

Upstream Stimulatory Factors Are Mediators of Ca^{2+} -Responsive Transcription in Neurons

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To identify molecular mechanisms that control activity-dependent gene expression in the CNS, we have characterized the factors that mediate activity-dependent transcription of *BDNF* promoter III. We report the identification of a Ca^{2+} -responsive E-box element, CaRE2, within *BDNF* promoter III that binds upstream stimulatory factors 1 and 2 (USF1/2) and show that USFs are required for the activation of CaRE2-dependent transcription from *BDNF* promoter III. We find that the transcriptional activity of the USFs is regulated by Ca^{2+} -activated signaling pathways in neurons and that the USFs bind to the promoters of a number of neuronal activity-regulated genes *in vivo*. These results suggest a new function for the USFs in the regulation of activity-dependent transcription in neurons.

Key words: upstream stimulatory factor; USF1; USF2; activity-dependent transcription; brain-derived neurotrophic factor; BDNF; calcium; neural plasticity; activity-dependent neural development

Introduction

Neural activity arising from sensory input induces the expression of new gene products that contribute to enduring adaptations in the CNS. These activity-dependent changes include the refinement of cortical circuitry during development (Katz and Shatz, 1996; Mao et al., 1999; Pallas, 2001; Sur and Leamey, 2001), the formation of long-term memories (Koenig and Lu, 1967; Nguyen et al., 1994), and the development of complex behaviors such as birdsong learning (Clayton, 1997). Neuronal activity and subsequent depolarization drive the influx of Ca^{2+} ions via L-type voltage-sensitive Ca^{2+} channels (L-VSCC) and the NMDA subtype of glutamate receptors, stimulating an intricate signaling network that regulates rapid transcriptional events at the promoters of downstream genes (Bito et al., 1997; Impey and Goodman, 2001; West et al., 2001). An especially interesting group of these activity-regulated genes encodes proteins that are expressed selectively in the nervous system and regulate synaptic maturation and function. Examples include the nicotinic acetylcholine receptor $\alpha 7$ subunit (nAChR $\alpha 7$) (Zhou et al., 2001), the major histocompatibility complex (MHC) class I genes (Corriveau et al., 1998; Huh et al., 2000), cyclooxygenase-2 (COX-2)/prostaglandin E synthase (PGES) (Yamagata et al., 1993), the neuropeptide preprotachykinin (Benson et al., 1994), and the neurotrophin brain-derived neurotrophic factor (BDNF). Invest-

igating the regulatory mechanisms that control the transcription of these genes in neurons may provide important insights into activity-dependent neural development and synaptic plasticity.

To identify molecular mechanisms that mediate activity-dependent gene expression in neurons, we have studied the transcriptional regulation of BDNF. BDNF is highly expressed in neurons and plays important roles in neuronal survival (Bonni et al., 1999), cortical development (Ghosh, 1996), and synaptic plasticity (Poo, 2001). Transcription of the *BDNF* gene is stimulated dramatically by membrane depolarization *in vitro* (Ghosh et al., 1994) and by neural activity during kindling, long-term potentiation (LTP) induction, and visual associative learning (Ernfors et al., 1991; Patterson et al., 1992; Tokuyama et al., 2000). The *BDNF* gene comprises five exons, the first four of which are spliced alternatively to a single 3' exon encoding the complete BDNF protein (Timmusk et al., 1993). Although the specific functions of the different BDNF transcripts are not yet clear, we have demonstrated previously that the promoter upstream of exon III is most responsive to membrane depolarization of cultured embryonic rat cortical neurons (Tao et al., 1998).

The cAMP/ Ca^{2+} -response element binding protein (CREB) is required for the activity-dependent transcription of a number of neuronal genes, including *BDNF* exon III (Shieh et al., 1998; Tao et al., 1998; Sasaki et al., 2000). However, activation of CREB alone is not sufficient to mediate the activity-dependent transcription of *BDNF* exon III. Mutations of *BDNF* promoter III 5' to the CRE sequence severely reduce the responsiveness of the promoter to Ca^{2+} influx (Shieh et al., 1998; Tao et al., 1998), indicating that there must exist additional transcription factors that cooperate with CREB to regulate *BDNF* promoter III in a Ca^{2+} -dependent manner in neurons. We have conducted detailed mutagenesis of the region 5' to the CRE in *BDNF* promoter III and find that it contains two distinct Ca^{2+} -response elements (CaREs). In this report we show that one of these elements (CaRE2) is a Ca^{2+} -responsive E-box. Using a yeast one-hybrid

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screen to identify factor(s) that bind this element, we found that upstream stimulatory factors 1 and 2 (USF1/2) bind to *BDNF* promoter III through the E-box sequence. The USFs are bound to *BDNF* promoter III *in vivo*, suggesting that they are likely to function as endogenous transcriptional regulators of *BDNF* expression in the brain. Consistent with a role for the USFs as regulators of Ca^{2+} -dependent transcription of *BDNF* exon III, we observe that the transcriptional activity of the USFs is activated by Ca^{2+} influx into neurons. In addition to the evidence that USFs regulate *BDNF* expression, we demonstrate by chromatin immunoprecipitation that the USFs are bound *in vivo* to a number of other activity-regulated promoters. These data suggest a new function for the USFs as activity-dependent transcriptional regulators in the brain that play a role in orchestrating neural development and synaptic plasticity.

Materials and Methods

Plasmids. *BDNF* pIII(170)-Luc, EF- β -gal, pSG424 (Gal4 only), Gal4-Luc, Gal4-USF2, pSG5-USF2, pSG5-USF2DN, 3 \times UBE-Luc (pU3ML-Luc), CRE-Luc, CMV-A-USF1, and the control vectors pML-Luc and CMV were described previously (Sheng et al., 1991; Luo and Sawadogo, 1996a,b; Abdollah et al., 1997; Tao et al., 1998). TK-pRL was purchased from Promega (Madison, WI). We generated 2 bp substitutions of *BDNF* pIII(170)-Luc by QuickChange site-directed mutagenesis (Stratagene, La Jolla, CA) with a pair of complimentary 5' oligos containing two random nucleotides at 2 bp intervals from nucleotides -54 to -41 in *BDNF* promoter III.

Cell culture, transfection, stimulation, and luciferase assay. Cortical neurons from embryonic day 18 (E18) Long-Evans rats (Charles River, Wilmington, MA) and E16 C57/Black 6 mice were cultured as described (Tao et al., 1998). Neurons were transfected at 3 d *in vitro* (3 DIV) by the Ca^{2+} phosphate precipitation method (Xia et al., 1996). At 2 d after transfection the neurons were depolarized with 50 mM KCl (Tao et al., 1998) for 7–9 hr. Cell extracts were used for a luciferase reporter assay or a dual luciferase reporter assay if the cells were cotransfected with TK-pRL (Promega); 80 μ l of extract was used for a β -galactosidase assay with *ortho*-nitrophenyl β -D-galactopyranoside as the substrate. The normalized luciferase activity was obtained by dividing the *firefly luciferase* activity by either the renilla luciferase activity or the β -galactosidase activity. Each normalized value represents the average of at least three independent determinations, and the error bars indicate the SEM. L-VSCCs were blocked by the addition of 5 μ M nimodipine (Roche Molecular Biochemicals, Indianapolis, IN), and NMDA receptors were blocked by 100 μ M D-APV (Sigma, St. Louis, MO) for 5 min before depolarization. Glutamate receptors were activated by the addition of 20 μ M glutamate.

