Published in final edited form as:

Mol Cell Neurosci. 2007 July ; 35(3): 447–455.

Ca²⁺, CREB and Krüppel: A novel KLF7-binding element conserved in mouse and human *TRKB* promoters is required for CREB-dependent transcription

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Abstract

Brain-derived neurotrophic factor (BDNF) signaling through its receptor, trkB, is essential for the proper development and function of the nervous system. Here we identify a novel regulatory element designated *TCaRE3* (*TRKB* Ca²⁺ Response Element 3) required for CREB-dependent *TRKB* transcription in neurons. *TCaRE3*-inactivating mutations abolished both Ca²⁺- and cAMP-stimulated *TRKB* expression, despite the presence of upstream *CREs*. *TCaRE3* mutations also reduced basal expression by at least 80%. Electrophoretic mobility shift assays revealed the presence of a neuronal nuclear factor able to bind *TCaRE3* in a sequence-specific manner and we have identified krüppel-like factor 7 (KLF7) as a candidate *TCaRE3* transcription factor. Importantly, despite limited overall sequence homology between the promoter regions of the human and mouse *TRKB* genes, *TCaRE3* exhibits 100% sequence identity. Mutation analysis of the human *TRKB* promoter region demonstrated that the role of *TCaRE3* is also conserved, suggesting that the functional interaction between CREB bound to the *CREs* and KLF7 bound to *TCaRE3* is essential for the proper regulation of *TRKB* in neurons.

The neurotrophin, BDNF, signals through its receptor trkB to mediate neuron survival and differentiation, neurite outgrowth, axon guidance, and activity-dependent synaptic plasticity (for reviews, see Huang and Reichardt, 2001; 2003). Studies of mice with forebrain-targeted deletions in the genes coding for BDNF or trkB displayed defects in neuron survival, long term potentiation, and learning and memory (Korte et al., 1996; Patterson et al., 1996; Xu et al., 2000). Abnormal BDNF/trkB signaling has been linked to neurological and psychiatric disorders such as Alzheimer's and Parkinson's diseases (Ferrer et al., 1999; Allen et al., 1999; Murer et al., 2001; Holsinger et al., 2000; Fumagalli et al., 2006a,b), as well as depression and schizophrenia (Nestler et al., 2002; Angelucci et al., 2005; Hashimoto et al., 2004;).

The expression of BDNF in cultured cortical neurons is upregulated by depolarization (Ghosh et al., 1994) due to the activation of Ca²⁺-dependent regulatory promoter elements in *BDNF*, the gene encoding BDNF (Tao et al., 1998; Shieh et al., 1998). Similarly, we demonstrated that depolarization-induced Ca²⁺ signaling specifically stimulated expression of catalytically-active, full-length trkB without affecting the inactive, truncated (T1) isoform (Kingsbury et al., 2003). The increased full-length trkB was readily auto-phosphorylated in response to

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BDNF, indicating that the newly synthesized receptors were at the plasma membrane and functionally active. Ca^{2+} -dependent up-regulation of full-length trkB was accompanied by preferential stimulation of the downstream *TRKB* promoter, P2 (Kingsbury et al., 2003). Thus, Ca^{2+} -dependent activation of *TRKB* P2 can increase neuronal responsiveness to BDNF by preferentially upregulating expression of the active isoform trkB. This coordinated regulation of both ligand (BDNF) and receptor (trkB) expression by Ca^{2+} -dependent activation of gene transcription may lead to synergistic enhancement of the strength of BDNF trkB signaling in response to neuronal activity (Nagappan and Lu, 2005).

Stimulation of *BDNF* and *TRKB* transcription by Ca^{2+} depends on a Ca^{2+} /cAMP response element binding protein (CREB) family member acting via a *CRE* in *BDNF* promoter III (Tao et al., 1998; Shieh et al., 1998) or a pair of tandem *CREs* in *TRKB* P2 (Kingsbury et al., 2003). Ca^{2+} -responsive promoters, including *BDNF* PIII (Chen et al., 2003), frequently possess additional regulatory elements that modulate the transcriptional responses of the *CRE* (e.g. Robertson, 1995; West et al., 2001). It is this functional interaction between multiple promoter elements that allows ubiquitous second messengers such as Ca^{2+} and cAMP to be translated into gene- and cell-specific transcriptional responses. We therefore investigated the possibility that additional regulatory elements within the *TRKB* promoter shape the response of *TRKB* P2 to Ca^{2+} signaling. Here we report that a novel DNA element, designated *TCaRE3*, is required for *TRKB* P2 to respond to Ca^{2+} signals, and identify krüppel-like factor 7 (KLF7) as a candidate transcription factor for *TCaRE3*. We also show that the sequence and function of *TCaRE3* are conserved in the human *TRKB* promoter.

