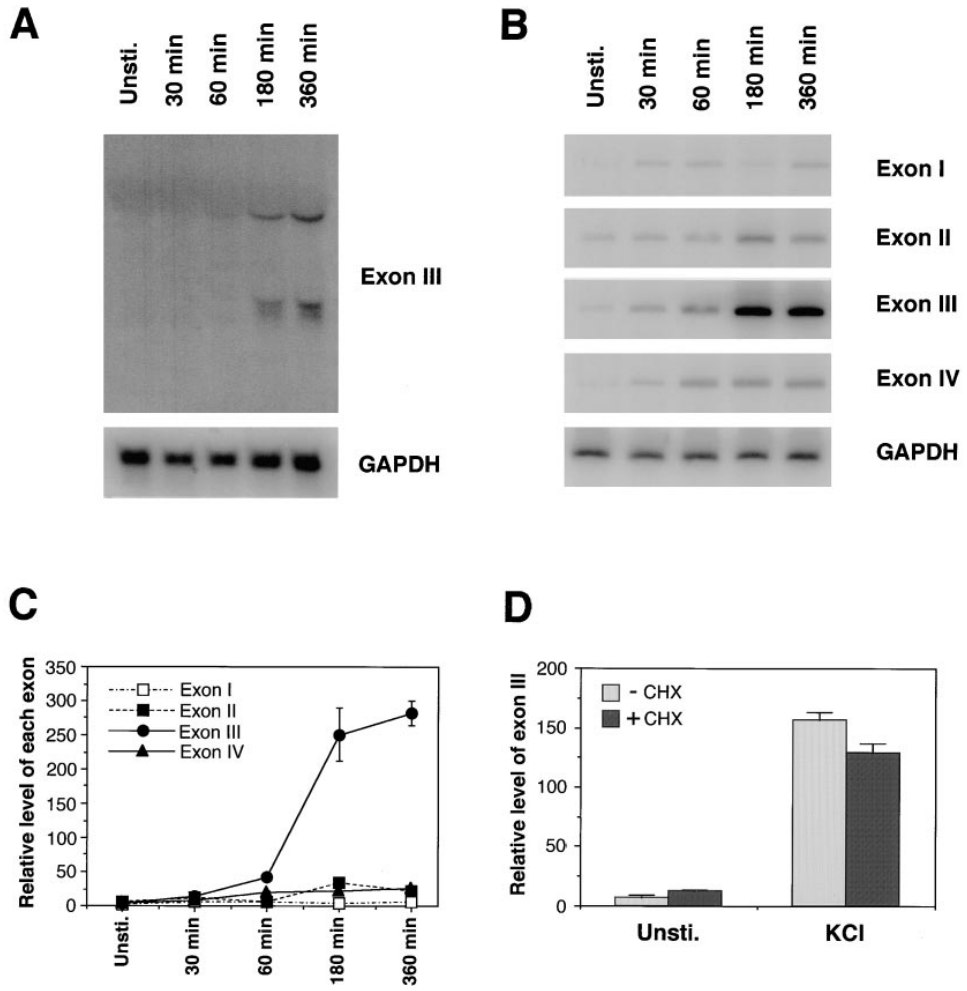

Figure 3. The Expression of Exon-Specific *BDNF* Transcripts

Total RNA was isolated from cultured cortical neurons that were left unstimulated or stimulated for the indicated times with 50 mM KCl and subjected to analysis.

(A) Northern blot analysis of *BDNF* transcripts. Twenty micrograms of total RNA were separated on a denaturing agarose gel and transferred to a nylon filter. *BDNF* transcripts were detected with the exon III–specific probe from the rat *BDNF* gene. The stripped filter was also hybridized with a probe to *GAPDH* to indicate that an equivalent amount of RNA was loaded in each lane.

(B) Detection of *BDNF* 5′ exons with RT-PCR. One microgram of total RNA was reverse transcribed into single-stranded cDNA, and a portion of the cDNA was amplified by PCR with exon-specific primers. The PCR products were separated on a polyacrylamide gel and visualized by PhosophorImager analysis. *GAPDH* transcripts were also RT-PCR–amplified and used as a control for RNA input and reverse transcription efficiency.

(C) Quantification of each exon-specific PCR product. The levels of radioactivity were obtained from the image. The relative level of each exon was normalized against the *GAPDH* level of the same sample. Each value is an average from two independent determinations.

(D) Inhibition of protein synthesis does not block *BDNF* exon III mRNA induction. Cycloheximide was added to the culture 30 min prior to the KCl stimulation. RNA was isolated after 1 hr of membrane depolarization. The level of exon III–containing transcripts was measured by RT-PCR. Each value represents the average of three independent determinations, and the error bars indicate the standard error of each mean.

the *BDNF* regulatory region 5′ of exon III. A comparison of the time course of *BDNF* transcription (measured by the run-on assay) and the time course of mature *BDNF* mRNA transcription (Northern and PCR assays) revealed that the induction of *BDNF* transcription occurs in parallel with an increase in the mature *BDNF* mRNA. However, the *BDNF* mRNA persists after *BDNF* transcription has shut off, suggesting that the *BDNF* mRNA is relatively stable.

Our analysis of the kinetics of *BDNF* mRNA induction by the nuclear run-on transcription assay, by Northern blotting, and by RT-PCR revealed that Ca^{2+} influx triggers *BDNF* transcription more slowly than it stimulates the transcription of IEGs such as c-*fos* and c-*jun*. The finding that *BDNF* mRNA induction is delayed relative to the induction of many IEGs raised the possibility that *BDNF* transcriptional activation might require the prior induction and expression of certain IEGs. To address this possibility, we asked if Ca^{2+} influx was still capable of inducing *BDNF* mRNA expression in the presence of cycloheximide, an inhibitor of protein synthesis that is known to block expression of IEG-encoded proteins. Cycloheximide (35 μg/ml) was added to cortical neuron cultures 30 min prior to membrane depolarization. At this concentration, cycloheximide was found to inhibit greater than 99% of protein synthesis in cortical neurons

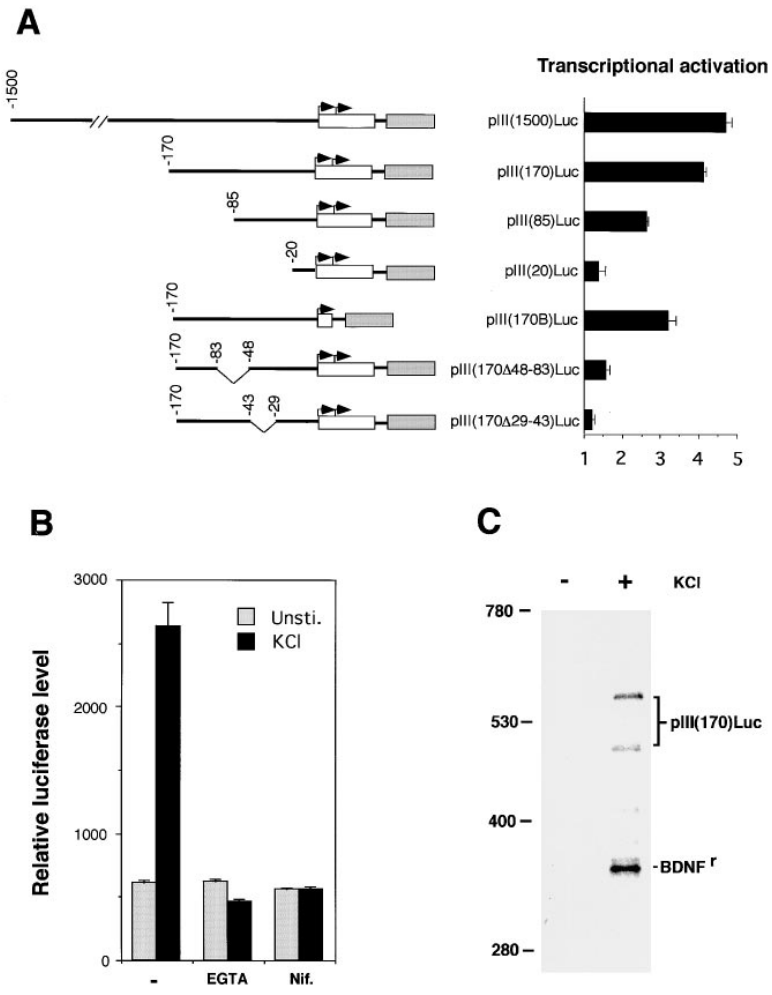

Figure 4. The Effect of *BDNF* Promoter III Mutations on Luciferase Reporter Gene Expression

(A) Analysis of *BDNF* promoter III activation by the luciferase assay. Plasmids with variant lengths of *BDNF* promoter III and exon III sequences fused to a luciferase gene were transiently transfected into cultured cortical neurons. Plasmid pEF-LacZ was cotransfected to monitor the transfection efficiency. Forty hours after transfection, cells were left unstimulated or stimulated with 50 mM KCl for 9 hr. The level of transcriptional activation is determined as the ratio of the luciferase activity from stimulated cell extracts relative to the luciferase activity from unstimulated cell extracts. Each value is the average of at least three independent determinations. Arrows indicate the transcriptional initiation sites. The numbers indicate the position of the promoter 5′ end relative to the first initiation site of transcription. The open boxes indicate *BDNF* exon III. The hatched boxes indicate the luciferase reporter gene.

(B) Blocking Ca^{2+} influx inhibits reporter gene expression. Cortical neurons were transfected with the pIII(170)Luc construct. Prior to membrane depolarization, cells were left untreated (−) or treated with either 2 mM EGTA or 5 μM nifedipine (Nif.). The luciferase assays were performed as described in (A).