Nuclear extracts and electrophoretic mobility shift assays. Nuclear extracts and electrophoretic mobility shift assays (EMSA) were performed as described (Tao et al., 1998). The radiolabeled CaRE2 probe was synthesized by Klenow (3'→5' exo⁻; New England Biolabs, Beverly, MA) with α -³²P-dCTP (NEN Life Science, Boston, MA), using complementary oligonucleotides: 5'-GTG AGC TGT CAT ATG ATA CCT CCT CTG CCT C-3' and 5'-GAG GCA GAG GAG G-3' (Invitrogen, San Diego, CA). Wild-type and mutant unlabeled probes were synthesized by PCR with primers 5'-GGT AAT TCG TGC ACT AGA G-3' and 5'-CGA GAG GGC TCC ACG CTG C-3', using the wild-type or mutant *BDNF* pIII(170)-Luc vectors as templates. Anti-USF1 (*sc*-229X), anti-USF2 (*sc*-862X), and anti-c-Myc (*sc*-42X) antibodies for supershift were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Yeast one-hybrid screen. The yeast one-hybrid screen for CaRE2 binding proteins was performed by using the Matchmaker Yeast one-hybrid system (Clontech, Palo Alto, CA). Six repeats of the CaRE2 sequence (from nucleotides -56 to -39) were cloned upstream of the *HIS3* gene. Then the plasmid was integrated into yeast genome to generate a yeast reporter strain, and the strain was transfected with a rat brain cDNA library containing $\sim 1 \times 10^6$ independent clones fused to the transcriptional activation domain of the yeast Gal4 protein (Clontech). Colonies

that grew on minimal medium lacking histidine were selected, and the cDNA-containing plasmids were recovered and sequenced from these colonies.

Immunocytochemistry. Anti-USF1 (C-20) and anti-USF2 (C-20) antibodies were purchased from Santa Cruz Biotechnology. Both antibodies were used at 1:400 for cell staining. The mouse TuJ1 anti- β -tubulin III antibody (Babco, Richmond, CA) was used at 1:300.

Chromatin immunoprecipitation assay. E18 rat cortical neurons (2×10^7 cells) at 5 DIV were treated with 1% formaldehyde at room temperature for 20 min. After two washes with $1 \times$ PBS, the cross-linked neurons were scraped off the plates. The neurons were pelleted, resuspended in 200 μ l of lysis buffer, and processed for chromatin immunoprecipitation with the chromatin immunoprecipitation (ChIP) assay kit (Upstate Biotechnology, Lake Placid, NY) with the following modifications: (1) the cell lysates were sheared by sonication for a total of 100 sec at 20 sec per interval; (2) the protein A agarose/antibody/transcription factor complex was washed in the low-salt immune complex wash buffer twice, high-salt immune complex wash buffer twice, LiCl immune complex wash buffer twice, and then $1 \times$ TE three times; and (3) the chromatin fragments that were pulled down with specific antibodies were resuspended in 200 μ l of H_2O , 10 μ l of which was used for PCR for 28–30 cycles. Anti-Bad (N-20), anti-c-Myc (C-33), and anti-Id (Z-8) antibodies were purchased from Santa Cruz Biotechnology. PCR primer sequences for *BDNF* exon III were 5'-GCG CGG AAT TCT GAT TCT GGT AAT-3' and 5'-GAG AGG GCT CCA CGC TGC CTT GAC G-3'; for *BDNF* exon V were 5'-AAG TGT AAT CCC ATG GGT TAC ACG-3' and 5'-CAG GAA GTG TCT ATC CTT ATG AAC CG-3'; for *COX-2* promoter were 5'-CCT GCC CCT ATG GGT ATT ATG C-3' and 5'-TTC GTG ACT GTG TCT TTC CGC-3'; for *nAChR α 7* promoter were 5'-ATT AAA CTG CAG GCG GGA CAG-3' and 5'-GCG GCC AAG CTT GGC TAT-3'; for *Nur77* promoter were 5'-CCT GGT CGG TTA TTT CGG-3' and 5'-AGC GCG GAT TGT TTG ATC-3'; for *GAP-43* promoter were 5'-AGT GTG GAA GCA TAA ATG AGA TGT TTG-3' and 5'-GGA GAT TTT GTG TGC AGT TGA TAA TTG-3'.

Quantitative real-time RT-PCR. RNA was prepared with the Absolutely RNA kit (Stratagene). Total RNA (1 μ g) was used for reverse transcription with the First Strand Superscript II kit (Invitrogen). PCR was performed in an iCycler (Bio-Rad, Hercules, CA) with the use of SYBR-green (PE Applied Biosystems, Foster City, CA). Each independent sample was assayed in triplicate. The threshold cycle for each sample was chosen from the linear range and converted to a starting quantity by interpolation from a standard curve run on the same plate for each set of primers. The *firefly luciferase* mRNA levels were normalized for each well to cotransfected α -globin mRNA levels. Single PCR products were verified both by assessing that the melting temperature of the product had a single value and by viewing the PCR product on an agarose gel. The primer sequences for *firefly luciferase* were 5'-GAG GTG AAC ATC ACG TAC GCG-3' and 5'-AAG AGA GTT TTC ACT GCA TAC GAC G-3' and for α -globin were 5'-CAA GAC CTA CTT CCC GCA CTT-3' and 5'-GCT CAG GTC GAA GTG CGG-3'.

Results

An E-box sequence in *BDNF* promoter III is a Ca^{2+} response element

Previously, we found that 170 bp of the 5' flanking sequence of *BDNF* exon III is sufficient to activate reporter gene expression in response to membrane depolarization-induced Ca^{2+} influx via L-VSCCs in cultured embryonic rat cortical neurons (Tao et al., 1998), suggesting that key CaREs reside within this region. To identify these CaREs, we have made systematic deletions and mutations of the 170 bp of *BDNF* promoter III and assessed the effects of these mutations on the Ca^{2+} inducibility of a luciferase reporter. Initially, we and others reported that both a CRE-like sequence bound by the transcription factor CREB as well as a discrete region 5' to this element are required for induction of reporter gene expression (Shieh et al., 1998; Tao et al., 1998). With further mutagenesis we have determined that this distal

regulatory region consists of two distinct elements. The most 5' element (CaRE1) lies between nucleotides –73 to –64 relative to the transcriptional initiation site of *BDNF* exon III and is bound by a novel transcription factor, calcium-responsive transcription factor (CaRF), which regulates *BDNF* exon III transcription in a Ca^{2+} - and neural-selective manner (Tao et al., 2002).

To characterize the element lying between CaRE1 and the CaRE3/CRE, we made two-nucleotide substitutions of the sequence between these elements in the context of the *BDNF* promoter III reporter gene construct. These mutant plasmids were transiently transfected into cultured cortical neurons, the cells were depolarized by exposure to elevated levels of KCl, and the induction of luciferase expression from the reporter gene was measured. As shown in Figure 1*a*, transcription from the wild-type *BDNF* pIII(170)-Luc reporter gene was induced significantly in response to membrane depolarization. Most mutations of the nucleotide sequence between –52 and –43 bp 5' to the *BDNF* exon III transcription initiation site severely reduced the ability of membrane depolarization to induce reporter gene expression, whereas mutations just outside this 10 bp region had little effect. These data identify the 10 bp nucleotide sequence from –52 to –43 bp relative to the *BDNF* exon III transcription initiation site as a critical Ca^{2+} response element that we have named CaRE2.

Characterization and cloning of the CaRE2 binding protein

The core of the CaRE2 sequence (ATCATATGAC) fits the consensus for an E-box element (CANNTG). E-box elements are bound by members of the basic helix-loop-helix (bHLH) family of transcription factors. However, analysis of the TRANSFAC database (<http://www.cbil.upenn.edu/tess/index.html>) indicated that none of the previously characterized factors favor the specific E-box sequence we had identified as CaRE2 in *BDNF* promoter III. Therefore, to identify the transcription factor(s) that mediate Ca^{2+} -dependent expression of *BDNF* exon III through CaRE2, we cloned and characterized the protein(s) that bind to this element.