RESULTS

Elements downstream of the P2 transcription initiation site modulate Ca^{2+} -dependent *TRKB* P2 expression

Deletion analysis of *TRKB* P2 revealed the presence of additional regulatory sites important for Ca^{2+} -dependent P2 expression located downstream from the P2 transcription initiation site. For the *TRKB* promoter, the translation initiation site has been numbered +1, placing the P2 transcription initiation site at -448 (Baretino et al., 1999). Beginning with the -944/+2 *TRKB*-luciferase construct, which we previously showed responds to depolarization-induced Ca^{2+} influx with a 2-fold increase in luciferase activity (Kingsbury et al., 2003), we generated a series of P2 *TRKB*-luciferase reporter constructs by progressive 3' truncation (Figure 1). Each reporter was transiently transfected into mouse cortical neurons and assayed for Ca^{2+} responsiveness following depolarization with elevated KCl (50 mM). Neurons transfected with -944/+2, -944/-77 or -944/-162 *TRKB*-luciferase constructs all responded with a 2-fold stimulation following depolarization, suggesting that the sequences downstream from bp -162 do not participate in the Ca^{2+} responsiveness of *TRKB* P2. Constructs -944/-241, -944/-325 and -944/-339 exhibited a 3-fold increase in reporter activity in response to depolarization. This increased stimulation suggests that there is a Ca^{2+} -dependent inhibitory element located between -162 and -241; we have not characterized this element further. As previously reported for -944/+2, removal of extracellular Ca^{2+} with EGTA blocked all depolarization-induced expression of these promoter constructs (data not shown). Further truncation to generate -944/-363, -944/-389 and -944/-444 resulted in constructs that were no longer stimulated in response to KCl-induced Ca^{2+} signaling. Thus, removal of the sequences between -339 and -363 eliminated Ca^{2+} -stimulated P2 expression. Since all of these constructs contain the tandem *CREs* located between -922 and -904, these findings indicate that both the *CREs* and an additional site located downstream from the transcription start site (-448) are required for Ca^{2+} -stimulated P2 expression. In addition to the loss of Ca^{2+} -stimulated expression, the -944/-363, -944/-389 and -944/-444 constructs also exhibited a 92–96% reduction in basal

TRKB P2-luciferase (* in Fig. 1), as compared to the original -944/+2 *TRKB*-luciferase construct (see below). In contrast, -944/-325 and -944/-339 showed wild type basal activity.

In light of the multiple elements involved in Ca^{2+} stimulated *TRKB* P2 expression, we have adopted a nomenclature similar to that used for Ca^{2+} response elements (*CaREs*) in rat *BDNF* promoter III (West et al., 2001) referring to *TRKB* promoter elements as *TCaREs* (*TRKB* Ca^{2+} Response Elements). Using this nomenclature, the new element located between -363 and -339 is designated *TCaRE3* (*TRKB* Ca^{2+} response element #3) and the pair of tandem *CREs* between -926 and -904 is now designated *TCaRE2*.

***TCaRE3* is a novel element enabling Ca^{2+} -dependent gene expression**

The sequence of the region from -363 to -339 required for *TCaRE3* function is shown in Figure 2. Using a scanning mutagenesis approach, a series of -944/-325 *TRKB*-luciferase constructs harboring the indicated point mutations was generated in order to more precisely delineate the location of *TCaRE3*. The basepair substitutions introduced in M1 were the same mutations introduced into -944/+2 to generate deletion construct -944/-363. In contrast to the -944/-363 deletion mutation, M1 had no effect on Ca^{2+} -dependent P2 expression, indicating that truncation to -363 failed to respond due to the removal of downstream sequences (Fig. 2, M1), rather than introduction of mutations into a critical site. Mutations that were found to disrupt Ca^{2+} responsiveness of *TRKB* P2 mapped the critical sequence of *TCaRE3* to 16 basepairs (Fig. 2, bold). Analysis of this sequence using TESS (Transcription Element Search Software, Univ. of Pennsylvania, Philadelphia, PA; URL: <http://www.cbil.upenn.edu/tess>), failed to identify a known Ca^{2+} response element, suggesting it contains a novel promoter element required for *TRKB* P2 Ca^{2+} -stimulated expression.

Mutation of *TCaRE3* also reduced basal *TRKB* expression. Figure 3 (left) shows the effect of mutation M4 (c.f., Fig. 2) on *TRKB* P2 luciferase activity. Note that, in addition to blocking Ca^{2+} -stimulated expression, introduction of M4 into the -944/-325 P2-luciferase construct decreased activity to less than 5% of wild type expression in unstimulated samples. All of the other *TCaRE3* mutations that abolished Ca^{2+} -dependent P2 stimulation (M3-M7) also reduced basal *TRKB* expression by 80-95% (not shown). Mutations of the *CREs*, located within *TCaRE2*, also reduced basal *TRKB* transcription (see below).

Analysis of the sequences flanking *TCaRE3* using TESS revealed a potential TBP binding site (GTCTATTAG), located between bp -379 and -371 (c.f., Fig. 8). Since TBP functions to enable transcription of numerous promoters (Lee and Young, 1998), we examined the possibility that the TBP site is also involved in *TRKB* P2 expression. Mutations (GTCTAAGCT) were introduced into the -944/-325 P2-luciferase construct to disrupt the candidate TBP site. As observed for mutations in *TCaRE3*, mutation of the TBP site resulted in a 94% reduction in basal *TRKB* P2-luciferase expression (Fig. 3, right); in contrast to *TCaRE3* mutations, however, the construct containing the TBP mutations still exhibited a 3.1-fold stimulation in response to elevated KCl, similar to the 3.2-fold increase observed in the -944/-325 wild type construct (Fig 1). Thus, although the TBP site is required for basal P2 transcription, it is not essential for the ability of Ca^{2+} to stimulate P2 activity.

***TCaRE3* is also required for cAMP responsiveness of *TRKB* P2**

We (Kingsbury et al., 2003) and others (Deogracias et al., 2004) have shown that P2 *TRKB*-luciferase reporters can also be stimulated cAMP signaling induced by forskolin. The requirement for *TCaRE3* in mediating P2 induction following forskolin treatment was tested using the *TRKB*-luciferase constructs generated to map *TCaRE3*. *TRKB* reporters lacking a functional *TCaRE3* failed to respond to cAMP signaling (Fig. 4), indicating that *TCaRE3* is

required for both Ca^{2+} and cAMP stimulation of *TRKB* P2. Thus, CREB cannot stimulate *TRKB* P2 via the upstream tandem *CREs* in the absence of a functional *TCaRE3*.