(C) Mapping of transcription initiation sites of the transfected *BDNF*-Luc gene. Cortical neurons were transfected with the pIII(170)-Luc construct. Total RNA was isolated from cells left untreated (−) and cells treated with 50 mM KCl (+) for 6 hr and then subjected to an RNase protection assay. The exogenous *BDNF*-Luc and endogenous rat *BDNF* transcripts are indicated by pIII(170)Luc and $BDNF^r$, respectively. The sizes (bp) of the RNA ladder are indicated by the numbers in the left margin.

as measured by ^{14}C-leucine incorporation into protein (data not shown). However, by PCR analysis cycloheximide treatment was found to have only a very small effect on the level of exon III–containing *BDNF* mRNAs between 1–2 hr after membrane depolarization (Figure 3D). This finding suggests that *BDNF* transcription is regulated by a mechanism that does not require new protein synthesis and can occur independently of the de novo expression of IEG protein products such as Fos and Jun. Rather, *BDNF* transcription, like that of well-characterized IEGs such as c-*fos* and c-*jun*, appears to be regulated by a preexisting cellular machinery that transmits the Ca^{2+} signal from the cytoplasm to the nucleus where gene induction occurs.

Unlike the c-*fos* and c-*jun* mRNAs, expression of the *BDNF* message was not superinduced in the presence of cycloheximide. Cycloheximide and other protein synthesis inhibitors have previously been shown to inhibit the rapid shut-off of IEG transcription that occurs subsequent to IEG induction, and also to lead to a dramatic stabilization of IEG mRNAs. As a consequence, although exposure to protein synthesis inhibitors effectively blocks the production of IEG-encoded proteins, the IEG mRNAs are typically superinduced in the presence of protein synthesis inhibitors (Greenberg et al., 1986). The fact that cycloheximide treatment does not lead to a superinduction of *BDNF* mRNA levels in membrane-depolarized cells indicates that some aspect of the regulation of *BDNF* transcriptional shut-off or *BDNF* mRNA stability control differs from that of typical IEGs such as c-*fos* and c-*jun*.

A Calcium/Cyclic AMP Response Element (CRE) -Like Sequence Is Present in *BDNF* Exon III Promoter

Having obtained evidence that Ca^{2+} influx triggers the induction of exon III–containing *BDNF* mRNAs at the transcriptional level, we sought next to identify the *cis*–acting DNA sequences that direct Ca^{2+}-dependent *BDNF* transcription. Toward this end, a series of reporter plasmids containing varying lengths of *BDNF* exon III 5′ flanking region and exon III were fused to the firefly luciferase reporter gene (see Figure 4A). Each reporter plasmid was transiently transfected into cortical neurons together with the plasmid pEF-LacZ in which *lacZ* gene is driven by the promoter of elongation factor. Since expression of the *lacZ* reporter is unaffected by membrane depolarization, this gene serves as a good internal control for variations in transfection efficiency and sample handling. After 40 hr, transfected cortical

cultures were subjected to membrane depolarization by exposure to elevated levels of KCl. As shown in Figure 4A, luciferase activity from pIII(1500)Luc, a plasmid carrying ~1.5 kb of exon III 5′ flanking region and exon III, was reproducibly induced 4- to 5-fold in response to membrane depolarization. This result suggests that a regulatory element(s) is present within the 1.5 kb of sequence 5′ of *BDNF* exon III or within exon III itself and confers the Ca^{2+} response. A reporter construct, pIII(170)Luc, which contains only 170 nucleotides of exon III 5′ flanking sequence, was found to be nearly as effective as pIII(1500)Luc in conferring a Ca^{2+} response, suggesting that key Ca^{2+} responsive elements reside within the 170 nucleotides that are immediately 5′ to the initiation site for *BDNF* exon III mRNA synthesis.

It has been previously shown that membrane depolarization–induced *BDNF* expression is mediated by Ca^{2+} influx through L-type VSCCs (Zafra et al., 1990; Ghosh et al., 1994). To determine if the induction of the *BDNF* promoter III–driven luciferase gene by membrane depolarization requires Ca^{2+} influx through L-type VSCCs, we tested the promoter's response when Ca^{2+} influx was blocked. As shown in Figure 4B, either removal of extracellular Ca^{2+} by chelation with EGTA or the addition of nifedipine, a specific L-type channel antagonist, blocked reporter gene induction. These findings indicate that KCl induction of the *BDNF* promoter III–driven luciferase gene is dependent upon Ca^{2+} influx through L-type VSCCs.

To verify that functional Ca^{2+}-responsive elements reside within the 5′ proximal region of the *BDNF* III promoter, we used an RNase protection assay to assess whether the 170 bp region of *BDNF* promoter III present in the pIII(170)Luc construct is capable of directing the production of the two correctly initiated *BDNF* exon III–containing mRNA transcripts. For the RNase protection assay an anti-sense RNA probe was designed that detects transcripts synthesized from either the transfected *BDNF*-luciferase reporter gene or the endogenous *BDNF* gene. *BDNF*-Luc transcripts that initiate correctly within *BDNF* exon III should protect 565 and 470 nucleotides of the probe. Correctly initiated endogenous rat *BDNF* exon III transcripts should protect 345 and 250 nucleotides of the ^{32}P-labeled RNA probe. As shown in Figure 4C, when pIII(170)Luc was transfected into cortical neurons, membrane depolarization of the neurons led to a robust increase in correctly initiated *BDNF*-luciferase transcripts as well as correctly initiated endogenous *BDNF* exon III mRNA transcripts. Prior to membrane depolarization, the levels of *BDNF*-luciferase transcripts and *BDNF* exon III mRNA were very low but the expression of the correctly initiated mRNAs was dramatically increased within 6 hr of membrane depolarization. We conclude that within a 170 bp DNA sequence element just 5′ of the initiation sites of *BDNF* exon III mRNA synthesis there are essential regulatory element(s) that mediate *BDNF*'s response to Ca^{2+} influx.

To identify the DNA sequences within the 170 bp *BDNF* promoter III element that mediate the Ca^{2+} response, promoter deletion mutants were generated and the effect of the deletions on luciferase reporter gene expression was determined. Deletion of sequences between −170 and −20 5′ of the *BDNF* exon III start site completely abolished the inducibility of the luciferase reporter gene, suggesting that a key Ca^{2+}-responsive regulatory element(s) resides within the −170 to −20 region. To determine if a regulatory element that works together with the −170 to −20 element is present within exon III, an additional deletion mutant was generated in which most of exon III was deleted but the *BDNF* exon III 5′ flanking sequences were left intact [plasmid pIII(170B)Luc]. The pIII(170B)Luc construct was found to be inducible by membrane depolarization, although the level of induction was less than that of a comparable construct, pIII(170)Luc, in which *BDNF* exon III is intact. This finding suggests that in addition to the −170 to −20 element, sequences within exon III make a modest contribution to Ca^{2+}-regulated *BDNF* transcription.

To identify the DNA sequences within the −170 to −20 element that mediate Ca^{2+} induction of *BDNF* transcription, a series of 5′ end deletion mutants were generated and tested for their ability to confer a Ca^{2+} response when inserted just 5′ of the nonresponsive pIII(20)Luc reporter construct. We found that as the extent of the 5′ deletion within the −170 to −20 element increased, there was a gradual decrease in the efficacy with which this element conferred a Ca^{2+} response (Figure 4A). Between nucleotides −85 to −20, the most significant decreases in promoter responsiveness were detected, suggesting that this region contains a DNA sequence(s) that binds a critical regulatory factor(s) that mediates the Ca^{2+} response.

Since the deletion analysis suggested that DNA sequences between −85 and −20 were important for the *BDNF* Ca^{2+} response, we compared this DNA sequence with other promoter sequences that are known to contain regulatory elements that can confer a Ca^{2+} response in the hope of identifying a more limited sequence motif that mediates the Ca^{2+} response. An analysis of the *BDNF* genomic sequence of rat reported by Timmusk et al. (1993) revealed the presence of a sequence 5′-TCACGTCA-3′ located 35 bp 5′ of the first exon III initiation site. This sequence is nearly identical to the consensus sequence of the CRE, which is 5′-TGACGTCA-3′ (Montminy et al., 1986). The CRE has previously been shown to be present in many other Ca^{2+} responsive genes and to mediate Ca^{2+} responses by binding members of the CREB transcription factor family (Ghosh and Greenberg, 1995). The presence of a potential CREB family binding site within the critical Ca^{2+} response element of the *BDNF* gene suggested that Ca^{2+} induction of *BDNF* transcription might be mediated by binding of a CREB family member to the TCACGTCA sequence. However, in a second study that reported the sequence of the *BDNF* gene of Sprague-Dawley rats (Nakayama et al., 1994), the 5′-TCACGTCA-3′ sequence or related sequences were not found within the *BDNF* III promoter region, raising the possibility that a different site might actually be the critical mediator of the Ca^{2+} response.