Because *BDNF* is highly expressed in neurons, we asked whether there is a protein within the nucleus of cortical neurons that binds specifically to the CaRE2 sequence. Nuclear protein extracts were prepared from embryonic rat cortex, mixed with a radiolabeled probe encompassing the CaRE2 sequence, and then subjected to an EMSA. A protein in the cortical neuron nuclear extract was found to bind to and retard the mobility of the radiolabeled CaRE2 probe in a non-denaturing polyacrylamide gel (Fig. 1*c*). The association of this nuclear protein with CaRE2 was specific, because its binding to CaRE2 could be competed by the addition of an excess of unlabeled wild-type CaRE2 probe, but not with the addition of excess CaRE2 sequences that failed to support Ca^{2+} -dependent induction in the context of the *BDNF* promoter III reporter gene (Fig. 1*a*). This correlation between the ability of the neuronal nuclear protein to bind CaRE2 sequences and the ability of these CaRE2 sequences to drive Ca^{2+} -dependent transcription of *BDNF* promoter III supports the hypothesis that there exists a protein in cortical neurons for which the interaction with CaRE2 is required for Ca^{2+} -dependent induction of *BDNF* promoter III transcription.

To identify the protein that regulates *BDNF* transcription through CaRE2, we used a yeast one-hybrid system to screen a rat brain cDNA library for CaRE2 binding protein(s). After screening 250,000 clones, we obtained three positive colonies. Protein extracts from these yeast contained a protein that bound to CaRE2 with the same specificity for wild-type and mutant CaRE2 sequences as the endogenous CaRE2 binding protein from neu-

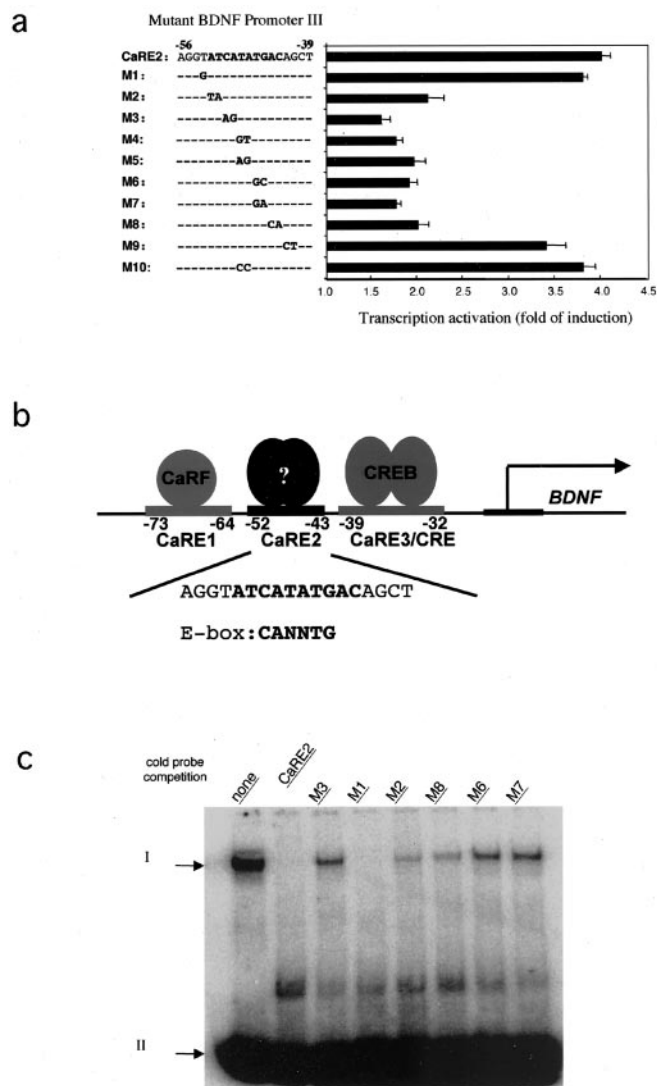


Figure 1. CaRE2 is a Ca^{2+} -responsive E-box element in *BDNF* promoter III. *a*, Characterization of CaRE2. A luciferase reporter plasmid driven by *BDNF* promoter III with either wild-type or mutant sequences was transfected into E18 + 3 DIV rat cortical neurons. The fold of induction equals the ratio of normalized luciferase activity from stimulated cells to that from the unstimulated neurons. The numbering indicates the position relative to the first transcription start site for *BDNF* exon III. In the mutant sequences the letters indicate the mutated bases, and the dashed lines represent unchanged nucleotides. *b*, Schematic diagram of Ca^{2+} response elements (CaREs) in *BDNF* promoter III. The CaRE2 sequence (bolded) from *BDNF* promoter III is shown and compared with the canonical E-box sequence. *c*, Characterization of the CaRE2 binding protein(s). Shown is the specific interaction of CaRE2 and its binding protein(s) in neurons. Nuclear extracts from P1 rat brain were mixed with radiolabeled probes containing the CaRE2 sequence before electrophoresis in a native polyacrylamide gel. Unbound radiolabeled CaRE2 probes (arrow II) migrated faster than the ones retarded by the CaRE2 binding protein(s) (arrow I). The specificity of the interaction was determined by the ability of excess unlabeled mutant or wild-type CaRE2-containing probes to compete away the radiolabeled CaRE2 probes from the DNA/protein complex. The sequences for the mutant probes (M1–M3, M6–M8) are shown in *a*.

ronal nuclear extracts (data not shown). After sequencing of the brain cDNA recovered from these clones, we were surprised to find that in each case the expressed protein was the mammalian bHLH transcription factor USF1.

USF1 is a 43 kDa bHLH family transcription factor (Sawadogo et al., 1988; Gregor et al., 1990) originally purified as a cellular protein that regulates the adenovirus major late promoter (Carthew et al., 1985; Sawadogo and Roeder, 1985; Moncollin et al., 1986). A highly homologous family member, USF2, of 44 kDa

also has been characterized (Sawadogo et al., 1988; Sirito et al., 1994). Both USFs are expressed ubiquitously (Sirito et al., 1994; Viollet et al., 1996) and bind to E-box elements as homo- or heterodimers (Gregor et al., 1990; Sirito et al., 1992). Preliminary characterization of the endogenous CaRE2 binding proteins from neurons had suggested that they were heat-stable proteins with molecular weights of ~45 kDa (data not shown), consistent with the properties of the USFs. Although USF1 and USF2 are known to be expressed in brain (Sirito et al., 1994), neither protein has been implicated previously in activity-dependent transcription. Therefore, we conducted an additional series of experiments to determine whether the USFs contribute to Ca^{2+} -regulated BDNF transcription.

USF1 and USF2 bind CaRE2

To examine whether the USFs bind CaRE2, we first asked whether the USFs are part of the CaRE2 binding complex that we observed by EMSA with cortical neuron nuclear extracts. The addition of anti-USF1 or USF2 antibodies to the CaRE2–neuronal nuclear protein complex caused an additional retardation of the complex by EMSA, suggesting that both USF1 and USF2 are in the CaRE2–protein complex (Fig. 2*a*). In contrast, the addition of antibodies that recognize a closely related bHLH family transcription factor, c-Myc, had no effect on the CaRE2–protein complex. These results indicate that endogenous USF1 and USF2 are part of the complex of proteins present in neuronal extracts that bind to CaRE2.

