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Calcium regulation of the brain-derived neurotrophic factor gene

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Abstract. Results from several laboratories have suggested that peptide factors known as neurotrophins may play roles coupling changes in synaptic activity to lasting changes in synaptic function. Consistent with this idea, increases in synaptic activity and intracellular calcium induce the expression of the gene that encodes the neurotrophin, brain-derived neurotrophic factor. Recently, a pathway has been elucidated in neurons by which the influx of extracellular calcium evokes brain-derived neurotrophic factor transcription (BDNF). Calcium activates BDNF transcription through two adjacent calcium response elements within one of the promoters of the BDNF gene. One of the two elements binds to the cyclic adenosine monophosphate (AMP) response element binding protein (CREB) transcription factor, and interfering with CREB or related family members inhibits calcium-dependent BDNF transcription. This review focuses on the mechanisms by which calcium influx regulates brain-derived neurotrophic factor expression and the implications that these results have for potential roles of neurotrophins in synaptic function.

Key words. Neurotrophins; plasticity; gene expression; synapse; CREB; calcium.

Introduction

The neurotrophins are a family of peptide growth factors that include nerve growth factor (NGF), brain-derived growth factor (BDNF), NT-3 and NT-4 [1]. Neurotrophins have wide-ranging effects in the nervous system; however, neurotrophins are best known as survival factors that are capable of sustaining specific neuronal subpopulations during critical periods in development [2–4]. More recently, several observations have suggested the possibility that neurotrophins might regulate synaptic plasticity, the property that couples features of a neuron's electrical activity to lasting changes in the structure and function of its synapses [5–10]. Intracerebral infusion of neurotrophins during critical periods of synaptogenesis in the visual cortex alters the patterns of synaptic connections that form [11–13]. Application of exogenous neurotrophins to brain slices produces neurotrophin-specific changes in the shape and length of dendritic branches and increases in the efficacy or 'strength' of synaptic transmission [14–16]. Conversely, animals in which the BDNF gene has been disrupted fail to undergo normal increases in synaptic strength following electrical stimulation [17]. These synaptic defects can be 'rescued' by the exogenous application of BDNF or by the reintroduction of the BDNF gene through transfection, suggesting that the defects in synaptic function in BDNF null mice are unlikely to be solely from defects in nervous system development [18, 19]. Taken together, these results have led to the idea that BDNF may play a critical role in the regulation of synaptic structure and function in the adult brain.

The finding that BDNF and other neurotrophins trigger biochemical pathways that regulate synaptic structure and function has implicated neurotrophins as molecular mediators of synaptic plasticity. That BDNF might play a role in synaptic plasticity has been further supported

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by the additional discovery of feedback pathways that allow synaptic activity, through the influx of extracellular Ca^{2+}, to regulate the expression of neurotrophins and their cognate receptors [20–23]. For example, stimulation of Schaffer collateral synaptic pathways in hippocampal slices or the induction of seizures in vivo induces increases in BDNF messenger RNA (mRNA) levels [24, 25]. Also, the simultaneous elevation of cyclic adenosine monophosphate (cAMP) and Ca^{2+} within cultured neurons increases levels of the BDNF receptor, TrkB, at the plasma membrane [26]. Thus, there exist within neurons reciprocal pathways by which neurotrophin signaling regulates synaptic activity, and, in turn, synaptic activity regulates neurotrophin signaling. The existence of reciprocal signal transduction pathways connecting neuronal activity to neurotrophin signaling has led to a simple feedforward model: neuronal activity induces the synthesis and secretion of neurotrophins which, in turn, activates signal transduction pathways within neurons to strengthen synapses and support neuronal activity (fig. 1) [27]. The model is attractive for two reasons. First, it suggests a mechanism to link transient and rapidly changing levels of neuronal activity to longer-lasting, more slowly varying neurotrophin signaling pathways and thereby might serve to convert short-term synaptic activity changes into long-lasting changes in synaptic physiology and morphology. Another reason that the model is attractive is that it suggests a way that neurotrophins could exert bidirectional control over synaptic strength. If synapses require a specific range of BDNF concentrations to remain stable, then changes in neuronal activity that produce BDNF levels that are above or below such a range could lead to increases or decreases respectively in synaptic strength. Most important, the model provides a framework for formulating hypotheses to test directly the role that neurotrophins play in plasticity.