We considered the possibility that the failure to detect the 5′-TCACGTCA-3′ sequence in the Sprague-Dawley rat *BDNF* gene might reflect a minor sequencing error rather than a true sequence difference between two rat strains. To address this issue, we PCR amplified and then sequenced a fragment of genomic DNA from a Long Evans rat or a Sprague-Dawley rat. DNA sequencing

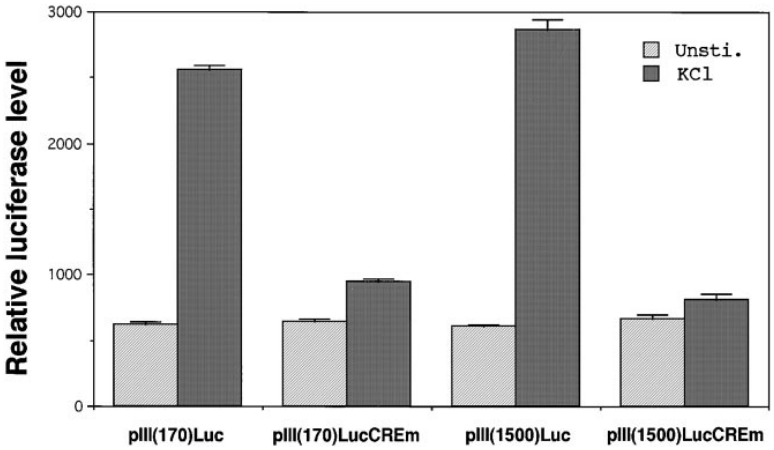

Figure 5. Disruption of the *BDNF* CRE Site Abolished *BDNF* Promoter III Activation in Cultured Cortical Neurons

Cultured cortical neurons were transfected with the indicated *BDNF*-luciferase constructs and plasmid pEF-LacZ. Forty hours after transfection, cells were left unstimulated or stimulated with 50 mM KCl for 9 hr. Cell extracts were assayed for luciferase and β-galactosidase activities. The relative luciferase activity was normalized for variation in transfection efficiency by dividing luciferase activity by β-galactosidase activity. Each value represents the average of at least three independent determinations, and the error bars indicate the standard error of each mean.

revealed that the sequence of the *BDNF* III promoter region is identical in the two rat strains and verified that the 5′-TCACGTCA-3′ sequence is present within the Ca^{2+} response element. It should be noted that the sequence within the 170 bp rat *BDNF* promoter III is highly conserved. A CRE-like element, 5′-GCACGTCA-3′, is located at similar position in human *BDNF* promoter (Shintani et al., 1992). The presence of the 5′-TCACG TCA-3′ sequence within the *BDNF* Ca^{2+} response element strongly suggested the possibility that CREB or a related factor might be a critical regulator of *BDNF* transcription.

To determine the importance of the 5′-TCACGTCAT-3′ element and other sequences within the −85 to −20 region of *BDNF* promoter III, several in-context deletion or substitution mutants were generated that would be expected to disrupt factor binding to *BDNF* promoter III. The effect of the mutations on depolarization induction of the pIII(1500)Luc or pIII(170)Luc reporter constructs was then assessed. A mutation that deleted the 5′-TCACGTCA-3′ sequence and five nucleotides of adjacent sequence but left the rest of the *BDNF* promoter intact [pIII(170Δ29–43)Luc] effectively abolished the reporter gene induction by membrane depolarization (Figure 4A). Likewise, the introduction of substitution mutations that simply changed the 5′-TCACGTCA-3′ sequence to 5′-CAGCTGCA-3′ resulted in *BDNF* promoter III luciferase reporter constructs that were nonresponsive to membrane depolarization (Figure 5). These findings strongly suggest that the *BDNF* CRE is an important mediator of Ca^{2+}-regulated *BDNF* transcription.

The importance of the 5′-TCACGTCA-3′ sequence for membrane depolarization induction of *BDNF* promoter III-driven transcription was also examined in cortical neurons that had maintained their synaptic connections in the context of a cortical slice. Cortical slice cultures were prepared, and *BDNF* exon III luciferase reporter genes together with plasmid pEF-LacZ were introduced into the slice by biolistic transfection (Arnold and Heintz, 1997). Transfected neurons within the slice were identified by staining with an anti-β-galactosidase antibody. When transfected cortical slices were bathed in membrane depolarizing concentrations of KCl for 9 hr, the level of luciferase expression was significantly enhanced in neurons that had been transfected with wild-type *BDNF* exon III promoter luciferase reporter construct pIII(170)-Luc. By contrast, a very low level of luciferase expression was detected in neurons within the depolarized cortical slice that had been transfected with pIII(170)-LucCREm (Figure 6). We conclude that in cortical neurons that have retained their synaptic connections, membrane depolarization induction of *BDNF* transcription is mediated at least in part by the 5′-TCACGTCA-3′ element.

A Second Element Is Also Critical for Ca^{2+}-Dependent *BDNF* Induction

In addition to the 5′-TCACGTCA-3′ sequence, we found by generating an additional in-context deletion mutant that the presence of a 36 bp sequence immediately 5′ of the 5′-TCACGTCA-3′ is critical for Ca^{2+} induction of BDNF transcription. Deletion of the 36 bp that span nucleotides −48 to −83 within *BDNF* promoter III rendered the *BDNF* promoter luciferase construct pIII(170Δ48–83)Luc nonresponsive to membrane depolarization (Figure 4A). To identify the critical element(s) in the −48 to −83 region, substitution mutations were introduced by random mutagenesis at different positions along this region within the context of pIII(170)Luc. The pIII(170)Luc mutant constructs were then transfected into cultured cortical neurons to test the responsiveness of the mutated promoters to membrane depolarization. As shown in Figure 7, most of the mutations within the regions of −53 to −59 and −77 to −83 did not affect the promoter's inducibility. Interestingly, the promoter inducibility was dramatically increased in mutant 5–1. This may be due to the introduction of the sequence TGACGA, which resembles the CRE. However, most of the mutations within −60 to −77 largely reduced the promoter's inducibility. These findings suggest that there is a second regulatory element located between −60 and −77 nucleotides relative to the start site of the *BDNF* promoter that is ~18 bp in length. This element may bind a transcription factor(s) that functions cooperatively with factors bound to the 5′-TCACGTCA-3′ element to regulate Ca^{2+}-dependent *BDNF* transcription.

A CREB Family Member Interacts with *BDNF* CRE and Mediates *BDNF* Induction

We next investigated the nature of the factors present in nuclear extracts that interact with the 5′-TCACGTCA-3′

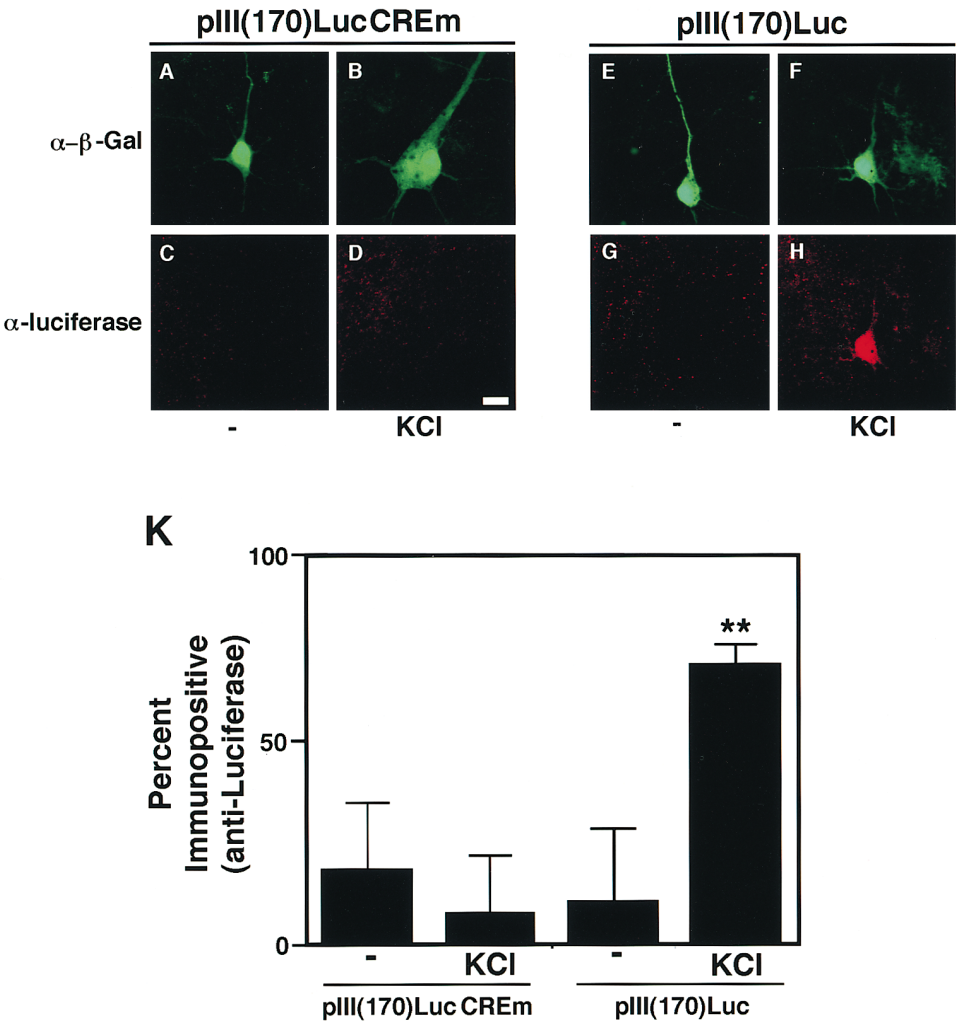

Figure 6. Disruption of the CRE Site Abolished pIII(170)Luc Promoter Activation in Neurons Within a Cortical Slice

Wild-type and mutant promoter constructs were cotransfected with pEF-LacZ into neurons of P14 cortical slices using the biolistic transfection method. Transfected slices were either stimulated with 50 mM KCl or left unstimulated. After 9 hr, the slices were fixed and immunostained for luciferase (red) and β-galactosidase (green).

(A–H) The panels show typical luciferase reporter responses mediated by intact or CRE-mutated promoters within single transfected neurons from cortical slices (successful transfection is indicated by the presence of β-galactosidase immunoreactivity). Neurons transfected with a *BDNF* reporter containing a mutated CRE [pIII(170)LucCREm; A–D] typically showed little or no reporter induction upon exposure to 50 mM KCl as indicated by the absence of an increase in luciferase immunoreactivity (cf. 6C and 6D). However, KCl did induce the expression of a *BDNF* reporter that contained an intact CRE [pIII(170)Luc] as indicated by the increase in luciferase immunoreactivity detected in these cells (cf. 6G and 6H).

