Ca\textsuperscript{2+}-dependent Regulation of TrkB Expression in Neurons*

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The neurotrophin brain-derived neurotrophic factor (BDNF), via activation of its receptor, tyrosine receptor kinase B (trkB), regulates a wide variety of cellular processes in the nervous system, including neuron survival and synaptic plasticity. Although the expression of BDNF is known to be Ca\textsuperscript{2+}-dependent, the regulation of trkB expression has not been extensively studied. Here we report that depolarization of cultured mouse cortical neurons increased the expression of the full-length, catalytically active isoform of trkB without affecting expression of the truncated isoform. This increase in protein expression was accompanied by increased levels of transcripts encoding full-length, but not truncated, trkB. Depolarization also regulated transcription of the gene, TRKB, via entry of Ca\textsuperscript{2+} through voltage-gated Ca\textsuperscript{2+} channels and subsequent activation of Ca\textsuperscript{2+}-responsive elements in the two TRKB promoters. Using transient transfection of neurons with TRKB promoter-luciferase constructs, we found that Ca\textsuperscript{2+} inhibited the upstream promoter P1 but activated the downstream promoter P2. Ca\textsuperscript{2+}-dependent stimulation of TRKB expression requires two adjacent, non-identical CRE sites located within P2. The coordinated regulation of BDNF and trkB by Ca\textsuperscript{2+} may play a role in activity-dependent survival and synaptic plasticity by enhancing BDNF signaling in electrically active neurons.

The neurotrophin, brain-derived neurotrophic factor (BDNF), mediates numerous functions in both the developing and mature nervous systems, including the survival of postmitotic neurons, axon growth and guidance, and synaptic plasticity (1). These effects of BDNF are mediated by the tyrosine kinase receptor, trkB. Binding of BDNF to trkB initiates dimerization and trans-autophosphorylation of tyrosine residues in the intracellular domain of trkB (2). These phosphotyrosine residues act as docking sites for effector proteins that activate downstream signaling pathways, leading to the activation of protein kinase cascades, Ca\textsuperscript{2+} mobilization, and gene expression, which orchestrate the cellular responses to BDNF (3). Excitatory synaptic input and the resulting elevation in intracellular [Ca\textsuperscript{2+}] have been shown to increase the synthesis and release of BDNF (4–9). This BDNF activates trkB receptors in the same or neighboring cells to promote their survival and may also enhance synaptic plasticity (1, 10). Although trkB levels change during development and exhibit cell-specific expression patterns (11–13), very little is known about the mechanisms that regulate TRKB expression.

At least four isoforms of trkB are produced by alternative splicing of the primary transcripts of the TRKB gene (14–16). Of these, only the full-length isoform, which contains an intracellular tyrosine kinase domain, is known to be capable of mediating BDNF signaling. Three truncated isoforms (T1, T2, and T\textsubscript{full}), which lack the intracellular kinase domain but possess the same extracellular BDNF binding domain as full-length receptors, can also be generated by alternative splicing. T1 is prominently expressed in the brain (14) and can act as a dominant negative inhibitor of BDNF signaling (17–21) by forming heterodimers with full-length trkB (17). These observations raise the possibility that the relative expression of full-length and truncated trkB isoforms in normal neurons can modulate cellular responsiveness to BDNF. Dysregulation of trkB isoform expression may also underlie some nervous system abnormalities. For example, overexpression of truncated trkB has been reported in cortical neurons in Alzheimer’s disease brain (22), where it may contribute to neurodegeneration, and in the trisomy 16 mouse model of Down syndrome, where it results in failure of BDNF-mediated neuron survival (20).

The TRKB gene can be transcribed from two promoters, P1 and P2 (23). Within the TRKB upstream sequence are multiple potential regulatory elements, including several Ca\textsuperscript{2+}/cAMP-response elements (CREs), suggesting that elevated Ca\textsuperscript{2+} and/or cAMP may regulate TRKB expression.