To determine whether USF1 and USF2 are sufficient to bind to CaRE2 directly, we tested the ability of *in vitro* transcribed and translated USF1 and USF2 to bind to CaRE2 in an EMSA. As shown in Figure 2*b*, both USF1 and USF2 are capable of binding to CaRE2, as indicated by a retardation of the radiolabeled CaRE2 probe. Moreover, this binding shows the same specificity for CaRE2 sequences as we observed for the endogenous CaRE2 binding protein (Fig. 1*c*). This correlation between the ability of USFs to bind CaRE2 sequences and the ability of these sequences to support activity-dependent transcription from BDNF promoter III suggests that USF binding to CaRE2 is relevant for activity-dependent BDNF exon III transcription in neurons.

USFs functionally regulate CaRE2-dependent Ca^{2+} -inducible BDNF exon III transcription

To determine whether the USFs mediate Ca^{2+} -inducible BDNF exon III transcription, we asked whether dominant-negative versions of USF block the activity-dependent induction of BDNF promoter III reporter gene transcription. Both USF1 and USF2 are expressed constitutively in the nuclei of cultured embryonic cortical neurons (Fig. 3*a–h*). To disrupt their function, we used a deletion mutant of USF2 (DN-USF2) that lacks the N-terminal transcriptional activation domain and effectively competes with endogenous USF1 and USF2 for binding to promoter E-boxes (Qyang et al., 1999) without activating USF-dependent transcription. Coexpression of DN-USF2 with the BDNF promoter III reporter gene resulted in a significant reduction of the activity-dependent induction of luciferase expression, whereas overexpression of wild-type USF2 had no effect (Fig. 3*i*). These data indicate that a nonfunctional USF bound to CaRE2 blocks activity-dependent BDNF exon III expression. To rule out the possibility that DN-USF2 was blocking BDNF transcription nonspecifically by occluding the binding of another bHLH protein to CaRE2, we examined the effect of a second dominant-negative USF construct that works via a different mechanism. A-USF is a dominant-negative USF construct that contains an acidic exten-

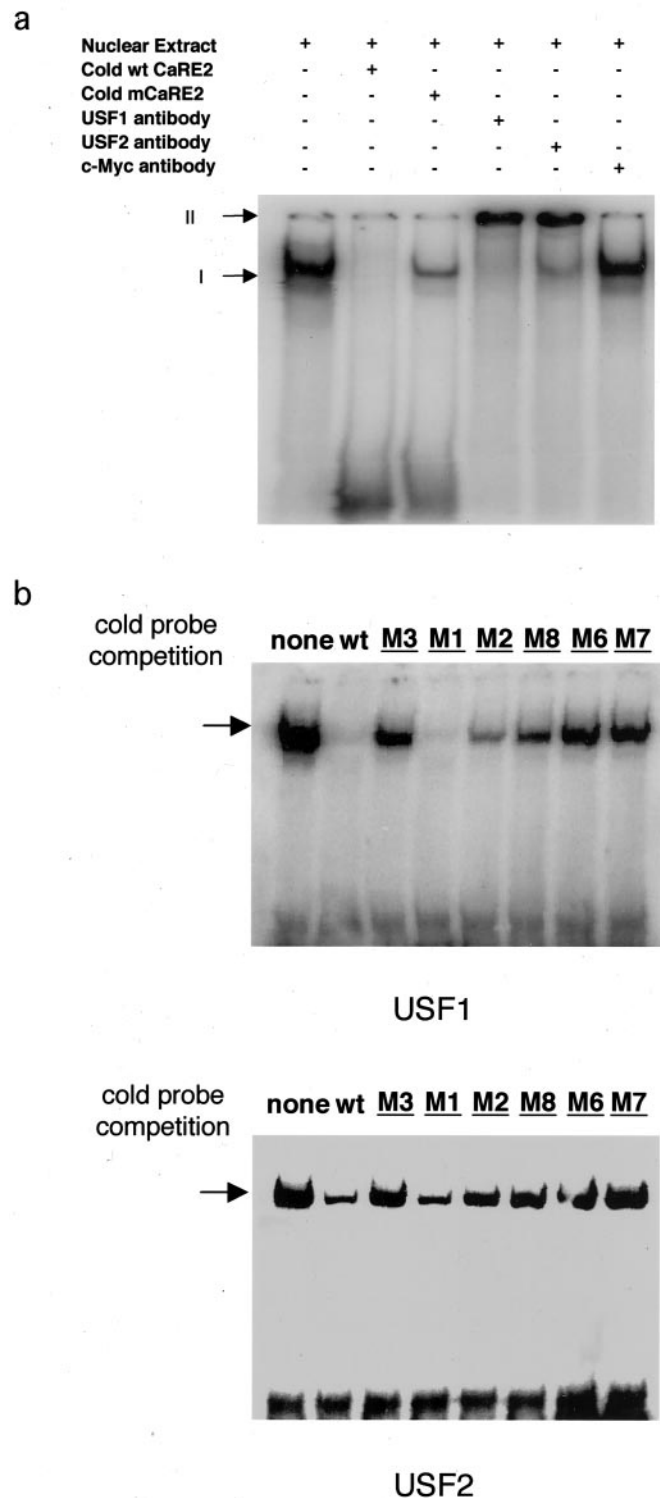


Figure 2. Upstream stimulatory factors (USFs) bind CaRE2. *a*, Endogenous USFs in neurons bind CaRE2. Nuclear extracts from P1 rat brain were incubated with excess unlabeled wild-type or mutant CaRE2 probes, anti-USF1 antibody, anti-USF2 antibody, or anti-c-Myc antibody on ice for 1 hr. Then radiolabeled CaRE2 probes were added to the mixture, which subsequently was subjected to EMSA. Arrow I marks the complex formed by the radiolabeled CaRE2 probes with the endogenous CaRE2 binding proteins. Arrow II indicates the supershifted complexes formed by anti-USF antibodies, the endogenous CaRE2 binding protein, and the radiolabeled CaRE2 probe. *b*, Recombinant USFs are sufficient to bind CaRE2. *In vitro* translated USF1 or USF2 proteins were mixed with radiolabeled CaRE2 probes and an excess of unlabeled wild-type or mutant CaRE2 probes and then subjected to EMSA. The sequences for the mutant CaRE2 probes are the same as those in Figure 1*a*. The arrow indicates the specifically retarded band.

sion in its DNA binding domain and that, when overexpressed in neurons, dimerizes with both endogenous USF1 and USF2, thereby preventing these molecules from binding to CaRE2 (Qyang et al., 1999). Consistent with the effects of DN-USF2, overexpression of A-USF significantly inhibited membrane depolarization induction of the *BDNF* promoter III-driven reporter gene, whereas transfection of the empty plasmid vector alone had no effect (Fig. 3j). In total, these data indicate that the USFs are required for activity-dependent transcription from *BDNF* promoter III.

Both USF1 and USF2 can regulate activity-dependent *BDNF* exon III transcription

The dominant-negative experiments suggest that USFs are required for Ca^{2+} regulation of *BDNF* transcription. However, both dominant negatives inhibit USF1 and USF2 and therefore do not distinguish whether one family member or both are the key regulators of Ca^{2+} -dependent *BDNF* transcription. To ask whether either USF1 or USF2 is sufficient for activity-dependent *BDNF* exon III expression or whether both are required for the response, we cultured embryonic cortical neurons from either USF1 or USF2 null mice or their wild-type or heterozygous siblings. These cells were transfected with the *BDNF* reporter construct, and the induction of luciferase in response to depolarization was measured. We observed no significant difference in the membrane depolarization-mediated induction of *BDNF* promoter III activity in any of the genotypes (Fig. 4a). Neither did we observe significant alteration in the Ca^{2+} responsiveness of *BDNF* promoter III in the USF1(–/–)/USF2(+/-) and USF1(+/-)/USF2(–/–) mice (data not shown). Taken together, these data suggest that both USF1 and USF2 are independently capable of mediating activity-dependent induction of *BDNF* promoter III. Because the constitutive USF1/2 double knock-out mice die early in embryogenesis (Sirito et al., 1998), it is not possible at this stage to use these loss-of-function mutants to corroborate the role played by both USF1 and USF2 in *BDNF* transcription.