(K) Quantification of neuronal luciferase responses by confocal measurements of immunofluorescence showed that the CRE mutation significantly reduced the fraction of responding neurons to levels that were not significantly different from unstimulated levels (**$p < 0.03$; ANOVA). The percent indicates the luciferase-positive cells among the total transfected cells.

element. To identify the factors present in nuclear extracts obtained from membrane-depolarized neurons that bind to the *BDNF* 5′-TCACGTCA-3′ sequence, electrophoretic mobility shift assays were performed using a 22 bp fragment of ^{32}P-labeled DNA corresponding to *BDNF* promoter sequence that includes the 5′-TCACG TCA-3′ element (Figure 8A). When the DNA probe was incubated with nuclear extracts prepared from KCl-stimulated or unstimulated neurons, one major and several minor complexes were identified on a nondenaturing gel (Figure 8B). There was no reproducible change in the protein/DNA complexes that formed when extracts obtained from unstimulated and membrane depolarized cells were compared, suggesting that the components of the protein/DNA complexes are already present in unstimulated neurons and that membrane depolarization does not change their DNA binding activity.

The binding of proteins to B-CRE was specific, inasmuch as the formation of the major complex was completely inhibited by the addition of an excess of unlabeled sequence containing the 5′-TCACGTCA-3′ element but not by an excess of unlabeled sequence in which the 5′-TCACGTCA-3′ element was mutated to 5′-CAGC TGCA-3′ (Figure 8C). The proteins that bind specifically to the 5′-TCACGTCA-3′ element but not the mutated element 5′-CAGCTGCA-3′ are likely to be mediators of

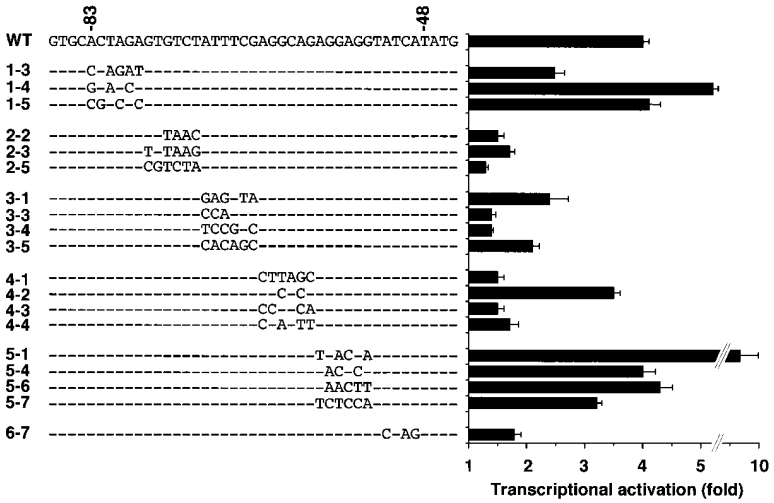

Figure 7. A 5′ 18 bp Element Is Critical for Ca^{2+}-Induced *BDNF* Promoter III Activation

Mutations were systematically introduced into the −48 to −83 region of *BDNF* promoter III and the transcriptional response of each mutant promoter was tested. The wild-type sequence is shown on the top (WT), and the vertical numbers indicate the positions relative to the first transcription start site. In the mutant sequences, letters indicate the mutated bases, and the dash lines indicate unchanged nucleotides. The assays were performed as described in the legend for Figure 4.

the Ca^{2+} response in cortical neurons, since the wild-type 5′-TCACGTCA-3′ element but not the mutant element 5′-CAGCTGCA-3′ was capable of conferring a Ca^{2+} response when present within *BDNF* promoter III.

To determine if CREB is a component of the protein complex that binds to the 5′-TCACGTCA-3′ element, a specific anti-CREB monoclonal antibody was added to the protein/DNA binding reaction and the effect on complex formation was assessed. We found that in the presence of the anti-CREB antibody the major protein/DNA complex (complex I) was supershifted to form complex II (Figure 8C). However, when control monoclonal antibodies (anti-ATF1 or anti-β-gal) were added to the protein/DNA binding reaction, no supershift was observed, indicating that the effect of the anti-CREB antibody is specific and reflects the presence of CREB in the complex. Taken together, these results suggest that CREB is a component of the protein complex that interacts with the 5′-TCACGTCA-3′ element, and that CREB or a related protein may be a critical mediator of membrane depolarization-induced *BDNF* promoter III transcription.

To address further the importance of a CREB family member as a mediator of Ca^{2+}-induced *BDNF* transcription, several CREB mutants were introduced into cortical neurons and their effect on *BDNF* promoter III–driven luciferase gene expression assessed. Previous studies have shown that Ca^{2+} influx through L-type VSCCs induces CREB phosphorylation at Ser-133 via a Ca^{2+}/calmodulin kinase (CaM kinase)–dependent mechanism, and that phosphorylation at Ser-133 activates CREB to stimulate CRE-dependent transcription (Yamamoto et al., 1988; Gonzalez and Montminy, 1989; Sheng et al., 1990; Sheng et al., 1991; Bito et al., 1996). A mutant CREB M1, in which Ser-133 is converted to an alanine, cannot be phosphorylated or activated but retains the ability to interact and bind effectively to the CRE. Thus, when overexpressed in cells, CREB M1 interferes with the function of CREB or a related family member by

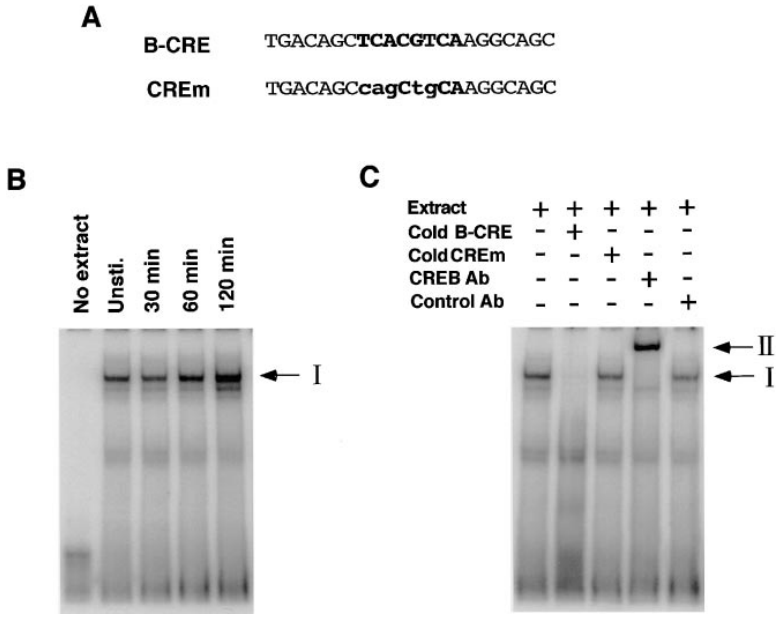

Figure 8. Interaction between the *BDNF* CRE-Like Sequence and CREB

(A) The nucleotide sequences of the *BDNF* CRE probe (B-CRE) and the mutant (CREm) probe. The CRE-like sequence is denoted by the bold letters. The CREm is identical to the B-CRE except for the substitution of five nucleotides at the CRE-like site. The B-CRE was used as a radiolabeled probe.

(B) Binding of nuclear proteins to radiolabeled B-CRE in an electrophoretic mobility shift assay. Nuclear extracts were obtained from cultured cortical neurons that were unstimulated (Unsti.) or stimulated with 50 mM KCl for the indicated times. "I" indicates the band later identified as the CRE-CREB complex.

(C) Specific interaction of the CRE and CREB in a gel shift assay. Nuclear extracts from cells stimulated for 120 min were incubated with the radiolabeled probe in the presence of competitors, or an anti-CREB–specific antibody, or a control antibody. Competitor DNA, B-CRE, or CREm was added to the reaction at 100-fold molar excess relative to the ^{32}P-labeled probe. "I" indicates the CRE-CREB complex and "II" indicates the supershifted complex.

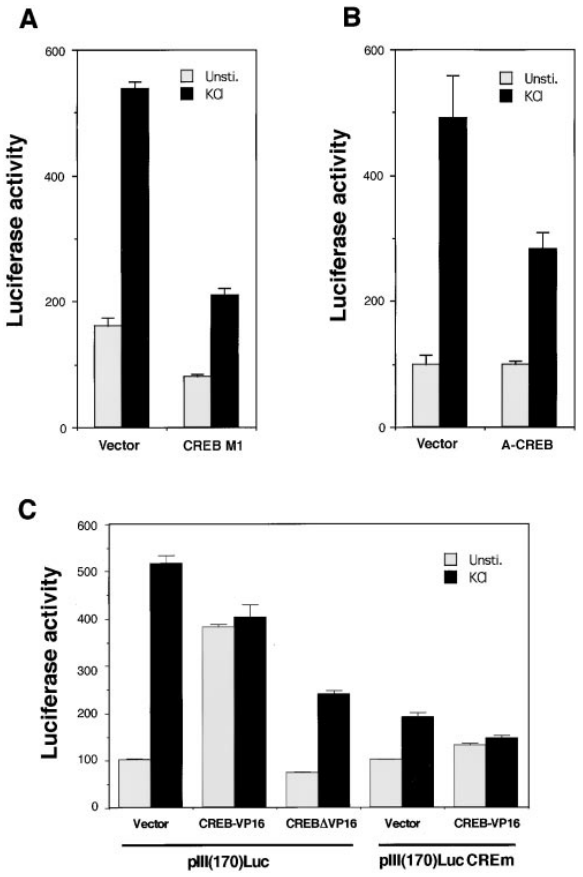

Figure 9. A CREB Family Member Binds to the *BDNF*-CRE and Mediates *BDNF* Transcription

(A and B) Overexpression of dominant negative CREB M1 or A-CREB inhibits promoter activation. Control vectors, or the CREB M1 expression vector, or the A-CREB expression vector was cotransfected with pIII(170)Luc into cultured cortical neurons. Forty hours after transfection, cells were left unstimulated or stimulated with 50 mM KCl for 9 hr. Cell extracts were then assayed for luciferase activity.