***TCaRE3* functions as a DNA element regulating *TRKB* P2 transcription**

Previous studies (Baretino et al., 1999) demonstrated that P2 generates a single transcript, which does not undergo splicing in the 5' UTR. Since *TCaRE3* is located in this 5' UTR sequence of the mRNA, our results could be explained if *TCaRE3* acted to promote Ca^{2+} -dependent RNA stability or translation, rather than to mediate Ca^{2+} -stimulated transcription. To distinguish between such transcriptional and post-transcriptional effects, the ability of *TCaRE3* to mediate Ca^{2+} -stimulated P2 *TRKB*-luciferase activity was analyzed in the presence of the transcription inhibitor actinomycin D (actD). Cortical neurons transiently transfected with -944/+2, -944/-325 or -944/-444 were depolarized with 50 mM KCl in either the presence or absence of actD (Fig. 5A). Ca^{2+} -dependent stimulation of P2 was blocked by actD in all constructs containing *TCaRE3* including the -944/-325 *TRKB* P2-luciferase construct, which lacks the downstream Ca^{2+} -responsive inhibitory element between -241 and -162 (Fig. 1). This demonstrates that a post-transcriptional role for *TCaRE3* was not being masked by the presence of this additional inhibitory element and that *TCaRE3* acts by regulating transcription. For each of the three constructs, there was a similar, minimal effect of actD on basal P2-luciferase activity over the 5 hour time course of the experiment, suggesting that the absence of *TCaRE3* did not increase RNA turnover.

In order to provide further evidence that *TCaRE3* functions as a DNA element, we tested the effect of *CRE* mutations on the ability of Ca^{2+} to stimulate the -944/-325 *TRKB* luciferase construct. We previously showed that Ca^{2+} -dependent transcription of *TRKB* P2 -944/+2 was abolished by introduction point mutations in either *CRE* in *TCaRE2* (Kingsbury et al., 2003). Mutation of either *CRE* abolished Ca^{2+} -dependent stimulation of *TRKB* expression, despite the continued presence of *TCaRE3* (Fig. 5B). Both *TCaRE2* mutations also reduced basal *TRKB* transcription by 70–80%, similar to what was observed with *TCaRE3* mutations (c.f., Fig. 3). The absence of any residual Ca^{2+} -dependent increase further supports the conclusion that *TCaRE3* functions at the level of DNA transcription, rather than RNA stability or translation, since any effect of *TCaRE3* at the level of *TRKB* RNA should still be functional, independently of the transcriptional blockade due to mutation of the *CREs*.

Neurons contain an endogenous transcription factor for *TCaRE3*

If *TCaRE3* functions as a promoter element, DNA sequences containing *TCaRE3* should interact with endogenous transcription factors. Nuclear extracts were prepared from cortical neurons that were either left untreated, or depolarized with high KCl for 30 minutes. Nuclear extracts were then subjected to electrophoretic mobility shift assays (EMSAs) with P-32 labeled oligonucleotide sequences containing either wildtype or mutant *TCaRE3*. The wild type sequence used is indicated in Figure 2 (bold). In the presence of nuclear extract, the wild type *TCaRE3*-containing probe underwent a mobility shift, resulting in a slower migrating band (arrow, Fig. 6A, lanes 1–2). The shift was observed in extracts generated from both control and depolarized neurons, suggesting that depolarization-induced Ca^{2+} signaling is not essential for the interaction of *TCaRE3* with its binding factor. Mutations that disrupt *TCaRE3* function in *TRKB*-luciferase reporter assays (Fig. 2) inhibited the mobility shift (Fig. 6A, lanes 3–4). Thus, a factor in neuronal nuclei interacts with the *TCaRE3* sequence in a sequence-dependent manner.

KLF7 binds *TCaRE3* *in vitro*

In order to identify *TCaRE3*-interacting factors, we performed a yeast one-hybrid (Y1H) screen against a human fetal brain library using a construct containing three tandem copies of *TCaRE3* as bait. We identified krüppel-like factor 7 (KLF7) as a factor interacting with

TCaRE3 in Y1H screens. Cultured mouse cortical neurons contain KLF7 mRNA as determined by PCR (Fig. 6E; see below). The *in vitro* interaction of KLF7 with *TCaRE3* was investigated using recombinant KLF7. Purified GST-KLF7c, which consists of the DNA binding domain of KLF7 fused to GST (Lei et al., 2001), was examined for its ability to bind *TCaRE3* in electrophoretic mobility shift assays (Fig 6B). In the presence of GST-KLF7, the wild type *TCaRE3* probe exhibited reduced mobility. GST alone had no effect on probe mobility. Introduction of mutations shown to inactivate *TCaRE3* inhibited the appearance of this slower migrating band, indicating that the interaction with GST-KLF7c required intact *TCaRE3*.