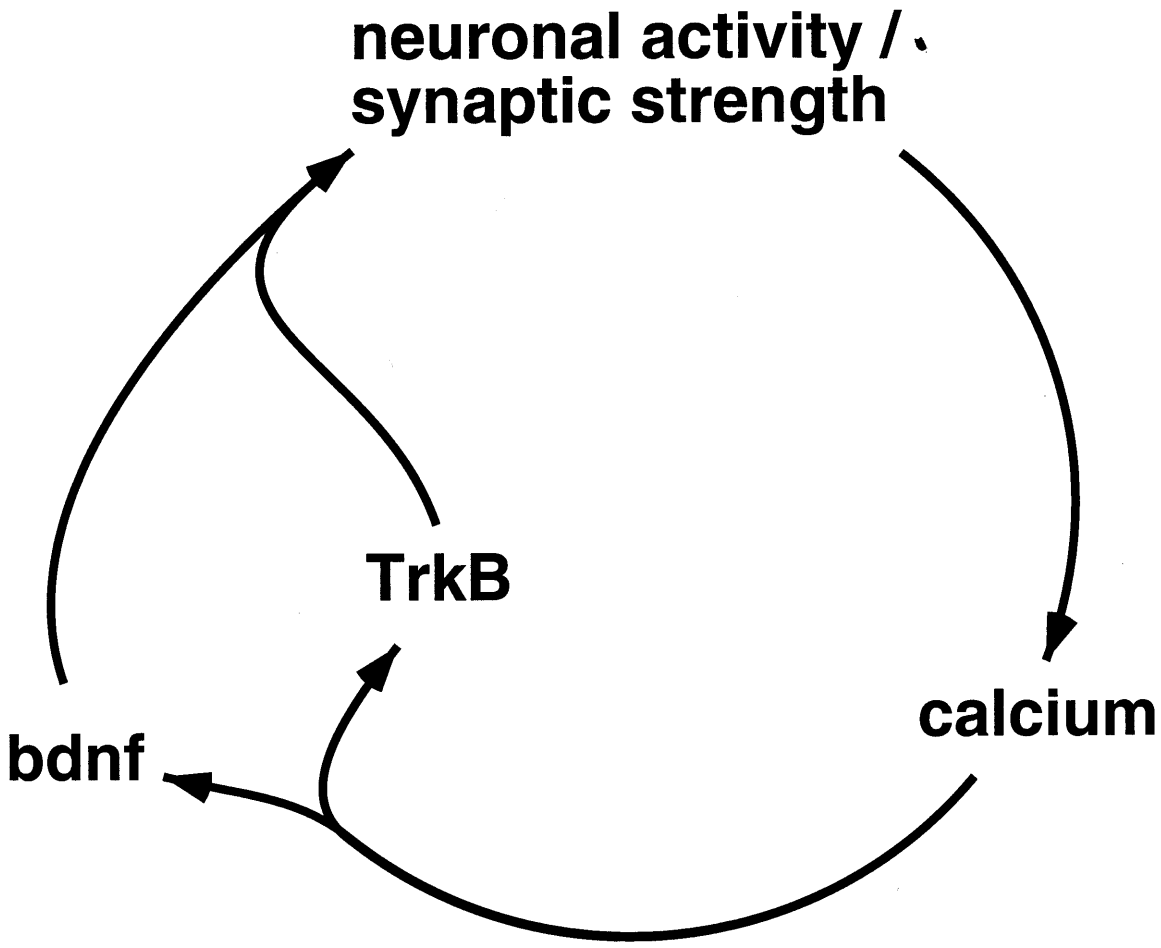

Figure 1. A model of a potential role for the neurotrophin brain-derived neurotrophic factor (bdnf) and its cognate receptor (TrkB) in the regulation of synaptic strength.