(C) A constitutively active CREB triggers Ca^{2+}-independent promoter activation. Cultured cortical neurons were transfected with the indicated constructs, and luciferase activities were assayed as previously described. CREB-VP16 contains full-length CREB and the transcriptional activation domain of VP16. CREBΔVP16 is similar to CREB-VP16 except that the DNA binding domain and dimerization domain of CREB is deleted. Each value represents the average of at least three independent determinations, and the error bars indicate the standard error of each mean.

competing with these proteins for binding to CREs (Gonzalez and Montminy, 1989). When a CREB M1 expression vector was cotransfected into cortical neurons along with a *BDNF* promoter III–luciferase construct, the presence of CREB M1 effectively inhibited membrane depolarization–mediated induction of the *BDNF*-luciferase gene (Figure 9A). We also tested the effect of A-CREB, a dominant negative form of CREB that works by a mechanism distinct from CREB M1. A-CREB has an acidic amphipathic extension onto the N terminus of the CREB leucine zipper domain. As a consequence, A-CREB dimerizes with CREB family members with a high affinity and prevents them from binding to CREs (Ahn et al., 1998). We found that A-CREB is able effectively to inhibit membrane depolarization–mediated induction of *BDNF* promoter III–driven luciferase expression (Figure 9B). In addition, K-CREB, a dominant negative form of CREB with a mutation in the DNA binding domain (Walton et al., 1992), was also found to inhibit membrane depolarization–mediated induction of the *BDNF*-luciferase gene (data not shown). The finding that the expression of any of three distinct dominant negative CREBs blocks *BDNF* promoter III reporter gene activity supports the conclusion that a CREB family member plays a critical role in mediating the *BDNF* promoter's response to membrane depolarization.

In contrast to the finding with the three dominant negative CREB mutants, cotransfection of a plasmid that encodes a constitutively active CREB (CREB-VP16) along with the *BDNF* promoter III luciferase gene resulted in the potent activation of the *BDNF*-luciferase reporter gene even in the absence of membrane depolarization (Figure 9C). CREB-VP16 is a chimeric protein in which CREB is fused at its N terminus to the transcriptional activation domain of a potent herpes virus transcriptional activator. The induction of the *BDNF*-luciferase gene by CREB-VP16 was due to the specific interaction between CREB and the CRE. Deletion of the DNA binding domain of CREB (CREBΔVP16) abolished the constitutive activation of the reporter gene. CREBΔVP16 also decreased the promoter's activity in both unstimulated and stimulated conditions, perhaps due to a squelching effect. An intact CRE was also required for *BDNF* promoter induction, since pIII(170)LucCREm was not responsive to CREB-VP16 (Figure 9C). The finding that constitutively active CREB-VP16 triggers Ca^{2+}-independent *BDNF* promoter III–dependent transcription and the findings that both a CRE mutation and dominant inhibitory forms of CREB block *BDNF* promoter III–dependent transcription strongly suggest that CREB or a closely related family member binds to the *BDNF* 5′-TCACGTCA-3′ element and mediates Ca^{2+}-dependent *BDNF* transcription.

We next investigated whether the phosphorylation of CREB at Ser-133 is important for Ca^{2+} regulation of *BDNF* transcription. It has been shown previously in a variety of cell types that membrane depolarization effectively induces CREB phosphorylation at Ser-133 (Sheng et al., 1990; Thompson et al., 1995; Deisseroth et al., 1996). To determine if the time course of CREB Ser-133 phosphorylation parallels the kinetics of *BDNF* transcriptional induction in membrane-depolarized cortical neurons, the phosphorylation of CREB at Ser-133 at various times after membrane depolarization was monitored by Western blotting using an antibody that specifically recognizes the Ser-133 phosphorylated form of CREB. As shown in Figure 10A, upon membrane depolarization of cortical neurons CREB Ser-133 phosphorylation was induced within 15 min and a high level of phosphorylation at Ser-133 was maintained for at least 3 hr. Using an antibody that recognizes CREB whether or not it is phosphorylated at Ser-133, we found that the total amount of CREB protein in cortical extracts is the same before and after membrane depolarization, suggesting that the increase in CREB Ser-133 phosphorylation is not due to an increase in the total amount of CREB protein (Figure 10A). The analysis of CREB Ser-133 phosphorylation revealed that at times when CREB

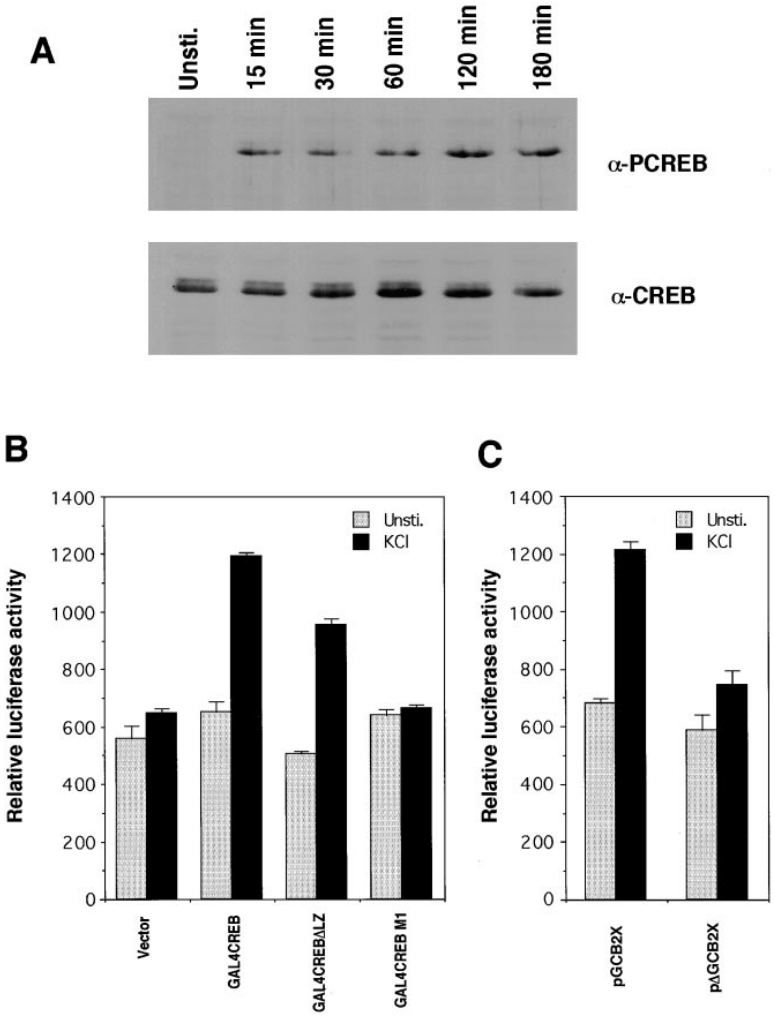

Figure 10. Phosphorylation of CREB at Ser-133 Is Critical for *BDNF* Promoter III Activation

(A) CREB Ser-133 phosphorylation was induced by membrane depolarization. Western blots of cell extracts from cultured cortical neurons were left unstimulated (Unsti.) or stimulated with 50 mM KCl for the indicated times. The blots were probed with anti-phosphoCREB(133) antibodies (α-PCREB) or anti-CREB antibodies (α-CREB).

(B) *BDNF* promoter III activation requires the phosphorylation of CREB at Ser-133. Construct pGCB2X, which contains two tandem GAL4 consensus binding sites that are inserted into the CREm site of pIII(170)Luc-CREm, was transfected into cultured cortical neurons with the indicated plasmids. Luciferase activity was assayed after preparation of cell extracts and was normalized to β-galactosidase activity. Each value represents the average of three independent determinations, and the error bars indicate the standard error of each mean.

(C) Optimal promoter activation requires cooperation of GAL4-CREB and upstream element binding proteins. pGCB2X or pDGCB2X was transfected into cortical neurons along with a GAL4-CREB expression vector. The luciferase assay was performed as described in (B).

is not phosphorylated at Ser-133 the level of *BDNF* transcription is undetectable, and at all times when *BDNF* transcription is high CREB is maximally phosphorylated. However, at early times after membrane depolarization CREB is highly phosphorylated at Ser-133 but *BDNF* transcription is not yet induced, and at times when *BDNF* transcription has shut off again CREB phosphorylation is still maintained. This suggests that the phosphorylation of CREB at Ser-133 may be important, but not sufficient, for Ca^{2+} induction of *BDNF* transcription. A second event that occurs more slowly is also likely to be important. One possibility is that a factor(s) that binds to the 18 bp element adjacent to the 5′-TCACGTCA-3′ element may also need to be modified and this modification may occur more slowly, more transiently, and may be rate limiting.

To determine if the phosphorylation of CREB at Ser-133 is required for membrane depolarization-mediated induction of *BDNF* reporter gene transcription, we targeted CREB to a different DNA regulatory sequence by fusing CREB to the DNA binding and dimerization domain of the yeast transcriptional activator GAL4 to generate GAL4-CREB. We then replaced the 5′-TCACG TCA-3′ element within the *BDNF* promoter III luciferase reporter gene with two GAL4 binding sites (pGCB2X) and asked if pGCB2X, upon transfection into cortical neurons, was responsive to membrane depolarization in a GAL4-CREB–dependent manner. As shown in Figure 10B, we found that in the absence of GAL4-CREB the amount of luciferase activity generated from the pGCB2X reporter was not increased by membrane depolarization. In contrast, in the presence of GAL4-CREB membrane depolarization led to an increase in pGCB2X reporter expression. Similar results were obtained using a reporter construct that contained only a single GAL4 site (data not shown). These experiments indicate that GAL4-CREB bound to GAL4 sites within pGCB2X mediates depolarization induction of luciferase expression.