KLF7 can bind to the chromosomal *TRKB* promoter

The ability of KLF7 to interact with the endogenous *TRKB* promoter was assayed using chromatin immunoprecipitation (ChIP). Since specific antibodies to KLF7 are not available, HEK293T or PC12 cells were transiently transfected with either KLF7-myc expression vector or empty control vector (c.f., Lei et al., 2005). Three days after transfection, cells were subjected to formaldehyde cross-linking and the nuclear chromatin was harvested and processed for ChIP. Anti-myc antibody was used to immunoprecipitate the KLF7. Following immunoprecipitation, PCR amplification of the region surrounding *TCaRE3* was conducted to test for the presence of *TRKB* P2. In cells transfected with KLF7-myc, anti-myc antibody, but not IgG, immunoprecipitated *TCaRE3*-containing *TRKB* chromosomal DNA (Fig. 6C). In contrast, anti-myc-immunoprecipitates from cells transfected with empty vector were not enriched for *TRKB* P2 promoter DNA. Thus, KLF7 can interact with the chromosomal *TRKB* promoter.

Dominant-negative KLF7 inhibits Ca^{2+} -dependent *TRKB* P2 expression

In order to examine the role of KLF7 in regulating *TCaRE3*, we generated a dominant-negative KLF7 allele (dn-KLF7) by deleting the activation domain of KLF7. The effect of dn-KLF7 on Ca^{2+} -stimulated *TRKB* P2 expression was analyzed by co-transfecting the dn-KLF7 expression vector with the -944/-325 *TRKB*-luciferase vector into cortical neurons. Ca^{2+} -stimulated expression of P2 was inhibited by the presence of dn-KLF7 (Fig. 6D). In contrast, co-transfection with empty vector had no effect on *TRKB* P2 transcription. We did not observe a significant decrease in basal P2-luciferase expression in the presence of dn-KLF7.

KLF7 and KLF6 mRNAs are expressed in cultured mouse cortical neurons

We used RT-PCR to confirm that KLF7 message is expressed in cultured mouse cortical neurons and to test for the presence of the closely-related KLF6. KLF7 and KLF6 are not only highly homologous in their DNA binding domain, but they also share a nearly identical stretch of 47 amino acids sequence at their amino terminus, required for transcriptional activation (Matsumoto et al., 1998). RNA was subjected to reverse-transcription followed by PCR using either KLF7 or KLF6-specific oligonucleotide primers. Both KLF7 and KLF6 transcripts were readily detected (Fig. 6E). The PCR products were absent from samples not treated with reverse transcriptase, demonstrating that the amplification of the two KLF cDNAs was due to the presence of RNA transcripts and not contaminating genomic DNA.

TCaRE3 is a conserved promoter element

Comparison of the genomic sequence of *TRKB* P2 (-944 to +2) with 1500 bp of the human *TRKB* (h*TRKB*; *NTRK2*) promoter region revealed only five blocks of high sequence conservation from mouse to human. As illustrated in Fig 7A, *TCaRE2* (CTGCGTCAGcccTCACGTCA) is located within a block of 60 bp that is identical between mouse and human *TRKB*. Several additional domains of 46, 99, 37, and 28 bp with 77–93% conservation are also present in the h*TRKB* promoter region. Within one of these, the sequence we have identified as *TCaRE3* is 100% conserved (Fig. 7A). In the mouse and the human

promoters, *TCaRE2* and *TCaRE3* are separated by 545 and 634 bases, respectively. To test the ability of the *hTRKB* promoter to respond Ca^{2+} , 1100 bp of the human promoter encompassing *TCaRE2* and *TCaRE3* were cloned into pGL3Basic to generate an *hTRKB*-luciferase reporter gene. Transient transfection of *hTRKB*-luciferase into mouse cortical neurons demonstrated that *hTRKB* could also be stimulated by either depolarization-induced Ca^{2+} signaling or forskolin-induced cAMP signaling (Fig 7B). Introduction of the *TCaRE3* mutation, M4, inhibited both Ca^{2+} -stimulated and cAMP-stimulated *hTRKB*-luciferase expression. Interestingly, mutation of *TCaRE3* also reduced basal *hTRKB*-dependent luciferase activity by approximately 80%. Thus, both the sequence and function of *TCaRE3* in *TRKB* expression are conserved in the human gene.

Requirement for *TCaRE3* in Ca^{2+} -stimulated *TRKB* P2 expression is cell type-specific

The requirement for *TCaRE3* to enable Ca^{2+} -dependent *TRKB* P2 expression was tested in several neuronal and non-neuronal cell types. Cells were transiently transfected with either wild type or mutated (M6 c.f., Fig. 2) -944/-325 luciferase reporter constructs and assayed for the ability of depolarization-induced Ca^{2+} signaling to stimulate reporter activity. Our results revealed that both the responsiveness of *TRKB* P2 to Ca^{2+} and the requirement for *TCaRE3* to mediate Ca^{2+} -dependent expression are cell-type dependent. The results of these analyses are summarized in Table 1. Depolarization of primary E18 rat cortical neurons and PC12 cells stimulated P2 to the same extent as in primary E16 mouse neurons and this stimulation was abolished by mutation of *TCaRE3*. Treatment of human embryonic kidney (HEK293) cells with 2 μM ionomycin to elevate $[\text{Ca}^{2+}]$, stimulated both wild-type and mutant (M6; c.f., Fig. 2) *TRKB* P2 expression. In contrast, neither mouse cortical astrocytes nor the rat insulin secreting cell line, RIN-1046, exhibited any stimulation of *TRKB* P2 in response to ionomycin or KCl, respectively. Interestingly, however, mutation of *TCaRE3* reduced basal *TRKB* P2-luciferase expression in all cell types (Table 1).