The BDNF gene is regulated by Ca^{2+}

To begin to understand better what role neurotrophin signaling might play in plasticity, two groups have begun to elucidate the signal transduction pathways by which the influx of extracellular calcium regulates BDNF mRNA levels [22, 21]. Both groups found that Ca^{2+} influx couples to pathways that lead to the activation of the transcription factor, cAMP-response element binding protein (CREB) or closely related family members and that CREB activation increases BDNF transcription through a Ca^{2+}/cAMP-response element (Ca/CRE) within one portion of the regulatory region of the BDNF gene. In addition, both groups found evidence for the existence of one or more additional response elements within the BDNF promoter near the CRE that must also be intact for the influx of extracellular Ca^{2+} to evoke BDNF transcription. The following review discusses our current understanding of the pathways by which neuronal activity-induced calcium influx regulates BDNF expression and how these findings might relate to models of neurotrophin function in plasticity.

The observation that the influx of extracellular calcium increases BDNF mRNA levels in neurons could occur because of increases in BDNF mRNA transcription, increases in BDNF mRNA stability or increases in both transcription and stability. To date, the effects of Ca^{2+} on BDNF message stability are unknown. However, it has been shown recently by nuclear run-on assay that depolarization-evoked extracellular Ca^{2+} influx induces increases in the transcription of BDNF mRNA in cultured cortical neurons [21]. Increased BDNF transcription occurred in the presence of protein synthesis inhibitors suggesting that all of the protein components of the signal transduction pathways that mediate Ca^{2+}-dependent BDNF transcription are present in neurons before stimulation. On this basis, BDNF can be classified as an immediate early gene; however, the kinetics of the induction of BDNF transcription appear slow compared with the prototypic immediate-early gene, c-*fos*. The delay likely reflects unknown differences in the precise mechanisms by which Ca^{2+} regulates the transcription of these two genes.

The fact that BDNF behaved as an immediate-early gene and that Ca^{2+} influx increased its transcription suggested that there were one or more Ca^{2+} response elements within the regulatory regions of the BDNF gene. However, the BDNF gene is large (40 kb) and complex, making the identification of these small putative response elements difficult (fig. 2A) [28–30]. Mature BDNF transcripts are formed from the five exons (exons I–V) that make up the BDNF gene by splicing one of the first four exons to the fifth (exon V) (fig. 2B). Each of the first four exons is driven by a unique promoter and contains a 3′ splice donor site. Exon V contains a 5′ splice acceptor site, the entire coding region of the BDNF gene and two alternative polyadenylation sites. Altogether, eight mature BDNF transcripts are possible. Since each of the splice variants encodes identical BDNF proteins, the significance of the different spliced forms remains unclear (fig. 2C).

Exon III of the BDNF gene contains a cAMP and Ca^{2+} response element

Since transcription could be initiated within each of the first four exons, putative Ca^{2+} response elements could be present in any or all of the regulatory regions associated with these exons. Initial experiments aimed at mapping the Ca^{2+} response elements began by determining whether the influx of extracellular Ca^{2+} led to the formation of mature BDNF messages that preferentially contained one of the first four exons [21]. Both Northern analysis and reverse transcriptase polymerase chain reaction (RT-PCR) showed that isotonic depolarization of cortical neurons led to the synthesis of mature BDNF mRNA that contained primarily exon III ($\sim 80\%$). Since Ca^{2+} influx led primarily to the formation of exon III-containing transcripts, it was likely that the putative Ca^{2+} response element(s) would be found within the regulatory region of exon III. Subsequently, both groups made reporter gene constructs using portions of the exon III promoter and showed that it was possible to delete all but approximately the first 100 nucleotides 5′ to the transcription start site and still retain Ca^{2+} responsiveness. Finer deletional and mutational analyses refined the localization of the Ca^{2+} response elements to two sequences (-78 to -48 and -43 to -30) that are each required for Ca^{2+} influx to activate the reporter gene.

Although an initial review of the upstream sequence (-78 to -48) did not reveal obvious consensus binding sites for known Ca^{2+}-regulated transcription factors, a review of the downstream element revealed an eight-nucleotide sequence that bore significant homology to a consensus Ca/CRE (fig. 3). The identification of a putative Ca/CRE is important because Ca/CREs are capable of binding members of a family of transcription factors (e.g. CREB, ATF-1 and NFIL6) whose ability to activate transcription is regulated by Ca^{2+}. To test whether the putative Ca/CRE is necessary for Ca^{2+}-induced BDNF transcription, single nucleotides within the Ca/CRE of the reporter gene constructs were mutated to disrupt the ability of the Ca/CRE to bind to CREB and to CREB family members. For Shieh et al., the effects of the Ca/CRE mutation on the ability of Ca^{2+} to induce BDNF transcription depended on the age of the rat pup from