To determine if GAL4-CREB functions by interacting with endogenous proteins through its leucine zipper, we examined pGCB2X activation by a mutant GAL4-CREB (GAL4CREBΔLZ) in which the CREB leucine zipper was deleted. Deletion of the CREB leucine zipper only modestly impaired the ability of GAL4-CREB to activate transcription from the pGCB2X reporter, indicating that GAL4-CREB does not need to dimerize with an endogenous leucine zipper containing protein to mediate *BDNF* reporter gene transcription in membrane-depolarized cells.

To determine if the phosphorylation of GAL4-CREB

at Ser-133 is critical for membrane depolarization induction of *BDNF* promoter III–driven reporter gene transcription, activation of the pGCB2X reporter by a mutant GAL4-CREB in which Ser-133 was converted to an alanine was assessed. In contrast to wild-type GAL4-CREB, GAL4-CREB M1 was ineffective at mediating pGCB2X reporter gene transcription in membrane-depolarized cortical neurons. We conclude from these experiments that CREB bound to *BDNF* promoter III mediates Ca^{2+}-dependent *BDNF* transcription by a mechanism that requires the phosphorylation of CREB at Ser-133.

To determine whether the regulation of reporter gene expression by GAL4-CREB is dependent on the second regulatory element, reporter gene induction was examined from a derivative construct, pΔGCB2X, in which nucleotides −48 to −83 were deleted. As shown in Figure 10C, pΔGCB2X reporter gene expression was poorly induced by membrane depolarization even when GAL4-CREB was present, suggesting that optimal gene induction requires the cooperation between GAL4-CREB and factors that bind to the −48 to −83 element.

Expression of CREB M1 Attenuates Endogenous *BDNF* Exon III Expression

Having demonstrated the importance of CREB as a mediator of *BDNF* promoter III reporter gene transcription, we next asked if CREB is also a critical regulator of the response of the endogenous *BDNF* gene to membrane depolarization. To address this issue, a dominant interfering form of CREB, CREB M1, was introduced into cortical neurons by transfection, and the effect of CREB M1 expression on endogenous *BDNF* transcription determined by RT-PCR using *BDNF* exon III and coding exon primers. Since only a small fraction of the cortical neurons are effectively transfected by the calcium phosphate precipitation protocol used for these experiments (<10%), it was necessary to separate the transfected from the nontransfected cells prior to the PCR analysis. This was accomplished by cotransfecting CREB M1 with a plasmid that drives the expression of green fluorescent protein (GFP). Concentrations of CREB M1 and GFP plasmids were chosen so that essentially all of the neurons that express GFP also express CREB M1. Between 6 and 40 hr after transfection, the transfected neurons exhibited bright green fluorescence. No apparent morphological changes were observed when GFP-expressing neurons cotransfected with CREB M1 were compared with GFP-expressing neurons that had been cotransfected with a control vector. This suggests that the expression of CREB M1 in cortical neurons does not have a deleterious effect on these cells. In addition, neurons expressing CREB M1 and GFP were able to flux Ca^{2+} in response to membrane depolarization (data not shown), indicating that the presence of CREB M1 did not affect the expression of L-type VSCCs.

To enrich for neurons that express GFP and CREB M1 after 3 hr of continuous stimulation with KCl or no treatment, cortical neuron cultures were trypsinized and dissociated into single cells. The GFP-positive/CREB M1–expressing cells or GFP-positive/control vector–containing cells were isolated by fluorescence-activated cell sorting (FACS). After FACS, total RNA was isolated from the GFP-positive cells and subjected to RT-PCR analysis using *BDNF* exon III and coding region primers. As shown in Figure 11, in cells transfected with GFP and an empty vector, membrane depolarization elicited a strong induction of *BDNF* exon III–containing mRNA transcripts, indicating that in the sorted cells the *BDNF* gene was still responsive to Ca^{2+} influx. By contrast, the level of membrane depolarization-induced *BDNF* exon III mRNA was significantly reduced in GFP-expressing cells that had been cotransfected with CREB M1. Quantitative analysis revealed that CREM M1 reduced the expression of exon III–containing *BDNF* mRNAs by 75% (Figure 11B). Taken together, these experiments indicate that a CREB family member is a critical mediator of membrane depolarization-mediated induction of endogenous *BDNF* transcription.

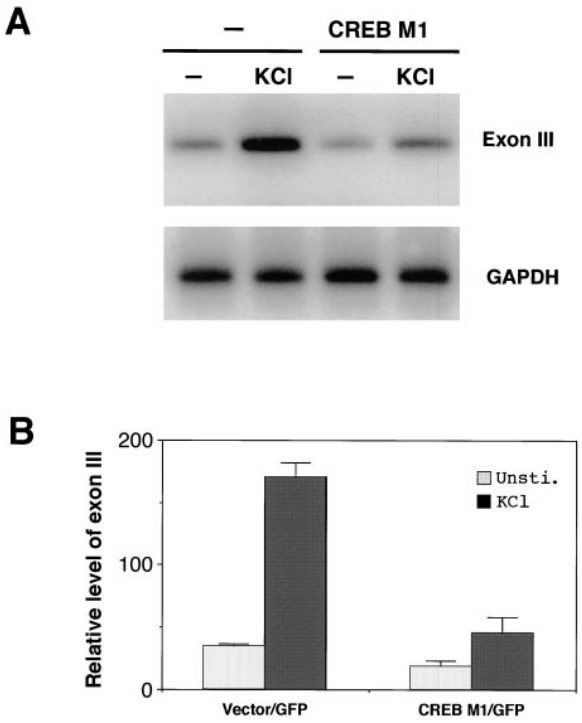

Figure 11. Dominant Negative CREB M1 Attenuates KCl-Induced Expression of the Endogenous *BDNF* Gene

Cultured cortical neurons were transfected with the CREB M1 expression vector and a GFP expression vector. Cells were left unstimulated or stimulated with 50 mM KCl. The transfected cells were isolated by FACS and *BDNF* exon III mRNA expression in these cells was analyzed by RT-PCR.

(A) Autoradiograph of PCR products of *BDNF* exon III and *GAPDH* transcripts.

(B) Quantification of *BDNF* exon III mRNA. The levels of radioactivity were obtained by PhosphoImager analysis. The relative level of each sample was normalized relative to the *GAPDH* level of the same sample. Each value is an average of three independent determinations.

Discussion

In this study, we have characterized the mechanisms by which neuronal activity regulates *BDNF* gene expression. We find in cultured embryonic cortical neurons

that membrane depolarization induces transcriptional activation of the *BDNF* gene, and that transcription from *BDNF* promoter III accounts for most *BDNF* gene expression under these conditions. We have characterized the minimal *BDNF* promoter region that confers the membrane depolarization response and within this region we have identified two elements that are highly conserved between rodents and human and are essential for *BDNF* transcriptional induction. One of the elements is a CRE located ~35 nucleotides 5′ of the first site of initiation of *BDNF* exon III transcription. The *BDNF* CRE binds CREB, which becomes newly phosphorylated on Ser-133 upon membrane depolarization. Within neurons, CREB, or a related family member, or both, then mediates *BDNF* transcription by a mechanism that appears to involve cooperation with a second critical regulatory element that is ~18 bp in length and is located just 5′ of the CRE.

The finding that a CREB family member is a critical mediator of Ca^{2+}-regulated *BDNF* transcription is likely to have considerable biological significance and may possibly explain how neuronal activity can effect changes in synaptic efficacy by inducing gene expression within the nucleus. Although the CREB family has been implicated as an important regulator of synaptic plasticity in a range of organisms from *Drosophila* to mammals (Dash et al., 1990; Frank and Greenberg, 1994; Bourtchuladze et al., 1994; Yin et al., 1994, 1995; Bartsch et al., 1995), an understanding of how this transcription factor acts in the nucleus to elicit a response at the synapse has remained elusive. One likely possibility is that the CREB family might regulate genes whose products act at synapses, and our finding that *BDNF* transcription is controlled by a CREB family member provides an example of a CREB target that is likely to function in this way. Other genes that have CREB binding sites within their regulatory regions and whose protein products are known to function at synapses include synapsin I and a particular subtype of potassium channel $K_V1.5$ (Sauerwald et al., 1990; Mori et al., 1993).

An interesting possibility is that Ca^{2+} and CREB family-dependent regulation of *BDNF* transcription may play a critical role in the strengthening of specific synapses that occurs in long-lasting forms of LTP (L-LTP). A recent study revealed that in L-LTP the selective potentiation of one of the many synapses on a postsynaptic neuron is due to a tagging mechanism in which the initial stimulus somehow marks the synapse (Frey and Morris, 1997; Martin et al., 1998). A consequence of tagging is that when the protein products of newly transcribed genes, such as the *BDNF* gene, are transported to all of the neuron's synapses, the newly synthesized proteins can only exert their effect on the specific synapse that has been recently potentiated. This would then result in long-lasting potentiation specifically at the tagged synapse. While the synaptic tagging model is quite compelling, the specific molecules that are the substrates for synaptic tagging and the newly synthesized proteins that act on the tagged synapse remain to be identified.