DISCUSSION

CREB is a ubiquitous regulator of gene expression, with thousands of mammalian genes containing one or more *CREs* in their promoters (Zhang et al., 2005). The response of a particular *CRE*-containing promoter to a Ca^{2+} or cAMP signal is a reflection of the functional interactions among promoter elements as determined by both developmental and cell-type specific expression of the corresponding transcriptional modulators (Mayr and Montminy, 2001). How the activation of CREB by specific intracellular signals or in different cell types translates to distinct gene activation patterns remains largely unknown and will require the systematic analysis of CREB-dependent activation of many genes in order to identify common themes (Conkright et al., 2003). Although the promoters of a number of CREB-dependent genes, including *cFos* and *BDNF*, have been shown to contain additional regulatory elements that impose specificity on CREB-mediated responses, our understanding of the *TRKB* promoter elements that function to shape the transcriptional response to Ca^{2+} and cAMP has been limited to the role of the tandem *CREs* (*TCaRE2*) (Kingsbury et al., 2003; Deogracias et al., 2004).

Here we report the identification of a novel DNA element, designated *TCaRE3*, that is required for the CREB-dependent stimulation of *TRKB* P2 transcription by Ca^{2+} and cAMP. Mutations of *TCaRE3* abolished depolarization-induced P2 reporter gene expression despite the continued presence of the pair of tandem *CREs* in *TCaRE2* (Figs. 1, 2), demonstrating that the tandem *CREs* (*TCaRE2*) are not sufficient to promote CREB-dependent activation of *TRKB* P2 by either Ca^{2+} (Fig. 1, 2) or cAMP (Fig. 4). Using both EMSA (Fig. 6B) and ChIP (Fig. 6C), we further show that KLF7, which is expressed in the neurons (Fig. 6E), can interact with *TCaRE3* and that a dominant-negative allele of KLF7 inhibits Ca^{2+} -dependent *TRKB* P2 expression (6D), suggesting that KLF7 functionally interacts with CREB to promote *TRKB* P2

transcription. Although this is the first report implicating KLF7 in Ca^{2+} -dependent transcription, KLF4 has previously been demonstrated to interact with CREB to regulate bradykinin B2 receptor expression during nephron differentiation (Saifudeen et al., 2005), suggesting that there may be functional interactions between CREB and multiple members of the KLF family during development.

KLF7, the mouse homolog of UKLF, belongs to the krüppel-like factor family of transcription factors (Turner and Crossley, 1999; Bieker 2001). KLF7 has been proposed to play a role in neurogenesis due to its early expression in postmitotic neurons during differentiation of the cerebral cortex (Laub et al., 2001). KLF7 has also been shown to regulate the expression of *TRKA*, the gene coding for the NGF receptor, trkA (Lei et al., 2001; Lei et al., 2005), although this regulation is mediated by a different regulatory element (*Ikaros2*) in the *TRKA* promoter. There is no sequence homology between *TCaRE3* and *Ikaros2*, moreover, mutation of a putative *Ikaros2* site located in *TRKB* P2 had no effect on P2 reporter gene expression in our assays (data not shown). Thus, despite differences in mechanism, these results reveal that KLF7 may play a general role in coordinately regulating the expression of different classes of trk receptors in the nervous system.

KLF7 knockout mice have recently been generated and reported to exhibit reduced trkA expression in dorsal root ganglion neurons (Lei et al., 2005); trkB expression levels in brain were not reported. It will be interesting to determine if trkB is altered in the KLF7 knockout brain and whether changes in trkB expression contribute to the reduced cortical thickness and decreased dendritic arborization observed by Laub et al. (2005). Future investigation of the role of KLF7 in regulating trkB expression *in vivo* will need to take into account the fact that *TRKB* transcription can be initiated from two distinct promoters (Baretino et al., 1999; Kingsbury et al., 2003) and that the presence of KLF6 may compensate for the loss of KLF7.

A summary of our current understanding of the promoter elements involved in shaping the Ca^{2+} responsiveness of *TRKB* P2 is shown in Figure 8. Since *TCaRE2* and *TCaRE3* are separated by more than 500 bp, the functional interaction we observed indicates that DNA looping would be necessary to promote a physical interaction between their corresponding transcription factors. In addition to *TCaRE2* (tandem *CREs*) and *TCaRE3*, *TRKB* P2 contains two other novel elements that can either enhance (*TCaRE1*, Kingsbury et al., 2003) or repress (*TCaRE4*; c.f., Fig. 1A) the amplitude of the promoter response, but are not absolutely required for Ca^{2+} - and cAMP-dependent transcription.

Mutation of either *TCaRE3* or the TBP site dramatically suppressed basal *TRKB* P2 expression (Fig. 3). *TCaRE2* mutations also suppressed basal transcription. This finding is not unique; a role for *CREs* in basal transcriptional activity was previously reported (Quinn et al., 1995). One possible explanation for these findings is that CREB, acting at *TCaRE2*, KLF7, acting at *TCaRE3*, and TBP all contribute to the formation of the basal *TRKB* P2 transcription initiation complex via Ca^{2+} -independent mechanisms, while CREB-KLF7 interactions, but not TBP, are required for Ca^{2+} -dependent transcription. An alternative explanation is that resting Ca^{2+} levels in unstimulated neurons may be sufficient to partially activate *TRKB* expression. In either case, the requirement for *TCaRE3* for basal *TRKB* transcription may explain triiodo-thyronine (T3)-dependent inhibition of *TRKB* reported in rat neuroblastoma cells (Pombo et al., 2000). *TCaRE3* encompasses one of four potential T3 half sites (TGTCCT) required for T3-dependent inhibition *TRKB*; activation of T3 receptors may inhibit *TRKB* expression by displacing KLF7.