The experiments with USF null mice suggest that both USF1 and USF2 may regulate *BDNF* promoter III. To seek evidence that both USF1 and USF2 when expressed at physiological levels are bound to the endogenous CaRE2 element of *BDNF* promoter III *in vivo*, we used a ChIP assay to study the *in vivo* occupancy of *BDNF* promoter III by USF1 and USF2. After first cross-linking DNA-bound proteins to chromatin in neuronal cultures, we lysed the cells and sheared the chromatin to an average of 150 bp in length. Next we used specific antibodies against USF1 and USF2 to immunoprecipitate these proteins along with the bound chromatin. After extensive washing and reversal of the cross-linking, we used specific PCR primers to test for the presence of

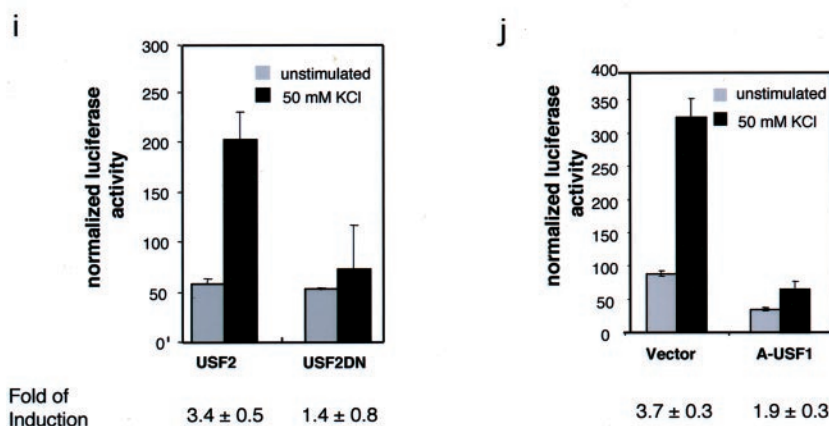
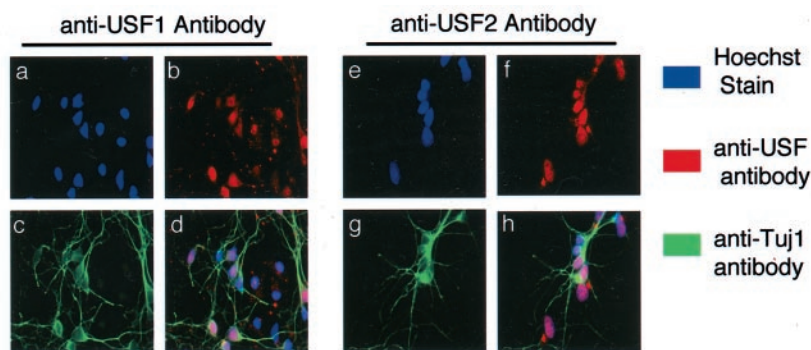


Figure 3. USFs mediate Ca^{2+} -dependent activation of *BDNF* promoter III. *a–h*, USFs are expressed in the nucleus of cultured cortical neurons. E18 cortical cultures were grown for 5 DIV and then fixed and stained with the anti-USF1 or anti-USF2 antibodies (*red*; *b, f*) and an antibody against the neuronal marker β -tubulin III (*anti-Tuj1*, *green*; *c, g*). Nuclei were stained with the Hoechst dye (*a, e*). Nuclear USFs in Tuj1-positive cells appear in *pink*, with *green* marking the neuronal processes (*d, h*). *i, j*, Dominant-negative forms of USF block Ca^{2+} -dependent activation of *BDNF* promoter III. E18 + 3 DIV rat cortical cultures were transfected with the *BDNF* promoter III luciferase reporter and a mammalian vector expressing either the dominant-negative forms of USF (*A-USF1* or *USF2DN*) or control vectors. A renilla luciferase reporter plasmid was cotransfected to normalize for transfection efficiency and sample handling.

BDNF promoter sequences that coimmunoprecipitated with the USF proteins. We were able to detect *BDNF* promoter III sequences in the USF immunoprecipitates (Fig. 4b). However, *BDNF* exon V, which should have been sheared away from promoter III, was not found in the pellet, indicating that immunoprecipitation of promoter III was specific (Fig. 4b). To control for antibody specificity, we performed the immunoprecipitation with a number of control antibodies and found that neither promoter III nor exon V of the *BDNF* gene immunoprecipitated with any of the control antibodies (Fig. 4b), although the anti-c-Myc antibody can precipitate its target promoters effectively in NIH 3T3 cells (data not shown). In total, these data strongly suggest that both USF1 and USF2 regulate transcription from promoter III of the endogenous *BDNF* gene *in vivo*.

USFs are activated by Ca^{2+} signals via L-VSCCs

The ability of USFs to regulate transcription of *BDNF* exon III through a Ca^{2+} -responsive element suggested to us that the transcriptional activity of USFs might be regulated by Ca^{2+} -activated signaling pathways in neurons. To isolate the activity of the USFs

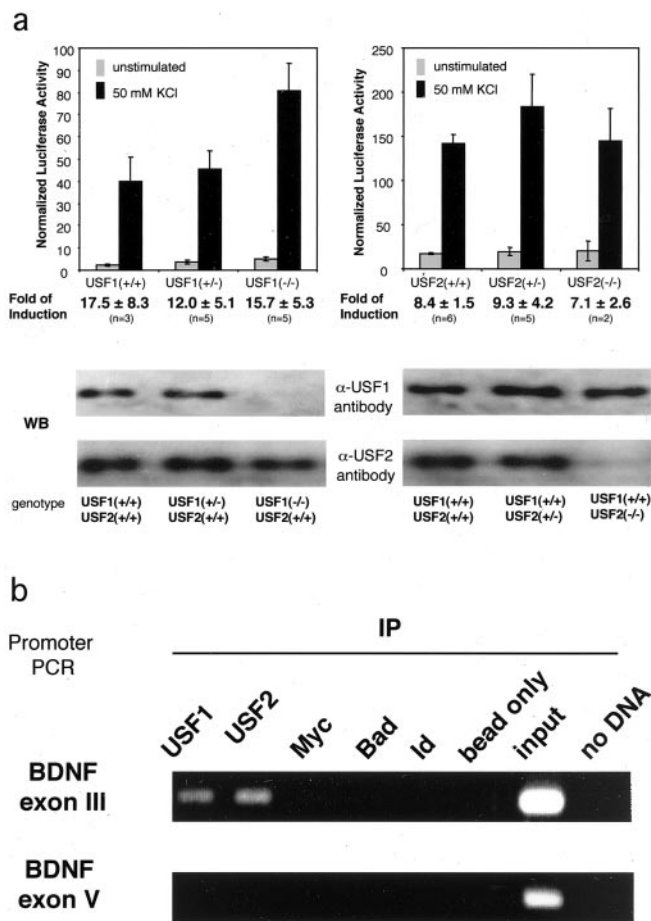


Figure 4. Endogenous USF1 and USF2 regulate *BDNF* promoter III *in vivo*. *a*, USF1 and USF2 are both capable of regulating the Ca^{2+} -inducible activation of *BDNF* promoter III. E16 cortical neurons were obtained from USF1 and USF2 wild-type, heterozygous, or homozygous null mice and cultured *in vitro* for 3 d before transfection with the *BDNF* promoter III luciferase reporter. *n* represents the number of pups used for the measurement. The genotypes for the mice were determined by Southern blot analysis. Western blot analysis shows the relative amounts of USF1 and USF2 in nuclear extracts from USF1 and USF2 wild-type, heterozygous, and homozygous mice with antibodies specific to the USF1 and USF2 proteins. *b*, Endogenous USF1 and USF2 bind *BDNF* promoter III *in vivo*. Cultured E18 + 5 DIV rat cortical neurons were treated with formaldehyde to cross-link DNA binding proteins to chromatin and then were subjected to chromatin immunoprecipitation with antibodies specific to USF1 and USF2 or a number of control antibodies. After reversing cross-links, we subjected eluted genomic DNA fragments to PCR with primers specific to *BDNF* promoter III or *BDNF* exon V. One percent of the input of the sheared chromatin before immunoprecipitation was used as a positive control for the PCR reaction. Negative controls include antibodies against c-Myc (a transcription factor), Bad (a cytoplasmic protein), Id (a bHLH protein lacking a DNA binding domain), or beads only.