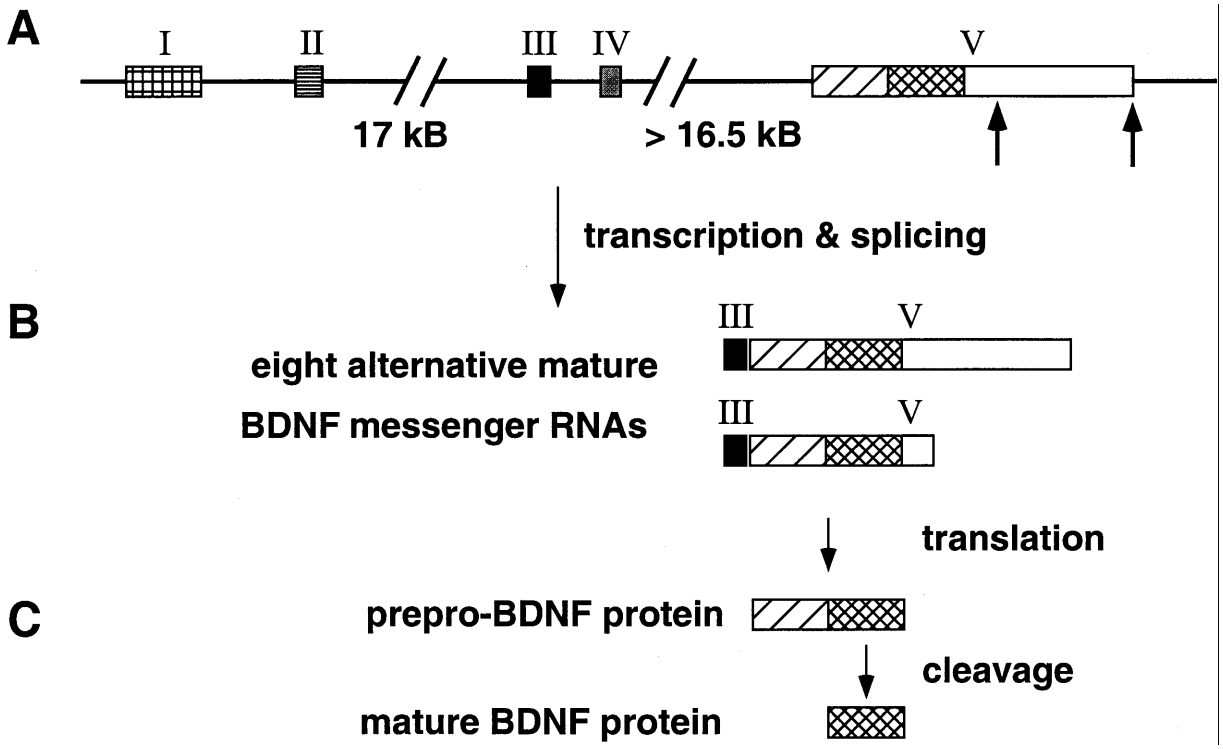

Figure 2. The organization of the BDNF gene. (*A*) The BDNF gene spans over 40 kB and contains five exons (I–V). Each of the first four exons contains its own promoter, one or more transcription initiation sites and a 3′ splice donor site. The fifth exon contains a 5′splice acceptor site and a 3′ UTR (open box) which has two alternative polyadenylation signals (black arrows). (*B*) Since BDNF messages can be transcribed from any of the first four promoters, and each message can be polyadenylated in two different positions, eight different mature BDNF transcripts are possible. Calcium influx leads to the preferential formation of mature BDNF transcripts that contain exon III (shown). (*C*) A precursor form of the BDNF protein (prepro-BDNF) is encoded within exon V and undergoes cleavage to yield the mature BDNF protein.

which the cortical neurons were cultured. Mutation of the Ca/CRE blocked completely Ca^{2+}-induced transcription of the reporter gene in neurons cultured from postnatal day 1 animals, whereas transcription of the mutated BDNF reporter gene was nearly normal in neurons cultured from rat embryos at the 18th day of gestation. By contrast, Tao et al. found that mutation of the Ca/CRE blocked reporter gene responses to Ca^{2+} influx in neurons cultured from rat embryos (gestational day 17/18), rat pups (postnatal day 1) and neurons in cortical slices from rats 14 days old [21]. The basis for these discrepant observations is not clear; however, the two groups did use reporter genes that were constructed and mutated in different ways that might be important.