The requirement for interactions among promoter elements provides opportunities for the integration of multiple intracellular signaling pathways and allows diversity in cellular responses to identical stimuli (Maniatis et al., 1987). The importance of the functional interaction between the *CREs*, located within *TCaRE2*, and *TCaRE3* is highlighted by the fact

that, despite only limited domains of homology between the mouse and human *TRKB* promoters, the sequences of *TCaRE2* and *TCaRE3* are identical in the two species (Fig. 7A). Mutation of *TCaRE3* in the human promoter abolished Ca^{2+} -stimulated *hTRKB* transcription and caused similar reductions in basal activity (Fig. 7B), demonstrating that the role of *TCaRE3* in *TRKB* expression is conserved in the human gene. This observation suggests that the interaction of CREB with a *TCaRE3* transcription factor such as KLF7 (or KLF6) is essential for the proper regulation of *TRKB* expression in neurons. Cell-specific regulation of *TCaRE3*, as could occur via expression of distinct KLF family members, may underlie the range of *TRKB* P2 responses reported in Table 1, which include the failure of Ca^{2+} to activate *TRKB* P2 in astrocytes and the activation of P2 by Ca^{2+} signaling in HEK cells even in the absence of *TCaRE3*.

EXPERIMENTAL METHODS

Cell culture and Transfection

Cortical neuron cultures were isolated from embryonic day 16 (E16) mouse embryos and plated on laminin-coated dishes at a density of $5\text{--}6 \times 10^5$ /well in 24-well plates for luciferase assays or 1.5×10^7 cells/100-mm dish for nuclear extract preparation. Neuron cultures were maintained in Neurobasal™ medium supplemented with 2% B27 (Invitrogen, Carlsbad, CA), 1 mM glutamine, and penicillin/streptomycin and incubated in 5% CO_2 at 37°C. Neurons were transiently transfected 3–5 days after plating using a Ca^{2+} -phosphate protocol (Xia et al, 1996). Typical transfection rates with this protocol range from 5–10% of the neurons.

Luciferase Assays

Luciferase assays were conducted as previously described (Kingsbury, et al., 2003). Individual luciferase measurements were normalized to co-transfected TK-Renilla values to correct for sample-to-sample variation in transfection efficiency. One day after transfection with reporter constructs, cells were stimulated for 5 hours by the addition of 50 mM KCl, 40 μM forskolin or 2 μM ionomycin (Sigma, St. Louis, MO). When indicated, 40 μM actD (Sigma) was added 30 min prior to stimulation.

Plasmids

The *TRKB* P2 –944/+2 luciferase reporter was previously described (Kingsbury, et al., 2003). Constructs –944/–77 and –944/–325 were generated by digesting –944/+2 with HindIII/StuI or HindIII/EV, respectively, treating with Klenow DNA polymerase to generate blunt ends and then re-ligating. All other truncations were produced by introduction of a HindIII site using QuickChange site-directed mutagenesis (Stratagene, La Jolla, CA) followed by HindIII digestion and re-ligation. The sequences of sense strand primers used were: –162: GGATCCTGAGCAGGTTG; –241: GGACATAGTCCTATAC; –339: GTCCCTCAGCCCTGAAGCTTGACCGTATCGATAT; 389: TCCAGTTGGTGAAGCTTGGGGTCTATTAGTC; 444: GGATCCAGAAGAATGTCTGAGTG. Scanning mutagenesis of –944/–325 was also conducted using Quickchange site-directed mutagenesis. The corresponding sense oligonucleotide sequences were: M1: CTATTAGTCTCATCCAAGCTTGTTTGTCTCAGC; M2: TCTATTAGTCTCATCGAATTCTGTTTGTCTCAGC; M3: ATTAGTCTCATCCATGCCGTTTGTCTCA-GCCC; M4: ATTAGTCTCATCCATGTAAGCTTGCTCTCAGCCCTG; M5: TCATCCATGTCTGTGAAGCTTCAGCCCTGAGGTGCG; M6: ATCCATGTCTGTTTGTCCGGAGCCCTGAGGTGCG; M7: ATGTCTGTTTGTCTCCGGCCTGAGGTGCGCAC; M8: GTCTGTTTGTCTCAGATCTGAGGTGCGCACC; M9:

TGTTTGTCTCAGCCGGGAGGTGCGCACCGAT; TBP Mutation:
GTGTGTGGGGTCTAAGCTTCTCATCCATGTCTG. A dominant-negative KLF7 allele was generated by amplifying the DNA-binding domain using the primers AAGCTTCTAGACCCCTTGCT and CTCGAGTTAGATATGTCTCTT and cloning into pcDNA3.1 EcoR1 to XhoI.

Electrophoretic Mobility Shift Assays (EMSAs)

Nuclear cortical neuron extracts were generated and EMSA conducted as previously described (Andrews and Faller, 1991). GST or GST-KLF7c were purified from *E. coli*. Single stranded oligonucleotides (−339 to −361 with and without mutations) were radiolabeled with P-32 using T4 kinase (Invitrogen) prior to annealing. Binding reactions were incubated at room temperature for 20 min prior to separation on a 6% TBE gel and autoradiography.

Yeast One-Hybrid (Y1H) Assay

Yeast one-hybrid assay was conducted using Matchmaker One-hybrid system from Clontech according to manufacturer's instructions. Three tandem copies of *TCaRE3* were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA):

GGATCCGAATTCGTTTGTCTCAGCCCTGAGGTGCGGTTTGTCTCAGCCCTGAG GTGCGGTTTGTCTCAGCCCTGAGGTG CGTCTAGATCT and cloned into target vectors using EcoRI and XbaI. Yeast were screened for His⁺ colonies following transformation with human fetal brain library (Clontech).