from that of other Ca^{2+} -responsive transcription factors on *BDNF* promoter III, we studied the effects of calcium signaling pathways on transcription from a plasmid containing three copies of a consensus USF binding element in front of a luciferase reporter gene (3×UBE-Luc) (Qyang et al., 1999). Although a single USF element alone is not sufficient to confer a Ca^{2+} response in the context of *BDNF* promoter III, many transcription factors that act cooperatively at complex promoters can drive transcription independently when their elements are present in multiple copies close to the start site of transcription of a luciferase reporter gene (Tao et al., 1998, 2002). Indeed, when transfected into cultured cortical neurons, membrane depolarization induced a significant increase in transcription from the 3×UBE-Luc reporter plasmid (Fig. 5*a*), whereas no induction of transcription was seen from a plasmid containing the luciferase re-

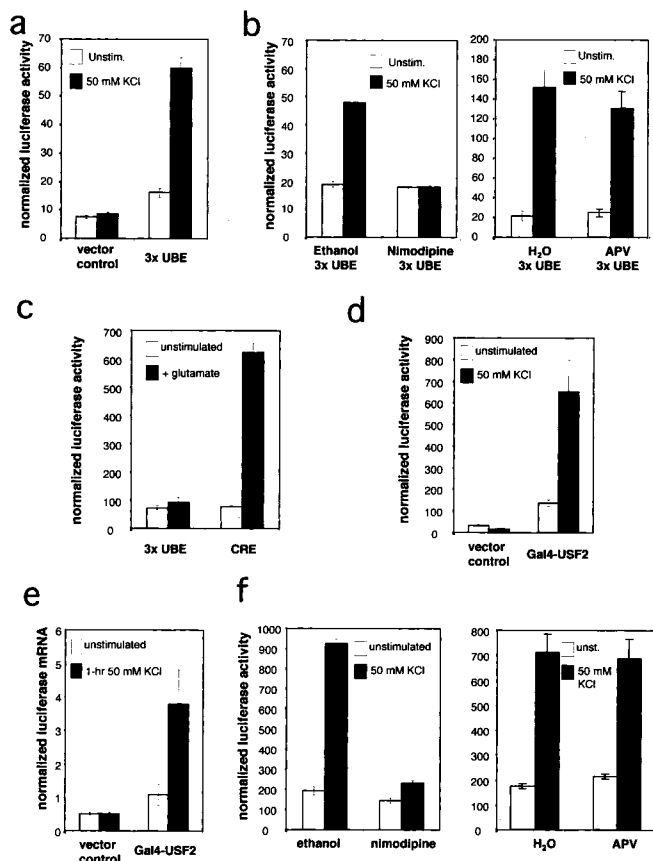


Figure 5. USFs are Ca^{2+} -regulated transcription factors activated via L-type VSCCs. *a*, USF binding element (UBE) is regulated by membrane depolarization. Cultured E18 + 3 DIV rat cortical neurons were transfected with either a firefly luciferase reporter gene driven by three repeats of the consensus USF binding element (3×UBE) or the control vector. *b*, *c*, USFs are activated by Ca^{2+} influx via L-type VSCC. Cultured E18 + 3 DIV rat cortical neurons were transfected with either the 3×UBE-Luc reporter plasmid or CRE-Luc plasmids. At 2 d after transfection the cells were treated with nimodipine, APV, or the carrier solution before depolarization with 50 mM KCl or glutamate stimulation. After 8 hr of stimulation the cells were lysed, and the luciferase activities were measured. *d*, *e*, The transcriptional activity of USF2 is regulated by membrane depolarization. Cultured E18 + 3 DIV rat cortical neurons were transfected with a Gal4-luciferase reporter plasmid and either an expression plasmid for the Gal4 DNA binding domain alone (control) or the Gal4-DNA binding domain fused to the transcriptional activation domain of USF2. *d*, A Renilla luciferase reporter plasmid was cotransfected as a control for transfection efficiency and sample handling. At 2 d after transfection the cells were depolarized with 50 mM KCl for 9 hr; then the cells were lysed, and the luciferase activities were measured. *e*, An α -globin expression vector was cotransfected to control for transfection efficiency and sample handling. At 2 d after transfection the neurons were depolarized with 50 mM KCl for 1 hr; then total RNA was purified. The total RNA was reverse transcribed into cDNA, and luciferase and α -globin cDNAs were measured by quantitative real-time PCR. *f*, The transcription activity of USF is regulated by Ca^{2+} influx via L-type VSCC. Cultured E18 + 3 DIV rat cortical neurons were cotransfected with Gal4-USF2 and Gal4-Luc plasmids. At 2 d after transfection the cells were treated with nimodipine, APV, or the carrier solution before depolarization with 50 mM KCl or glutamate stimulation. After 8 hr of stimulation the cells were lysed, and the luciferase activities were measured.

porter but lacking the USF binding enhancer sequence. Blocking L-VSCCs with nimodipine completely inhibited depolarization-induced transcription of the UBE reporter gene. In contrast, blockade of the NMDA type of glutamate receptors with APV had little effect (Fig. 5*b*). Furthermore, activation of glutamate receptors did not stimulate transcription from the USF reporter plasmid, although under these conditions glutamate effectively induced transcription from a CREB reporter gene (Fig. 5*c*). Taken together, these results suggest that Ca^{2+} influx via L-VSCCs reg-

ulates the transcriptional activation of the USFs in cortical neurons.

There are a number of mechanisms by which Ca^{2+} influx could regulate the USFs. In most cells surface receptor activation leads to nuclear translocation of USF2 (Frenkel et al., 1998). However, when we expressed USF1 or USF2 fused to green fluorescent protein (GFP) in cultured cortical neurons, we observed nuclear localization for both proteins independent of membrane depolarization (data not shown). In addition, immunofluorescent staining with anti-USF1- and anti-USF2-specific antibodies and preparation of nuclear extracts from cultured cortical neurons indicate that both USF1 and USF2 are primarily nuclear under both unstimulated and membrane-depolarized conditions (Fig. 3*a–h*; data not shown). A second possibility is that the USFs could undergo regulated binding to the CaRE2 element (Berger et al., 1998; Marmillot and Scovell, 1998; Cheung et al., 1999). However, we observed no change in the binding of the USFs to CaRE2 by EMSA, using nuclear extracts prepared from unstimulated or membrane-depolarized neurons (data not shown). In addition, using the ChIP assay, we found USF1 and USF2 bound to the endogenous *BDNF* promoter III under both unstimulated and membrane-depolarized conditions (data not shown), indicating that the DNA binding activities of USFs to *BDNF* promoter III are not regulated by Ca^{2+} influx into neurons.