In this report, we demonstrate that depolarization alters the relative expression of full-length and truncated trkB receptors in cultured cortical neurons and identify Ca\textsuperscript{2+}-dependent regulatory elements in the TRKB promoters involved in this response.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—Cortical neurons were isolated from embryonic day-16 mouse embryos and plated at a density of 6 × 10\textsuperscript{5}/well in 24-well dishes for luciferase assays and 3 × 10\textsuperscript{5}/well in 35-mm dishes for RNA and protein analysis. Neuron cultures were maintained in Neurobasal medium supplemented with 2% B27, 2 mM glutamine, and penicillin-streptomycin and incubated in 5% CO\textsubscript{2} at 37 °C. Neurons were transiently transfected 3–5 days after plating using a Ca\textsuperscript{2+}-phosphate protocol (24). TRKB luciferase reporters were introduced at a concentration of 1 μg/well; cells were cotransfected with 0.5 μg/well TK

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Depolarization Increases the Level of Full-length trkB in Cortical Neurons—Because of the importance of BDNF/trkB signaling in activity-dependent changes in neurons, we investigated the ability of depolarization to regulate trkB expression. Embryonic mouse cortical neurons grown in culture for 5–7 days were depolarized with 50 mM added KCl to induce Ca\(^{2+}\) influx. Cells were then harvested for Western blot analysis. As previously reported for hippocampal neurons (20), cortical neurons expressed primarily full-length trkB (Fig. 1). In the presence of 50 mM added KCl, the level of full-length trkB protein was elevated by 5 h and continued to increase up to 16 h, the longest time studied (Fig. 1A). The low level of truncated trkB did not change following KCl treatment. Depolarization also increased the amount of phospho-trkB observed in response to BDNF stimulation (Fig. 1B), demonstrating that the additional full-length trkB was functional.

Depolarization Increases the Level of mRNA Encoding Full-length trkB in Cortical Neurons—In light of the effects of depolarization on full-length trkB expression, real-time PCR was conducted to determine the effect of depolarization on trkB RNA expression. Following reverse transcription of total RNA, TRKB transcripts were analyzed using primer pairs specific for either full-length or truncated T1 trkB isoforms (Fig. 2A). Five hours of stimulation with elevated KCl produced an ~3-fold increase in full-length trkB transcripts, whereas there was no increase in T1 transcripts (Fig. 2B). The increase in full-length transcripts was sensitive to EGTA (data not shown).

Ca\(^{2+}\) Regulates TRKB Expression—The presence of three CRE sites in the TRKB promoter region (23) suggested that Ca\(^{2+}\) and/or CaM can modulate TRKB. Ca\(^{2+}\)-dependent TRKB transcription was investigated using a TRKB-luciferase reporter gene. Approximately 2.5 kb of the TRKB promoter region, including both P1 and P2, was cloned upstream of the luciferase gene and transiently transfected into cortical neurons using a Ca\(^{2+}\)-phosphate method (24). Depolarization of the neurons by the addition of 50 mM KCl resulted in a 2-fold increase in TRKB-dependent transcription as measured by luciferase activity (Fig. 3A). The presence of EGTA eliminated the response to KCl, indicating that Ca\(^{2+}\) influx was required to stimulate TRKB-luciferase expression.

To determine the pathway of Ca\(^{2+}\) entry in response to depolarization, neurons transfected with the ~2558-bp luciferase plasmid were stimulated with KCl in the absence and presence of Ca\(^{2+}\) channel or glutamate receptor (GluR) blockers. Western blot analysis using antibodies to the extracellular domain of trkB or to actin. The presence of three CRE sites in the TRKB promoter region (23) suggested that Ca\(^{2+}\) and/or CaM can modulate TRKB. Ca\(^{2+}\)-dependent TRKB transcription was investigated using a TRKB-luciferase reporter gene. Approximately 2.5 kb of the TRKB promoter region, including both P1 and P2, was cloned upstream of the luciferase gene and transiently transfected into cortical neurons using a Ca\(^{2+}\)-phosphate method (24). Depolarization of the neurons by the addition of 50 mM KCl resulted in a 2-fold increase in TRKB-dependent transcription as measured by luciferase activity (Fig. 3A). The presence of EGTA eliminated the response to KCl, indicating that Ca\(^{2+}\) influx was required to stimulate TRKB-luciferase expression.