KLF7 and KLF6 PCR

KLF7 and KLF6 mRNAs were detected by reverse transcriptase-PCR using the following primer pairs: KLF7: AAGAGGGTCCATCGCTGTCAGTTT and ACCTGTGTGTTTCTGTAGTGCCT; KLF6: TTTCTGCTCGGACTCCTGATCGTT and AAGATAGCGTTCCAACCTCCAGGCA. When reverse transcriptase was omitted from the reaction (−RT), no PCR product was observed demonstrating that the assay was not detecting genomic DNA.

Chromatin Immunoprecipitation (ChIP)

The interaction between epitope-tagged KLF7 and endogenous *TCaRE3* was examined as described by Lei et al. (2005) for the KLF7-*Ikaros*2 interaction. HEK-293T or PC12 cells were transfected with either KLF7-myc or empty vector using Lipofectamine 2000 three days prior to harvesting for ChIP assays. Cells were cross-linked with formaldehyde and chromatin extracted from isolated nuclei was digested with mung bean nuclease to an average size of approximately 300 base pairs. Immunoprecipitations were conducted using a kit from Upstate Biotechnology (Lake Placid, NY), with Protein G-agarose (Upstate) substituted for Protein A-agarose. Anti-myc (9E10) was purchased from Roche Applied Science (Indianapolis, IN). Reverse crosslinking was conducted overnight at 65°C. DNA was extracted with phenol-chloroform, ethanol-precipitated and re-suspended in water for PCR analysis. Oligonucleotide primers for PCR of the *TCaRE3* domain were: GCTCAGTC-TTACGCGTGTCTGTTT and CGGCATACGAACCGCTTTACA.

Acknowledgements

We thank L.L. Bambrick, S.G. Dorsey, D.D. Ginty and P.D. Murray for helpful discussions. We thank C.D. Roby and S. Connolly for technical support. Funding provided by grants NIH R01NS40492, NIH R01NS048095 and DOD W81XWH-04-1-0176 (BKK) and by NIH training grant T32 NS07375 (TJK). TJK is a BIRCWH Scholar supported by NICHD/ORWH/NIDDK (NICHD K12 HD43489).

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Figure 1. Identification of a domain required for Ca^{2+} -dependent *TRKB* P2 expression

TRKB P2-luciferase reporter genes containing progressive 3' truncations (left) were transiently transfected into cortical neurons. Two days later, cultures were treated with 50 mM KCl for 5 hours prior to harvesting for luciferase assays (right). Individual luciferase measurements were normalized to co-transfected TK-Renilla values to correct for sample-to-sample variation in transfection efficiency. For each construct, Renilla-normalized luciferase measurements from depolarized cells are presented relative to those of control, unstimulated cells. Bars show mean $\pm$ sem (N=4). Deletion of bp -162 to -241 increased the magnitude of Ca^{2+} stimulation, suggesting the presence of an inhibitory element within that sequence. Deletion of bp -339 to -363 abolished Ca^{2+} -stimulation of P2 luciferase activity, defining the domain of *TCaRE3* (*TRKB* Ca^{2+} -response element 3). * denotes constructs exhibiting reduced basal activity.

Figure 2. Mutational analysis of *TCaRE3*

The -944/-325 *TRKB* P2-luciferase construct was subjected to scanning mutagenesis from -399 to -363 to map the nucleotides essential for *TCaRE3* function. Cortical neurons were transiently transfected with corresponding P2-luciferase reporter constructs (left). Two days after transfection, cultures were stimulated with 50 mM KCl and harvested for luciferase assays (right). Bars show the averages of quadruplicate determinations $\pm$ sem. This mutation analysis revealed that the region from -344 to -339 (bold) is required for Ca^{2+} -stimulated expression of *TRKB* P2.

Figure 3. Mutation of *TCaRE3*, but not the neighboring TBP site disrupts Ca^{2+} -dependent P2 expression

–944/–325 *TRKB* P2-luciferase constructs were transiently transfected into cortical neurons and assayed for Ca^{2+} -stimulated expression following depolarization for 5 hours. Bars show the averages of quadruplicate determinations $\pm$ sem. Samples were normalized to basal expression of wild type –944/–325 construct assayed in parallel. Mutation of either *TCaRE3* (left) (M4, c.f., Fig. 2) or the TBP site (right) (GTCTATTAG→GTCTAAGCT) resulted in a similar decrease in basal P2 expression, but only the *TCaRE3* mutation abolished Ca^{2+} -stimulated *TRKB* P2 expression. Bars show mean $\pm$ sem (N=4).

Figure 4. *TCaRE3* mediates cAMP-dependent P2 expression

Cortical neurons were transiently transfected with -944/-325, -944/-339 or -944/-363 *TRKB* P2 luciferase constructs or -944/-325 with *TCaRE3* mutation M6 (c.f., Fig. 2). Two days after transfection, cultures were stimulated for 5 hours with 40 μ M forskolin. Bars show mean $\pm$ sem (N=4).

Figure 5. *TCaRE3* functions as a DNA element

A. Cortical neurons were transfected with -944/+2, -944/-325 (inhibitory element, *TCaRE4*, deleted) or -944/-363 (*TCaRE3*-deleted) constructs. The ability of 50 mM KCl to stimulate P2 expression was tested in the presence or absence of actD (40 μ M). Despite the presence of *TCaRE3* sequence in RNA transcripts, neither -944/+2 nor -944/-325 exhibited any stimulation of luciferase activity following 5 hours KCl treatment. **B.** Mutation of either of the upstream *CREs* in *TCaRE2* abolished depolarization-stimulated P2 expression even though *TCaRE3* was still present. Thus, the presence of *TCaRE3* in the RNA is not sufficient to mediate Ca^{2+} -dependent stimulation of *TRKB*. With both *CRE* mutations, unstimulated *TRKB* transcription was reduced to an extent similar to that observed with *TCaRE3* mutations (c.f., Fig. 3). In this figure, basal luciferase activity for each *CRE* mutation has been normalized to 1.0 to highlight the absence of Ca^{2+} dependence with the *CRE* mutations. Bars show mean $\pm$ sem (N=4).