Because Ca^{2+} influx does not appear to regulate the nuclear localization or DNA binding of the USFs, we considered the possibility that Ca^{2+} -dependent signaling pathways might regulate the transcriptional activation domains of the USFs directly. To determine whether Ca^{2+} influx directly regulates the ability of the USFs to activate transcription, we tethered the transcriptional activation domain of USF2 to the DNA binding domain of the yeast transcription factor Gal4 (Luo and Sawadogo, 1996b). When it was transfected into cortical neurons along with a reporter gene containing the Gal4 upstream-activating sequence driving expression of luciferase (Gal4-Luc), membrane depolarization induced a significant increase in luciferase expression (Fig. 5*d*). This induction requires the activation domain(s) of USF2 because the control vector that contains no USF2 showed no increase in response to membrane depolarization (Fig. 5*d*). Similar to the results observed for induction of the 3×UBE-Luc, pretreatment with nimodipine completely blocked the activation of Gal4-USF2, whereas APV had no significant effect (Fig. 5*f*). To evaluate whether Ca^{2+} induction of Gal4-USF2-dependent transcription occurs with a rapid time course that would be required for USFs to be Ca^{2+} -responsive regulators of *BDNF* transcription, we used a quantitative real-time RT-PCR assay to assess the induction of luciferase mRNA after 1 hr of membrane depolarization. Under these conditions we observed a significant membrane depolarization-dependent induction of the activity of Gal4-USF2 (Fig. 5*e*). These data indicate that the ability of USFs to activate transcription is enhanced by Ca^{2+} signaling pathways in neurons and suggest that a rapid biochemical modification of either the USFs or critical interacting proteins mediates Ca^{2+} induction of USF-dependent transcription.

USFs are general regulators of activity-dependent transcription

These experiments suggest that USFs are Ca^{2+} -regulated transcription factors in neurons and raise the possibility that USFs may contribute to the inducible expression of activity-regulated genes in addition to *BDNF*. In fact, a number of genes characterized *in vitro* by EMSA and by reporter gene assays as USF target genes (Paterson et al., 1995; Howcroft et al., 1999) also are known

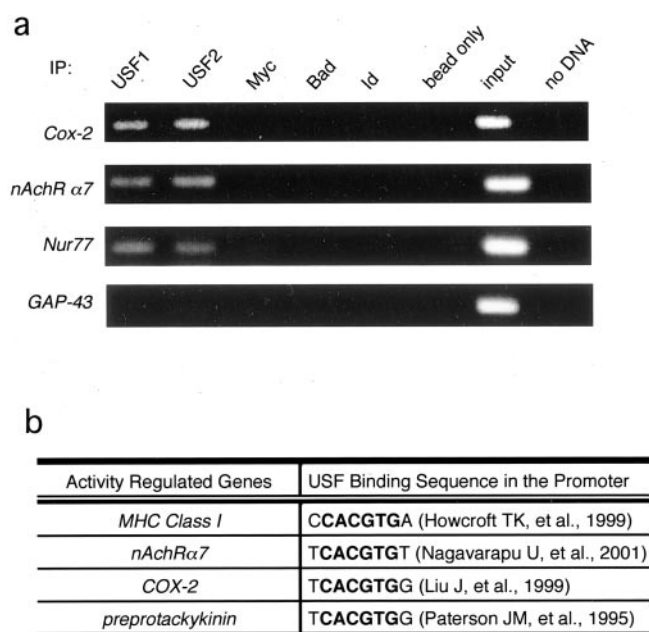


Figure 6. USFs are general regulators of Ca^{2+} -dependent gene transcription in the nervous system. *a*, USFs bind to the promoters of several activity-regulated neuronal genes *in vivo*. Cultured E18 + 5 DIV rat cortical neurons were treated with formaldehyde to cross-link DNA binding proteins to chromatin and were subjected to chromatin immunoprecipitation with antibodies specific to USF1 and USF2 or a number of control antibodies. After reversing the cross-linking, we subjected eluted genomic DNA fragments to PCR with primers specific for *COX-2*, *Nur77*, *nAChR α 7*, and *GAP-43* promoters. *b*, Neuronal activity-regulated genes that are known USF targets.

to be induced by neuronal activity *in vivo* (Fig. 6*b*), although the USFs previously have not been suggested to mediate this induction. We used the ChIP assay (described in Fig. 4*b*) to assess whether the USFs bind to the endogenous promoters of several of these activity-regulated genes in neurons. As shown in Figure 6*a*, anti-USF1 and anti-USF2 antibodies specifically precipitate the promoters for *COX-2* and *nAChR α 7*, which are known to be activity-regulated *in vivo* (Yamagata et al., 1993; Zhou et al., 2001) and to be USF target genes *in vitro* (Liu et al., 1999; Nagavarapu et al., 2001). Anti-USF antibodies also precipitated the promoter for the immediate early gene *Nur77*, suggesting that *Nur77* also may be a USF target gene. To determine the specificity of anti-USF antibodies in these ChIP experiments, we showed that these anti-USF antibodies do not pull down the *GAP-43* promoter, which is neither activity-regulated nor a known USF target gene. Given that the transcriptional activity of the USFs is regulated by Ca^{2+} signaling in neurons and that USFs are bound to the endogenous promoters of a number of activity-inducible genes, these findings suggest that USF1 and USF2 may orchestrate the activity-regulated expression of a sizable group of neuronal genes that are important for neural development and synaptic plasticity.

Discussion

USFs regulate activity-dependent transcription from *BDNF* promoter III

In this study we have defined a new Ca^{2+} -responsive E-box element, CaRE2 (ATCATATGAC), in *BDNF* promoter III. This element is required to confer Ca^{2+} -responsive transcriptional activation of *BDNF* promoter III. Using a yeast one-hybrid screen, we identified the upstream stimulatory factors (USF1 and USF2) as CaRE2 binding proteins. Several lines of evidence indicate that

the USFs are relevant regulators of activity-dependent *BDNF* transcription through CaRE2. We have shown that endogenous USF1 and USF2 from neuronal nuclear extracts bind to the CaRE2 sequence *in vitro*. The USFs are sufficient to bind directly to the CaRE2 sequence and only bind CaRE2 sequences that support activity-dependent transcription of the *BDNF* promoter III luciferase reporter. Overexpression of dominant-negative forms of the USFs block activity-dependent transcription from *BDNF* promoter III, suggesting that the transcriptional activity of the USFs is required for this induction. Finally, using a chromatin immunoprecipitation assay, we demonstrate that in neurons both USF1 and USF2 are bound to the CaRE2-containing region of promoter III in the endogenous *BDNF* gene. Either USF1 or USF2 appears to be sufficient to support activity-dependent *BDNF* expression, because we observe that depolarization-induced increases in *BDNF* promoter III activity occur normally in mice null for either USF1 or USF2, as well as in USF1(−/−)/USF2(+/−) and USF1(−/+)/USF2(−/−) mice. Because the constitutive USF1/2 double knock-out mice exhibit early embryonic lethality (Sirito et al., 1998), loss-of-function studies that corroborate the role played by the USFs in *BDNF* transcription will await the generation of conditional or brain-specific USF1/2 double knock-out mice. Nonetheless, in total, our data strongly support a critical role for the USFs in the induction of activity-dependent transcription from *BDNF* promoter III.