CREB or a closely related family member mediates Ca^{2+}-dependent BDNF transcription

Having shown that the BDNF-Ca/CRE is important for Ca^{2+}-evoked BDNF transcription, both groups performed a series of experiments to characterize further the signal transduction pathways that couple Ca^{2+} influx to the activation of the BDNF-Ca/CRE. Both groups showed by mobility shift assay that a protein(s) found in nuclear extracts prepared from cultured cortical neurons binds to the BDNF-Ca/CRE in a sequence-specific way and that this protein(s) is recognized by an antibody raised against CREB but not by antibodies raised against the closely related family members ATF-1 or ATF-2 [22, 21]. Tao et al. found that the ability of this protein(s) to bind to a BDNF-Ca/CRE did not depend on stimulation of the cultured neurons from which the nuclear extracts were prepared [21]. However, Shieh et al. made a different observation using a longer oligonucleotide that encompassed the BDNF-Ca/CRE as well as some or all of the upstream element (-72 to -30) [22]. In this case, virtually no binding was detected in extracts prepared from unstimulated neurons, whereas binding was robustly induced in extracts prepared from neurons that had been isotonically depolarized. The observation by Tao et al. that a radiolabeled BDNF-Ca/CRE bound nuclear factors equally well from either stimulated or unstimulated neurons is con-

sistent with known transactivation mechanisms for transcription factors that belong to the CREB family. CREB is thought to bind to DNA regulatory regions constitutively, but its ability to transactivate gene expression is strictly controlled through the phosphorylation of critical residues within its regulatory region, such as Ser-133.

Having shown that the BDNF-Ca/CRE could bind to CREB or a closely related CREB family member, both groups tested the effects of blocking CREB function on the ability of Ca^{2+} to induce BDNF transcription. To block CREB function, neurons were cotransfected with the BDNF reporter genes and one of three different dominant-interfering forms of CREB. The three domi-