Fig. 1. Depolarization induces full-length trkB protein expression. A, cortical neurons were incubated in medium supplemented with 50 mM KCl for 5 or 16 h as indicated and harvested for Western blot analysis using antibodies to the extracellular domain of trkB or to actin. B, cortical neurons were maintained in either normal or elevated KCl for 16 h and then exposed to 50 ng/ml BDNF for 10 min. Western blotting was carried out using anti-phospho-trkB, which recognizes phosphorylated Tyr-515 in trkB. The level of BDNF-stimulated phospho-trkB (P-trkB) was enhanced by exposure to elevated KCl, demonstrating that the additional full-length trkB induced by depolarization was functionally active.
Fig. 2. Depolarization induces full-length trkB mRNA expression. A, diagram illustrating the location of isoform-specific primers (arrows) for full-length and truncated (T1) trkB mRNAs. Both isoforms share a common extracellular BDNF binding domain and transmembrane domain (TM) but have different intracellular domains and their mRNAs have distinct 5'- and 3'-UTRs. B, cortical neurons were incubated for 5 h in the absence or presence of 50 mM added KCl. Levels of trkB RNA were quantified using real-time PCR as described under “Experimental Procedures” using primer pairs specific for RNA encoding either full-length (trkB.FL) or T1 truncated (trkB.TR) receptors (arrows in panel A). Data shown are mean ± S.E. (n = 4 experiments) mRNA levels in the presence of elevated KCl relative to unstimulated control.

Fig. 3. Ca²⁺ entry stimulates TRKB transcription. A, cortical neurons transiently transfected with −2558/+2 TRKB-luciferase plasmid were stimulated for 6 h with elevated KCl in the absence or presence of 2 mM extracellular EGTA. Luciferase activity was assayed as described under “Experimental Procedures” and is reported relative to unstimulated activity. B, transfected neurons were stimulated as in panel A in the absence or presence of 100 μM nifedipine or 80 μM APV plus 20 μM DNQX. Data shown are means ± S.E. (n = 4). The same results were obtained in five (A) or two (B) experiments.
also activated in response to cAMP, we investigated the ability of cAMP to activate the TRKB P2 promoter. Cortical neurons transfected with either the −1429/+2 or −944/+2 TRKB-luciferase reporter gene were treated with 50 μM forskolin, an adenylyl cyclase activator, for 6 h in either the absence or presence of 50 mM added KCl. Forskolin stimulated the luciferase activity of each TRKB reporter 2-fold, demonstrating that P2 can be stimulated by cAMP signaling (Fig. 6). The effects of depolarization-induced Ca2+ signaling and increased cAMP were additive for both −944/+2 and −1429/+2 P2-dependent reporter genes when the neurons were simultaneously treated with elevated KCl and forskolin.

**DISCUSSION**

Depolarization resulted in increased expression of full-length trkB protein and increased phosphorylation of trkB upon BDNF stimulation (Fig. 1). Depolarization also preferentially increased the level of endogenous full-length trkB mRNA without significantly affecting the level of truncated trkB message (Fig. 2B). These results led us to investigate the role of Ca2+ in the transcriptional regulation of TRKB. We show here that the two promoters of TRKB are differentially regulated by Ca2+. P1 reporter constructs were inhibited by Ca2+, whereas P2 reporters were stimulated by Ca2+ (Fig. 4, B and C). Although the quantitative contribution of P1- and P2-derived transcripts to specific trkB isoform expression has not yet been established, these observations suggest that Ca2+-regulated TRKB promoter selection can alter the relative expression levels of trkB isoforms and consequently modulate cellular BDNF responsiveness.

The stimulation of BDNF expression by Ca2+ has been extensively studied (4–6). Ca2+-dependent activation of BDNF promoters I and III is mediated by single CRE sites acting in concert with additional Ca2+-dependent regulatory sites. We show here that TRKB P2 contains a pair of CREs, separated by 4 bp, both of which are required for Ca2+-dependent expression of TRKB (Fig. 5B) via the activation of CREB or a related transcription factor (Fig. 5C). Because single CRE sites are sufficient to mediate Ca2+-dependent transcription in genes such as BDNF, it is unclear why both CREs in TRKB are necessary. Interestingly, a similar requirement for two tandem CRE sites has been previously reported in the nNOS promoter (27). In addition to the CRE sites, our data indicate that an additional upstream element located between −944 and −1429 functions to promote the Ca2+-stimulated expression of P2...
here add another level of regulation to this list: Depolarization selectively increases the expression of catalytically active, full-length trkB via activation of Ca\(^{2+}\)-responsive elements in the TRKB promoter.

Based on these findings, we propose that the coordination of ligand and receptor expression by Ca\(^{2+}\) can regulate efficacy of the BDNF/trkB signaling system in those neurons that receive appropriate levels of excitatory stimulation. Activity-dependent regulation of cellular responsiveness to BDNF via Ca\(^{2+}\)-dependent control of trkB expression could be a novel mechanism by which neuronal activity can modulate not only cell survival but also synaptic plasticity and may play important roles in the pathological as well as the normal nervous system.

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