Our finding that Ca^{2+} regulates *BDNF* transcription by a CREB family member–dependent mechanism, coupled with the previous findings from knockout mice that both CREB and BDNF are required for L-LTP, raises the possibility that BDNF may be an important component of the synaptic tagging mechanism. An intriguing possibility is that at least one synaptic tagging event initiated by L-LTP is the synapse-specific modification of the BDNF receptor TrkB by phosphorylation, so that TrkB's ability to mediate the BDNF response is somehow enhanced. Since L-LTP triggers the induction of *BDNF* transcription and likely its transport to all of the neuron's synapses, the selective long-lasting potentiation of the tagged synapse might reflect the ability of BDNF to bind TrkB or signal through TrkB most effectively at the synaptic site where L-LTP was initiated.

Whether or not BDNF and TrkB are critical components of the synaptic tagging mechanism, it is clear that BDNF plays a role in synaptic potentiation, and CREB's importance as a mediator of synaptic transmission in a variety of organisms could well reflect its function as a key regulator of *BDNF* transcription. In addition, since BDNF is known to regulate a variety of steps in the process of neuronal differentiation including proliferation, differentiation, and survival, it is likely that as a regulator of *BDNF* transcription CREB family members may be found to be important for these processes as well.

Given BDNF's importance for nervous system development and function, it will be useful to characterize further the mechanisms by which Ca^{2+} regulates CREB and CREB family members activate *BDNF* transcription. Ca^{2+} entry into neurons via the NMDA subtype of glutamate receptor or through L-type VSCCs induces CREB phosphorylation and *BDNF* transcription. However, the induction of *BDNF* mRNA synthesis by NMDA receptor agonists is less robust and more transient than the activation of *BDNF* transcription that is triggered by Ca^{2+} influx through L-type VSCCs (Ghosh et al., 1994). This difference in *BDNF* induction most likely reflects the fact that NMDA receptor agonists induce CREB phosphorylation at Ser-133 only transiently, while Ca^{2+} influx through L-type VSCCs leads to the rapid but prolonged phosphorylation of CREB at Ser-133 (S. F. et al., unpublished data). This difference in the kinetics of CREB phosphorylation and *BDNF* transcription appears to be of biological significance inasmuch as L-type VSCC activation promotes the survival of cortical neurons by BDNF-dependent mechanisms, whereas NMDA receptor stimulation has no significant effect on cortical neuron survival (Ghosh et al., 1994)

The signaling pathways that mediate Ca^{2+}-dependent phosphorylation of CREB and may thereby regulate gene transcription have been the subject of intense investigation, and a number of pathways have been identified that may contribute to the CREB Ser-133 phosphorylation event. Two Ca^{2+}/calmodulin–dependent kinases, CaM kinase II and IV, phosphorylate CREB at Ser-133 in vitro and in vivo (Sheng et al., 1991; Matthews et al., 1994; Sun et al., 1994; Bito et al., 1996). However, CaM kinase IV is more likely the mediator of the Ca^{2+} response, since it appears to be localized to the nucleus of neurons, whereas the major forms of CaM kinase II are mainly cytoplasmic (Nakamura et al., 1995; Bito et al., 1996). In addition to CaMK IV, other kinases may also contribute to Ca^{2+} regulation of CREB phosphorylation and *BDNF* transcription. Likely possibilities are

members of the pp90rsk family (RSK 1–3) and the MAP kinase–activated protein (MAPKAP) kinases 1–2 that have recently been shown to mediate growth factor–induced CREB Ser-133 phosphorylation (Xing et al., 1998). The RSKs and MAPKAP kinases are both activated by the Ras signaling pathway, and Ras is activated by Ca^{2+} influx through L-type VSCCs or NMDA receptors (Rosen et al., 1994; Xing et al., 1996; S. F., unpublished data), possibly via PYK2, Src, or Ras-GRF (Farnsworth et al., 1995; Lev et al., 1995; Rusanescu et al., 1995). In neurons that express RSKs and MAPKAP kinases, these enzymes might be activated and could catalyze CREB Ser-133 phosphorylation. Further experimentation will be required to determine which of the various kinases are critical mediators of Ca^{2+} regulation of *BDNF* transcription.

While our experiments indicate that CREB family function is required for Ca^{2+} induction of *BDNF* mRNA synthesis, it appears that CREB Ser-133 phosphorylation is not sufficient to lead to *BDNF* induction. Several findings suggest that an additional event is also required. First, we find that in addition to the CRE there is an 18 bp element whose presence is required for Ca^{2+} induction of *BDNF* transcription. Presumably, a transcription factor(s) bound to this element functions cooperatively with a CREB family member to mediate the BDNF response. The factor that binds to the 18 bp element may be activated by Ca^{2+}. This is suggested by our finding that although CREB is maximally phosphorylated at Ser-133 within minutes of membrane depolarization, *BDNF* transcription is not induced until somewhat later. It may be that the factor binding the 18 bp element is modified with delayed kinetics relative to CREB, and that *BDNF* is not induced until both factors are activated.

The *BDNF* 5′ 18 bp element may also confer neuronal specificity to *BDNF* induction. We find that membrane depolarization of PC12 cells leads to CREB Ser-133 phosphorylation and the induction of IEGs (such as c-*fos*) whose expression is regulated by CREB. However, membrane depolarization of PC12 cells does not induce transcription of the endogenous *BDNF* gene or transfected *BDNF* promoter luciferase constructs. A possible explanation for the inability to induce *BDNF* in PC12 cells might be the absence of the transcription factor(s) that binds to the *BDNF* 5′ 18 bp regulatory element. A critical next step will be to identify and characterize the factor(s) that binds to the *BDNF* 5′ 18 bp regulatory element. The characterization of this factor will likely provide insight into the mechanisms by which Ca^{2+} regulates gene expression and neuronal adaptive responses.

Experimental Procedures

Cell Culture and Stimulation

All cell culture experiments were performed using cortical neurons from embryonic day 17/18 (E17/E18) Long Evans rats. Cells were grown in basal medium Eagle with 5% calf serum, 15 mM glucose, 1 mM L-glutamine, and penicillin/streptomycin. The cells were seeded onto culture dishes at a density of 3×10^6 cells per well in a six-well plate (35 mm), 7×10^6 cells per 60-mm-diameter dish, and 2×10^7 cells per 100-mm-diameter dish and maintained in a humidified incubator with 5% CO_2 at 37°C. Dishes were coated with poly-D-lysine and laminin (Xia et al., 1996).

Cells were stimulated on 5 DIV (days in vitro) or 2 days after transfection. Tetrodotoxin (1 μM) was added the night before stimulation to reduce endogenous synaptic activity. Thirty minutes prior to stimulation, 100 μM amino-5-phosphonovaleric acid (APV) was added to block NMDA receptor activity. KCl was added to a final concentration of 50 mM to induce membrane depolarization as previously described (Bading et al., 1993).

Nuclear Run-On Assay, Northern Blot and RT-PCR

Cortical neurons were stimulated or left unstimulated on 5 DIV. Nuclear run-on assays were carried out according to a previously described procedure (Greenberg and Ziff, 1984). In each reaction, 1×10^8 nuclei were used. RNA was isolated by the guanidinium thiocyanate phenol-chloroform procedure (Chomczynski and Sacchi, 1987). The *BDNF* probe contained *BDNF* exon III, exon IV, and the coding region of exon V. The other probes were as previously described (Bartel et al., 1989). Northern blot analyses were performed according to a standard procedure (Ausubel et al., 1987).

For RT-PCR, mRNA was reverse transcribed into single-stranded cDNA with M-MLV reverse transcriptase using oligo$(dT)_{15}$ as primers in a 20-μl reaction containing 1 μg total RNA. The reaction mixtures were diluted 100-fold and then subjected to PCR amplification of *BDNF* 5′ exons. The PCR reactions contained 1 μM of the common reverse primer E5R that was ^{32}P-labeled and corresponded to the DNA sequence in the exon V and one of the following 5′exon specific primers: 1.5 μM of E1F or 1 μM of E2F; 1 μM of E3F or 0.75 μM of E4F. To increase the primers annealing stringency, 2.5% DMSO was added to the reaction. Amplification was carried out for 27 cycles. Each cycle consisted of the following steps: 94°C for 20 s, 92°C for 40 s, 55°C for 60 s, and 72°C for 60 s. The amount of total RNA in the samples was normalized to the amount of *GAPDH*, which was amplified for 23 cycles. The primers were as follows: E5R, 5′-GAGAA GAGTGATGACCATCCT-3′; E1F (exon I specific), 5′-CTCAAAGGGA AACGTGTCTCT-3′; E2F (exon II specific), 5′-CTAGCCACCGGGGTG GTGTAA-3′; E3F (Exon III specific), 5′-TGCGAGTATTACCTCCGC CAT-3′; and E4F (exon IV specific), 5′-TTGGGGCAGACGAGAAAG CGC-3′. *GAPDH* primers were: 5′-TCCATGACAACTTTGGCATCG TGG-3′ and 5′-GTTGCTGTTGAAGTCACAGGAGAC-3′.

The PCR products were separated by electrophoresis on 6% polyacrylamide gels and were visualized and quantified by PhosphorImager analysis.