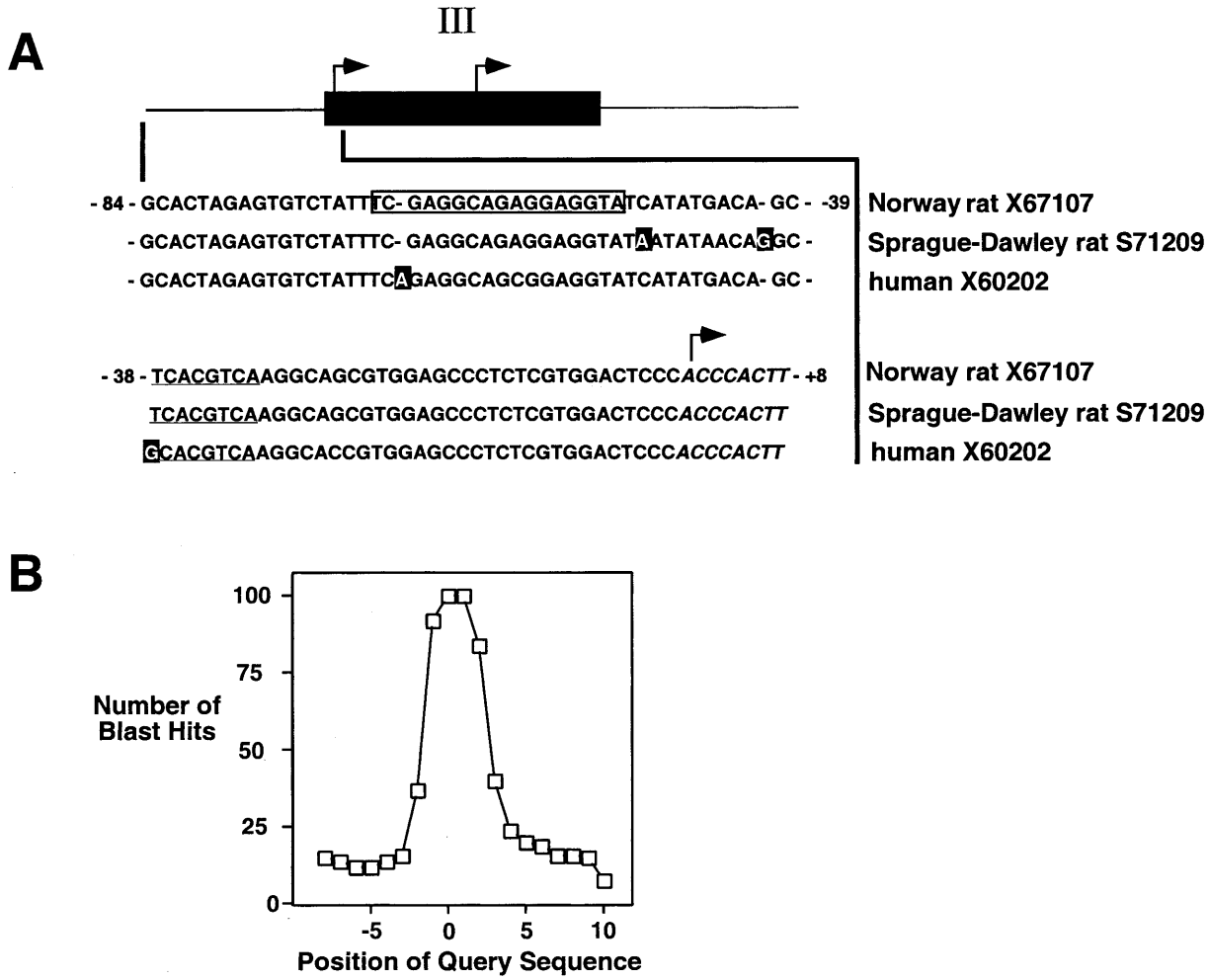

Figure 3. The BDNF exon III contains calcium response elements. (*A*) The cartoon depicts BDNF exon III (black rectangle) with its two transcription initiation sites indicated by arrows. Below the cartoon are shown three corresponding nucleotide sequences for the exon III promoter up to the first transcription initiation site (black arrow). The sequences come from two species of rat (Norway rat, accession number X67107; Sprague-Dawley rat S71209) and from human (accession number X60202) with one minor modification. The Sprague-Dawley sequence in the database contained an error, and the corrected sequence is shown. Otherwise, base-pair mismatches between the three sequences are shown as white letters against a black background. One sequence (underlined) homologous to the calcium and cyclic-AMP response element is required for calcium-induced BDNF transcription. Deletional and mutational analysis has shown that one or more additional calcium response elements ('upstream elements') are found 5′ to the BDNF Ca/CRE. (*B*) The region of the exon III promoter that is upstream of the BDNF Ca/CRE contains a sequence that is found within many other genes. Frames of 17 bp sequences from the BDNF upstream response element were sampled iteratively and compared to existing database sequences by BLAST analysis. The number of genes from the database that contains a homologous sequence (with less than 3 bp differences out of 17) is plotted versus the query sequence (the 17 bp portion of the upstream response element that was tested). The 17 base-pair sequence of the BDNF upstream response element that matched most commonly sequences from the database is indicated by a box in figure 3A. The boxed sequence represents query sequence position '0'; positions for other 17 bp query sequences are numbered relative to this sequence position.

nant-interfering forms of CREB differed in the mechanism by which they blocked CREB function. One form, CREBM1, can bind to Ca/CREs, but it contains a critical mutation (S133A) that prevents its phosphorylation and activation. CREBM1 probably blocks normal CREB function by preventing CREB or CREB family members from binding to Ca/CREs and mediating transcription. Another dominant-interfering CREB, KCREB, lacks a DNA binding domain and probably sequesters wild-type molecules of CREB or related family members by binding to them through its leucine zipper, thereby inhibiting their function. Recently, a third dominant-interfering CREB, ACREB, was designed. Similar to KCREB, ACREB sequesters CREB or closely related family members by specifically binding to them. However, because of special features of its design, ACREB preferentially sequesters a smaller subset of CREB family members such as CREB, ATF and CREM, and thus is potentially more specific. Cotransfection of either CREBM1, KCREB or ACREB together with the BDNF reporter gene inhibited depolarization-induced BDNF transcription. The fact that three different dominant-interfering CREBs have the same blocking effect strongly suggests that CREB or a very closely related family member mediates Ca^{2+}-induced BDNF transcription.