Figure 6. *TCaRE3* interacts with *KLF7*

A. Neurons contain a *TCaRE3* binding factor. Nuclear extracts from cortical neurons were subjected to electrophoretic mobility shift assays using either wild type or mutant *TCaRE3* sequences as probe. A gel shift (arrow) was observed with wild type probe but not with probes with *TCaRE3* mutation M4 (c.f., Fig. 2). Depolarization of the neurons prior to extraction did not affect the gel shift. **B.** Recombinant *KLF7* interacts with *TCaRE3*. GST or GST-*KLF7c* was purified from *E. coli*. Binding to *TCaRE3* was investigated assayed using EMSAs as described in **A**. *KLF7* caused a shift of wild-type *TCaRE3* probe, but not a probe containing an inactivating mutation (M4). No shift was observed with GST alone. **C.** *KLF7* binds chromosomal *TRKB* promoter. HEK293 cells (lanes 1–4) and PC12 cells (lanes 5–8) were transiently transfected with either empty vector or *KLF7-myc*. Three days after transfection, cells were crosslinked and subjected to ChIP with anti-myc to precipitate DNA sequences associated with *KLF7-myc*; ChIP with IgG served as control. Immunoprecipitation of *TCaRE3* containing DNA fragments was detected by PCR. **D.** Dominant-negative *KLF7* inhibits depolarization-induced *TRKB* P2 transcription. Cortical neurons were co-transfected with -944/-325 and either a dominant-negative *KLF7* construct or an empty (CMV) vector. Two days after transfection, cells were stimulated by addition of 50 mM KCl for 5 hours prior to harvesting for luciferase assays. Bars show mean $\pm$ sem (N=4). Similar results were obtained in two additional experiments. *, significantly different from control, $p < .05$. **E.** Mouse cortical neurons express both *KLF6* and *KLF7* mRNA. PCR using primers specific for *KLF6* or *KLF7* revealed single band at the predicted sizes. No bands were observed when reverse transcriptase (-RT) was omitted demonstrating that the products were not due to contaminating genomic DNA.

Figure 7. *TCaRE3* in the human *TRKB* promoter

A. Sequence comparison between mouse *TRKB* P2 (−944/+2) and the human *TRKB* promoter region revealed varying degrees of sequence homology. Upper panel: The sequences of *TCaRE2* and *TCaRE3* (black ovals) are identical in the mouse and human promoters and fall within 100- and 35-bp regions of sequence identity (gray). Lower panel: The nucleotide sequence bracketing *TCaRE3* is shown for both mouse and human *TRKB*; identity is indicated by the vertical lines. The *TCaRE3* sequence is shown in bold. **B.** Mouse cortical neurons were transiently trans-fected with human *TRKB*-luciferase reporter genes containing either wild type or mutated (M4; Fig. 1B) *TCaRE3*. One day later, cultures were treated with either 50 mM KCl or 50 μ M forskolin for 5 hours prior to harvesting for luciferase assays. Bars show mean $\pm$ sem (N=4). Similar results were obtained in two additional experiments.

Figure 8. Multiple promoter elements regulate *TRKB* P2 transcription

We have identified four promoter elements that are involved in regulating the responsiveness of *TRKB* P2 to Ca^{2+} signaling. We refer to these elements as *TCaREs* (*TRKB* Ca^{2+} -response elements). *TCaRE2* and *TCaRE3* are required for Ca^{2+} -dependent P2 expression. *TCaRE2* consists of a pair of tandem *CREs* (Kingsbury et al., 2003). *TCaRE3* is a novel element required for *CRE*-dependent expression (Figs. 1, 2, 4, 5). *TCaRE1* enhances Ca^{2+} -dependent *TRKB* P2 expression (Kingsbury, et al., 2003). *TCaRE4* is a suppressor site located downstream from *TCaRE3* (c.f., Fig. 1), which has not been further characterized. The TBP site is required for basal but not Ca^{2+} -dependent expression in mouse *TRKB* P2 (c.f., Fig 3).

Table 1**Cell-type specific regulation of *TRKB* P2-luciferase**

TRKB P2-luciferase constructs harboring either wild type *TCaRE3* sequence or mutated M6 (c.f., Fig. 2) sequence were transiently transfected into cells and assayed for expression following 5 hours stimulation with Ca^{2+} signaling triggered by either depolarization (neurons, RIN 1046 and PC12 cells) or ionomycin treatment (astrocytes and HEK 293 cells).

Cell Type	Ca ²⁺ -dependent Expression (+Ca ²⁺ /Untreated)		Basal Expression (M6/WT)
	<i>TCaRE3</i> WT	<i>TCaRE3</i> M6	
Cortical neurons (mouse)	3.0	0.5	0.15
Cortical neurons (rat)	2.5	0.7	0.12
Astrocytes (mouse)	1.0	1.0	0.13
PC12 (rat)	2.0	1.0	0.04
RIN 1046 (rat)	0.5	0.5	0.13
HEK 293 (human)	2.6	2.2	0.13