Plasmid Constructions

Rat genomic DNA was used as the template for PCR to amplify the ~500 bp *BDNF* promoter III fragment with the forward primer A (5′-CGCGGATCCAAAGCATGCAATGCCCTGGAA-3′) and the reverse primer A (5′-GCGGGATCCACCTTTTTCAGTCACTACTTG-3′). The fragment was cloned into the BglII site of pGL3-Basic luciferase reporter vector to generate plasmid pIII(170)Luc. pIII(1500)Luc was constructed by replacing the region between SmaI and NdeI sites of pIII(170)Luc with a 1 kb fragment between EcoRV and NdeI sites of p*BDNF* IIICAT (Timmusk et al., 1995). The fragment in plasmid pIII(20)Luc was amplified by PCR with forward primer B (5′-CATGCC CGGGAGCCCTATCGTGGACTC-3′) paired with reverse primer A. The fragment in plasmid pIII(170B)Luc was amplified with forward primer A paired and reverse primer B (5′-GTGGGATCCGGCAGCGC GAGCAGTCCTCT-3′). Plasmid pIII(170Δ48–83)Luc was generated by deleting the fragment between ApaLI and NdeI sites of the *BDNF* promoter in pIII(170)Luc. Plasmid pIII(170Δ29–43)Luc was generated by PCR using primers in which sequence from −29 to −43 was deleted. The CRE mutation was introduced into *BDNF* promoter by PCR amplification with reverse primer A and a forward primer (5′-GAAGGTATCATATGACAGCCAGCTGCAAGGCAGCGTG GAGCC-3′) in which the CRE site was mutated. The PCR-amplified fragment was then digested with NdeI and AgeI and used to replace the corresponding region in plasmid pIII(170)Luc to generate pIII(170)LucCREm. Plasmid pIII(1500)LucCREm was constructed by adding the EcoRV and NdeI fragment of p*BDNF* IIICAT into the SmaI and NdeI sites of pIII(170)LucCREm. Plasmid pGCB2X was constructed by inserting a synthetic double-stranded DNA sequence containing two GAL4 consensus binding sites (5′-TCGGAGGACTGT CCTCCGATCGGAGGACTGTCCTCCGA-3′) into the mutated CRE site of pIII(170)CREm. The 5′ element mutations within the −48 to −83 region were generated by first amplifying fragments with

primers containing randomized bases at particular regions and then swapping the fragments with the corresponding DNA sequences within pIII(170)Luc. Vent DNA polymerase was used in all PCR amplification reactions, and the PCR-amplified regions used to generate plasmid constructs were sequenced to confirm the correct sequence.

CREBΔVP16 has been described elsewhere (Sun and Maurer, 1995) and contains the first 283 amino acids of CREB fused in-frame to the 3′ end of the VP16 activation domain. CREB-VP16 was constructed by subcloning a BspEI/Xba fragment from full-length CREB (Loriaux et al., 1993) into the corresponding sites of CREBΔVP16, resulting in a fusion of the VP16 activation domain to full-length (1–341) CREB.

Cortical Neuron Transfection and Luciferase Assay

The cortical neuron transfections were carried out on 3 or 4 DIV according to a previously described procedure (Xia et al., 1996). For luciferase assays, one 35-mm well of cells was transfected with 1 μg of reporter plasmid and 0.7 μg of pEF-LacZ.

Forty hours after transfection, cells were stimulated with KCl for 9 hr. The plates were then washed twice with cold PBS, and the cells were lysed with 200 μl lysis buffer (Promega). Twenty microliters of cell extract were used for a luciferase reporter assay (Promega); 100 μl of extract were used for a β-galactosidase assay with *ortho*-nitrophenyl β-D-galactopyranoside as the substrate.

RNase Protection Analysis

Plasmid pBLuc was constructed to generate an anti-sense riboprobe. A 700 bp fragment between KpnI and BstBI sites of pIII(170)-Luc was ligated into the KpnI and ClaI sites of pBluescript KS. The plasmid was linearized with XhoI, and the anti-sense probe was generated using T7 polymerase. Cortical neurons in 60-mm dishes were transfected with 6 μg of pIII(170)Luc. Forty hours after transfection, cells were left unstimulated or stimulated with elevated KCl for 6 hr. Total RNA was isolated and subjected to RNase protection analysis (Finkbeiner et al., 1997). Protected RNA fragments were separated on 4% polyacrylamide gels. A radioactively labeled RNA ladder with a 0.16–1.77 kb range (GIBCO BRL) was used as size markers. Gels were dried and visualized by PhosphorImager analysis.

Biolistic Transfection and Quantification of Luciferase Expression in Cortical Slices

Coronal cortical slices 200 μM thick were prepared from postnatal day 14 (P14) Wistar rats. Slices were briefly collected in a solution of artificial cerebrospinal fluid and transferred to inserts in six-well plates each containing 1.3 ml of media. Four hours after slices were placed in inserts, they were transfected with 10 μg each of pEF-LacZ and either pIII(170)Luc or pIII(170)LucCREm using previously described biolistic transfection methods (Arnold and Heintz, 1997). Slices were incubated for 12 hr then depolarized (55 mM KCl) or mock stimulated. During treatments, 100 μM APV and 40 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) were included to isolate Ca^{2+} entry through voltage-dependent Ca^{2+} channels. Nine hours after stimulation, slices were washed with PBS and fixed for 1 hr at 4°C with a 2.5% paraformaldehyde/4% sucrose PBS solution. Subsequently, slices were incubated in a 30% sucrose/PBS solution for 2 hr at 4°C and then freeze-thawed. Slices were then sequentially incubated in a blocking solution of 4% bovine serum albumin and 1% normal goat serum in PBS for 2 hr, primary antibody (anti-LacZ [1:300, Promega] and anti-luciferase [1:200, Promega]) diluted in blocking solution for 2 hr, and secondary antibody (goat anti-mouse Cy2 [1:300, Jackson Labs] and goat anti-rabbit Cy3 [1:500, Jackson Labs]) diluted in blocking solution. Slices were washed with PBS between incubations with primary and secondary antibodies and after incubation with secondary antibodies. Slices were transferred to slides, air dried for ≥4 hr, dipped in Xylene for 3 min, and mounted with coverslips using Permount.

In a blinded fashion, immunofluorescence from β-galactosidase and luciferase staining was collected from each transfected neuron and digitized as a z-series of paired confocal images spaced 1 μm apart. Immunofluorescence was quantitated for each label by averaging the intensities of pixels within a 3 μm^2 area in the neuronal soma from the mid-plane section for each neuron. Each luciferase signal value was normalized for transfection efficiency by dividing the average pixel intensity of luciferase staining by the corresponding average pixel intensity of β-galactosidase staining. The distribution of the stimulated and unstimulated responses mediated by pIII(170)Luc was bimodal: most of the unstimulated responses were low and the stimulated responses were generally high. The normalized luciferase value that corresponded to a point of minimal overlap between the two distributions was then used to reclassify neurons from each slice as positive or negative. The fraction of responding neurons was calculated for each slice and averaged between slices for a given condition.

Electrophoretic Mobility Shift Assays

Cortical neurons were stimulated with elevated KCl on 5 DIV. Nuclear extracts for the in vitro DNA-binding assay were prepared by a procedure described previously (Ausubel et al., 1987). Binding of CREB to the labeled *BDNF* CRE probe was carried out in a 12 μl reaction mixture containing 20 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 40 mM KCl, 5 mM DTT, 10% glycerol, 1 μg poly(dI-dC), 3 μg BSA, 10 μg nuclear extract protein and 3–5 fmol ^{32}P-labeled probe. The probe corresponding to the *BDNF* promoter III sequence was synthesized as complementary oligodeoxyribonucleotide strands (5′-GTTGACAGCTCACGTCAAGGCAGC-3′, 5′-CTGCTGCCTTGACGTGAGCTGTC-3′) and labeled with ^{32}P-ATP with T4 polynucleotide kinase according to standard procedures. CREm probe was synthesized as complementary oligodeoxyribonucleotide strands (5′-GTTGACAGCCAGCTGCAAGGCAGC-3′, 5′-CTGCTGCCTTGCAGCTGGCTGTC-3′). In the competition assays, unlabeled probes were added to the reaction mixture 10 min prior to the addition of the labeled probe. In antibody supershift assays, reaction mixtures were preincubated with 1 μl of CREB-specific monoclonal antibody (2–4H-4B, kindly provided by Dr. Jim Hoeffler) or control antibodies (anti-ATF1[F1–1], kindly provided by Dr. Jim Hoeffler, or anti-β-gal, Promega) for 1 hr at 40°C prior to the addition of the labeled probe. After the labeled probe was added, the mixture was incubated for 20 min at 25°C. Ten microliters of the binding reaction mixture were then applied to a 6% polyacrylamide gel in 40 mM Tris-borate (pH 7.5) 2.5% and glycerol and electrophoresed in the same buffer without glycerol. After electrophoresis, the gels were dried and visualized using a PhosphorImager.

Immunoblotting

Cortical neurons (3.5×10^6 cells per 35-mm dish) were stimulated on 5 DIV with an elevated level of KCl (50 mM). Cells were lysed in SDS sample buffer. The experiments were carried out according to a previously described procedures (Ginty et al., 1993; Thompson et al., 1995). The blots were visualized by enhanced chemiluminescence.

Fluorescence-Activated Cell Sorting

Cortical neurons were transfected with dominant interfering CREM M1 expression vector and an enhanced GFP expression vector. Forty hours after transfection, cells were left untreated or stimulated with an elevated level of KCl (50 mM). Following 3 hr of stimulation, the medium was removed and chilled on ice. Cells were washed once with Hanks' balanced salt solution (HBSS), treated with trypsin-EDTA at room temperature for 10 min, and then washed once with HBSS containing 2 mg/ml trypsin inhibitor. The saved medium was filtered through a 0.2 μm filter. RNase inhibitor was added to the medium to a final concentration of 800 U/ml. Cells from three to six dishes were pooled, resuspended in the original medium, and sorted by florescence-activated cell sorter (FACS). During the process of cell sorting, the cells were kept on ice whenever possible. GFP-positive cells were collected and pelleted by centrifugation. Total RNA was isolated using an RNeasy mini kit (Qiagen) and then subjected to RT-PCR analysis. cDNA from ~5000 cells was used in each PCR reaction. *BDNF* exon III was amplified for 30 cycles, and *GAPDH* was amplified for 25 cycles.

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