However, an important general criticism of reporter gene assays is that they may not faithfully recapitulate the complex regulation of the full promoter of the endogenous gene that they are designed to model. To test whether the Ca/CRE is important for Ca^{2+}-induced transcription of the endogenous BDNF gene, Tao et al. cotransfected CREBM1 and the fluorescent transfection marker, green fluorescent protein [21]. After fluorescent-activated cell sorting of the transfected neurons, Ca^{2+}-induced transcription of the endogenous BDNF gene was examined by RT-PCR in the purified population of transfected neurons. CREBM1 blocked depolarization-induced BDNF transcription, suggesting that CREB or a closely related family member is essential to mediate Ca^{2+}-induced transcription of the endogenous BDNF gene.

If CREB or a related family member was necessary, could CREB, by itself, mediate Ca^{2+}-dependent BDNF transcription? Deletion of the element upstream of the Ca/CRE blocked Ca^{2+}-dependent BDNF transcription, suggesting that CREB may not function alone. However, promoter deletions can affect neighboring sequences indirectly by disrupting CREB binding, so it remained possible that CREB could function independently of the upstream element. To define the role that CREB plays, the Ca/CRE within the BDNF reporter gene was replaced with the DNA sequence that binds the yeast transcription factor GAL4. Likewise, CREB was modified to replace its DNA binding domain with the corresponding GAL4 DNA binding domain from yeast (GAL4-CREB). Since the GAL4 DNA sequence is not found in mammalian cells, neither the modified GAL4 BDNF reporter gene nor GAL4-CREB is affected by or interacts with any known mammalian DNA sequences or proteins. As expected, when GAL4-CREB and the GAL4 BDNF reporter gene are cotransfected into neurons, Ca^{2+}-dependent transcription of the reporter gene is reconstituted. However, if GAL4-CREB is omitted or if a mutated form is substituted (GAL4-CREBM1), Ca^{2+}-dependent BDNF transcription is blocked. Together, the experiments show that CREB must be phosphorylated at ser-133 to mediate BDNF transcription. Importantly, if the upstream element was deleted (-83 to -48), GAL4-CREB failed to mediate Ca^{2+}-dependent BDNF transcription. Although the possibility that the deletion of the upstream element affected the ability of GAL4-CREB to bind to the reporter gene cannot be excluded, these results are consistent with the idea that CREB interacts in some way with proteins bound to the upstream element to cooperatively mediate Ca^{2+}-dependent BDNF transcription.

How is Ca^{2+} influx coupled to the phosphorylation and activation of CREB? Obvious candidate Ca^{2+}-activated kinases known to phosphorylate and activate CREB are the Ca^{2+}/calmodulin kinases (CaM kinase) I, II and IV. Shieh et al. noted by immunocytochemistry that CaM kinase II levels are low in the nucleus compared with the cytoplasm but that CaMKIV was present in neuronal nuclei [22]. Shieh et al. went on to show that constitutively active forms of CaMIV, when transfected into neurons, were able to activate BDNF transcription in the absence of extracellular Ca^{2+} influx. Conversely, the introduction of a kinase-inactive version of CaMKIV attenuated Ca^{2+}-evoked BDNF reporter gene expression consistent with the idea that CaMKIV is an important mediator of Ca^{2+}-dependent BDNF expression.

Summary and remaining questions

Together these studies have revealed an intracellular pathway by which the influx of extracellular Ca^{2+} regulates BDNF gene transcription. Ca^{2+} influx phosphorylates and activates CREB or closely related family members, which act through a Ca/CRE sequence within exon III of the BDNF gene. Together with proteins bound to another element within exon III, CREB or a closely related family member regulates BDNF gene transcription. One reason that these results are important is that CREB-dependent gene expression has been

Table 1. Some potential transcription factor binding sites within BDNF exon III (−84 to −46).