A new role for the USFs in the CNS

Although the USFs were among the first bHLH transcription factors to be identified and they were shown to be expressed in brain (Sirito et al., 1994), little was known about their function in the nervous system. Our data now suggest that these transcription factors may play an important role in the regulation of activity-dependent gene expression in neurons. We find that both USF1 and USF2 are expressed in the nuclei of neurons throughout the brain, including both the neocortex and the hippocampus (Fig. 5*a*; data not shown), brain regions in which neuronal activity induces transcription of a large set of genes. In these neurons both USF1 and USF2 are bound to the endogenous promoters of a number of activity-induced genes for which the products are important regulators of synaptic maturation and function, including *BDNF*, *nAChR α 7*, and *COX-2*. In addition, we find that USF1 and USF2 are bound to the promoter for the *Nur-77* gene, an immediate early gene that encodes a transcription factor for which the expression is upregulated dramatically by neuronal activity.

On *BDNF* promoter III we have shown that the USFs cooperate with other transcription factors to regulate activity-dependent gene expression. We have identified three discrete Ca²⁺-response elements (CaRE1, CaRE2, and CaRE3/CRE) in *BDNF* promoter III that are required for the induction of exon III transcription in response to Ca²⁺ influx in neurons (Tao et al., 1998, 2002). Mutation of any one of the CaREs effectively blocks the activity-dependent induction of *BDNF* exon III transcription, indicating that within the context of *BDNF* promoter III these three elements cooperatively promote transcription. However, when isolated from *BDNF* promoter III, each of these elements is independently inducible by neuronal activity, and each is bound by distinct transcription factors (CaRF, USF1/2, or CREB) for which the transcriptional activity can be modulated by Ca²⁺ signaling in neurons (Tao et al., 1998, 2002). These observations raise the question as to why all three factors are required to promote transcription in the context of the intact *BDNF* promoter III.

One possibility is that each of the *BDNF* regulatory factors serves a unique but essential role in regulating Ca²⁺ induction of *BDNF* transcription. Like the induction of *BDNF* exon III expression itself, the activity of the CaRE1 binding protein CaRF is regulated in a Ca²⁺- and neural-selective manner, suggesting that this factor may confer stimulus and cell type selectivity with the expression of *BDNF* exon III (Tao et al., 2002). In contrast, the CaRE3/CRE binding protein CREB can be activated by a wide variety of stimuli, all of which lead to the phosphorylation of CREB at Ser¹³³. However, recent studies have shown that Ca²⁺ influx into neurons induces phosphorylation of CREB at two additional sites, Ser¹⁴² and Ser¹⁴³, both of which also are required for calcium-mediated CREB-dependent transcription in neurons (Kornhauser et al., 2002). Phosphorylation at these two additional sites appears to disrupt the interaction of CREB with CBP (Parker et al., 1999), raising the possibility that a factor other than CBP may mediate Ca²⁺ regulation of CREB-dependent transcription in neurons. USF activity together with CaRF and CREB may be required for the modification of chromatin structure and the recruitment of the basal transcriptional machinery to *BDNF* promoter III, thereby facilitating effective Ca²⁺ regulation of *BDNF* promoter III.

Interestingly, USF binding elements are found in tandem with CREB binding elements in a number of promoters (Cvekl et al., 1994; Durham et al., 1997; Kingsley-Kallesen et al., 1999), suggesting that cooperative activation of these two factors may be critical for the transcription of a number of Ca²⁺-inducible neuronal genes. A recent report suggests that the USFs may also be involved in the regulation of *BDNF* promoter I (Tabuchi et al., 2002). Anti-USF antibodies were able to supershift a CRE binding complex on *BDNF* promoter I, and a dominant-interfering form of USF (A-USF) was found to reduce moderately the calcium inducibility of *BDNF* promoter I. It remains to be determined whether the USFs directly interact with *BDNF* promoter I and whether USFs associate with this promoter *in vivo* as assessed by chromatin immunoprecipitation analysis. Because A-USF can act to sequester other USF interacting proteins such as E47 (Dear et al., 1997), the exact role of USF family members in *BDNF* promoter I regulation awaits further characterization. However, the potential involvement of USFs together with CREB in the regulation of alternative *BDNF* promoters provides support for the idea that cooperation between these two calcium-responsive factors plays a role in regulating activity-dependent transcription in neurons.

Regulation of USF transcriptional activity

In addition to showing that CaRE2 is required for activity-dependent induction of *BDNF* promoter III, our findings suggest that the activity of USFs may be induced by calcium influx into neurons. As shown in Figure 5, membrane depolarization induced robust activation of USF-dependent transcription. This effect was blocked completely by L-type VSCCs-specific inhibitors but was unaffected by NMDA receptor inhibitors, suggesting that Ca²⁺ entry via L-type VSCCs mediates the activation of USF-dependent transcription in membrane-depolarized neurons. In contrast to membrane depolarization with elevated levels of KCl, the addition of glutamate to cultured neurons failed to activate USF-dependent transcription. Under these conditions glutamate induces significant Ca²⁺ influx through NMDA receptors and a modest amount of calcium influx through the L-type VSCC (Bading et al., 1995). The failure of glutamate to induce USF-dependent transcription under these conditions suggests that Ca²⁺ influx through the NMDA receptor may not

activate USF activity. Glutamate-induced calcium influx through the L-type VSCC under these conditions appears not to be sufficient to activate the signaling molecules responsible for USF transcriptional activation. It is possible that the calcium signaling pathways activated by the NMDA receptors may be antagonistic to those activated by the L-VSCCs. Experiments designed to distinguish among these possibilities may yield insights into the integrative effect of calcium signals on activity-dependent neuronal gene expression.

Because the transcriptional activity of a Gal4-USF2 fusion protein also can be regulated rapidly by calcium signaling pathways in neurons, we postulate that a post-translational modification of either USFs or components of the transcriptional machinery that USFs bring to the promoters of Ca²⁺-responsive genes may mediate Ca²⁺ induction of USF-dependent transcription. Phosphorylation is a common means of regulating the activity of transcription factors, and USF1 has been reported to be a phosphoprotein in HeLa cells (Galibert et al., 1997). In melanocytes UV stress induction of the phosphorylation of USF1 by p38 MAP kinase is required for UV-induced expression of *Tyrosinase* (Galibert et al., 2001). Our preliminary data from two-dimensional isoelectric focusing experiments suggest that, when overexpressed in neurons, both USF1 and USF2 exist as multiple species with distinct isoelectric focusing points, consistent with the phosphorylation of the USFs at multiple sites (data not shown). Several kinases are known to be activated in neurons in response to calcium influx via L-VSCCs, including the MAP kinases Erk1/2 and p38, the calcium-calmodulin kinases II and IV, and protein kinase A (Mao et al., 1999; Shaywitz and Greenberg, 1999; Dolmetsch et al., 2001). However, whether phosphorylation of USFs or USF-associated factors is regulated by neuronal activity remains to be determined.

One mechanism by which neuronal activity might control the function of the USFs is by regulating the interaction between USFs and components of the basal transcription machinery. The USFs have been shown to interact physically with TAFII55, and via this interaction USFs can recruit the TATA-box binding complex TFIID (Workman et al., 1990; Reach et al., 1991; Kokubo et al., 1993; Chiang and Roeder, 1995). The USFs also have been shown to interact with TFII-I, a factor highly expressed in brain that is thought to mediate transcription from TATA-less promoters via binding to an Inr element (Roy et al., 1991, 1997; Cheriyaath et al., 1998). *BDNF* promoter III is a TATA-less promoter that contains an Inr element near the transcriptional initiation site (Timmusk et al., 1993), suggesting that *BDNF* promoter III could be regulated by a TFII-I-dependent mechanism. Future studies aimed at elucidating the molecular events that control the recruitment of these and other basal transcription factors to activity-dependent promoters such as *BDNF* promoter III may help to identify the mechanism by which USFs mediate Ca²⁺-dependent transcription in neurons.

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