Transcription Factor	Direction	Sequence	LLH	LLH Density
Upstream response element				
IUF-1	reverse-complement	CAtTAGAG	12	1.5
CBF	normal	CACGTgAA	12	1.5
Sp1	reverse-complement	GAGGAGGgAT	16	1.6
C/EBPα	reverse-complement	TtTGGTAAT	14	1.556
Downstream response element				
ATF	normal	ACGTCA	12	2
CREB	reverse-complement	TgACGTCA	12	1.5

The 'direction' column refers to the orientation of the putative response element within the BDNF exon III promoter. The 'sequence' column shows the consensus nucleotide binding site for the identified transcription factor: uppercase letters indicate nucleotides that match exactly those in the BDNF exon III promoter; lowercase letters indicate mismatches. The 'LLH' and 'LLH density' columns are measures of the quality of the potential binding site within the BDNF exon III promoter. LLH is defined as the base 2 log of the sum over all binding site bases of the ratio M/P, where M is 1 if a match occurred and 0 otherwise, and P is the probability of a match assuming a uniform distribution of bases. LLH density is defined simply as the average LLH over the matched sequence: 2.0 is the maximum possible; −1 is the minimum. Abbreviations: IUF-1, insulin upstream factor 1; CBF, CCAAT binding factor; C/EBPα, CCAAT/enhancer-binding protein; ATF, activated transcription factor; CREB, cAMP response element-binding protein.

implicated in the late phase of synaptic plasticity, but until these studies, examples of CREB-regulated gene products that act at synapses had been unknown. These studies suggest that the BDNF gene could be a CREB target that encodes a protein, BDNF, known to regulate synaptic function. Although these studies provide a variety of new insights into the regulation of the BDNF gene, these studies have also generated new questions. What is the nature of the upstream element(s) that mediate Ca^{2+}-dependent BDNF transcription? Since Ca/CREs can be found in the other BDNF exons (I, II and IV), why is it that the Ca/CRE within exon III appears especially Ca^{2+}-responsive? Why are there so many BDNF splice variants if they all encode the same mature protein? What role do nontranscriptional mechanisms play in the interplay between Ca^{2+} and neurotrophin signaling. It is difficult to even speculate about answers to most of these questions; however, a few observations about the possible nature of the upstream element(s) can be made.

Although both studies have identified a similar region of the BDNF exon III promoter as being important, the specific sequence of the upstream response element and the transcription factor(s) that binds to it remain to be identified. No perfect binding site for known transcription factors is present within this upstream region. However, portions of the sequence can be found in other genes, raising the possibility that the sequence(s) could be a common response element(s) that has yet to be identified. On a BLAST search, the most well represented sequence within the upstream region is 5′-TCGAGGCAGAGGAGGTA-3′ (fig. 3A and B). A similar sequence was first identified as a potential Ca^{2+} response element within the gene for D28k calbindin, a calcium binding protein [31]. However, a similar sequence can also be found within the gene for PI-3 kinase [32], transthyretin (prealbumin) [33], the nervous system homeobox gene Sax-1 [34], insulin receptor substrate-2 (IRS-2) [35, 36], and the promoters of the gene for calcineurin A α [37] and angiotensin-converting enzyme [38]. Using the TRANSFAC analysis program to look for the presence of any known transcription factor binding sites failed to identify any perfect sequence matches [39]. If an error rate is allowed of one nucleotide mismatch in eight—roughly the deviation of the BDNF Ca/CRE from a consensus Ca/CRE—several transcription factor binding sites were identified (table 1). However, using this analysis Sp1 (record T00752) is the only transcription factor that is capable of binding to a portion of the common sequence 5′-TCGAGGCAGAGGAGGTA-3′ [39]. Sp1 is not known to be Ca^{2+}-responsive; rather, it is believed to drive the constitutive expression of genes. Thus, the precise sequence within the upstream response element that cooperates with CREB or related family members to mediate Ca^{2+}-dependent BDNF expression remains to be defined. The fact that the upstream sequence within exon III bears no homology to any known Ca^{2+} response elements suggests the intriguing possibility that the Ca^{2+} response element(s) and the transcription factor(s) that binds to it could be new. Further work could lead to the identification of a completely new pathway of Ca^{2+}-dependent gene expression that works with CREB to mediate synaptic plasticity.

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