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Regulation of *cpg15* by signaling pathways that mediate synaptic plasticity

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Abstract

Transcriptional activation is a key link between neuronal activity and long-term synaptic plasticity. Little is known about genes responding to this activation whose products directly effect functional and structural changes at the synapse. *cpg15* is an activity-regulated gene encoding a membrane-bound ligand that regulates dendritic and axonal arbor growth and synaptic maturation. We report that *cpg15* is an immediate-early gene induced by Ca^{2+} influx through NMDA receptors and L-type voltage-sensitive calcium channels. Activity-dependent *cpg15* expression requires convergent activation of the CaM kinase and MAP kinase pathways. Although activation of PKA is not required for activity-dependent expression, *cpg15* is induced by cAMP in active neurons. CREB binds the *cpg15* promoter in vivo and partially regulates its activity-dependent expression. *cpg15* is an effector gene that is a target for signal transduction pathways that mediate synaptic plasticity and thus may take part in an activity-regulated transcriptional program that directs long-term changes in synaptic connections.

Introduction

Regulation of gene expression is critical for bringing about long-term changes in synaptic connections in response to neuronal activity (Goelet et al., 1986; Sheng and Greenberg, 1990). Synaptic activation results in Ca^{2+} influx through N-methyl-D-aspartate (NMDA) receptors and L-type voltage-sensitive calcium channels (VSCCs), triggering several kinase pathways (Sheng et al., 1991; Bading et al., 1993; Deisseroth et al., 1996). Three kinase pathways that play critical roles in various aspects of long-term plasticity are the Ca^{2+}/calmodulin-dependent protein kinase (CaMK), the mitogen-activated protein kinase (MAPK), and the protein kinase A (PKA) pathways. These pathways activate transcription factors and cofactors such as cAMP-responsive element binding protein (CREB), CREB-binding protein (CBP), the ternary complex factor Elk-1, and Ca^{2+} response factor CaRF, which in turn activate transcription of activity-regulated genes (Sheng et al., 1991; Xia et al., 1996; Chawla et al., 1998; Hu et al., 1999; Tao et al., 2002). Pharmacological and genetic studies have shown that the CaMK, MAPK, and PKA pathways and downstream factors like CREB are important for the electrophysiological paradigms of plasticity, long-term potentiation, and long-term depression, as well as for learning and memory (reviewed in Yin and Tully, 1996; Brandon et al., 1997; Silva et al., 1998; Impey et al., 1999; Orban et al., 1999; Soderling, 2000; Sweatt, 2001; Lisman et al., 2002). These pathways have also been shown to generate structural changes that can alter neuronal connectivity (Wu and Cline, 1998; Wu et al., 2001; Redmond et al., 2002; Vaillant et al., 2002). Despite strong evidence linking kinase pathways and transcription factors to long-term plasticity, little is known about how activation of these upstream regulatory molecules leads to long-term structural and functional changes at the synapse. Identifying effector genes regulated by these pathways is a crucial first step in elucidating the cellular processes that underlie plasticity. Among known activity-regulated genes, only a handful have been shown to function as effector genes whose products directly mediate functional and structural changes

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at the synapse. Of these, upstream regulation of only two genes, *brain-derived neurotrophic factor (BDNF)* and *homer/vesl*, has been studied (Shieh et al., 1998; Tao et al., 1998; Sato et al., 2001).

candidate plasticity gene 15 (cpg15) was isolated in a screen for activity-regulated genes induced by kainate-stimulated seizure in the rat dentate gyrus (Nedivi et al., 1993) and was subsequently shown to be sensitive to physiological stimuli such as light in the visual cortex (Nedivi et al., 1996). Its temporal and spatial expression patterns and its regulation by sensory input correlate with times and places of activity-dependent developmental plasticity (Corriveau et al., 1999; Nedivi et al., 2001; Lee and Nedivi, 2002). When overexpressed in *Xenopus* optic tectal neurons, CPG15 induces elaboration of dendritic and axonal arbors and synaptic maturation by AMPA receptor insertion (Nedivi et al., 1998; Cantallops et al., 2000). These properties make *cpg15* a potential target for activation by signal transduction pathways that lead to long-term plasticity.

Here we use cultured cortical neurons to investigate the pathways that lead to transcriptional activation of *cpg15* by synaptic activity. These studies demonstrate that *cpg15* is an immediate-early gene (IEG) regulated by multiple-signal transduction pathways, transcription factors, and promoter elements that have been strongly implicated in plasticity. Comparing the regulation of activity-induced effector genes will identify those salient properties of signaling pathways that mediate plasticity.

Results

cpg15 is an IEG induced by synaptic activity through NMDA receptors and L-type VSCCs

To study transcriptional regulation of the *cpg15* gene, we first examined whether *cpg15* expression in mouse primary neuronal cultures adequately reflects its in vivo regulation. Cortical neurons cultured for 14 div were treated for 9 h with the sodium channel blocker tetrodotoxin (TTX) to block action potential activity or with the γ-aminobutyric acid (GABA) antagonist picrotoxin (PTX) to stimulate neurons. GABA receptor blockade releases the tonic inhibition imposed by inhibitory neurons in the culture, causing excitatory neurons to fire synchronous bursts of action potentials (Hardingham et al., 2001). A single band of 2.0 kb was detected on Northern blots with a *cpg15* probe (Fig. 1A), consistent with the predicted transcript length (Naeve et al., 1997). TTX-treated cultures showed low levels of *cpg15* mRNA, indicating that basal *cpg15* expression is maintained in the absence of action potential firing. Nontreated cultures showed an approximately two-fold higher level of *cpg15* expression than TTX-treated cultures (Fig. 1B), likely due to spontaneous activity. PTX-treated cultures showed an approximately five-fold higher expression of *cpg15* than TTX-treated cultures. These results show that *cpg15* levels in primary cortical cultures reflect a combina-

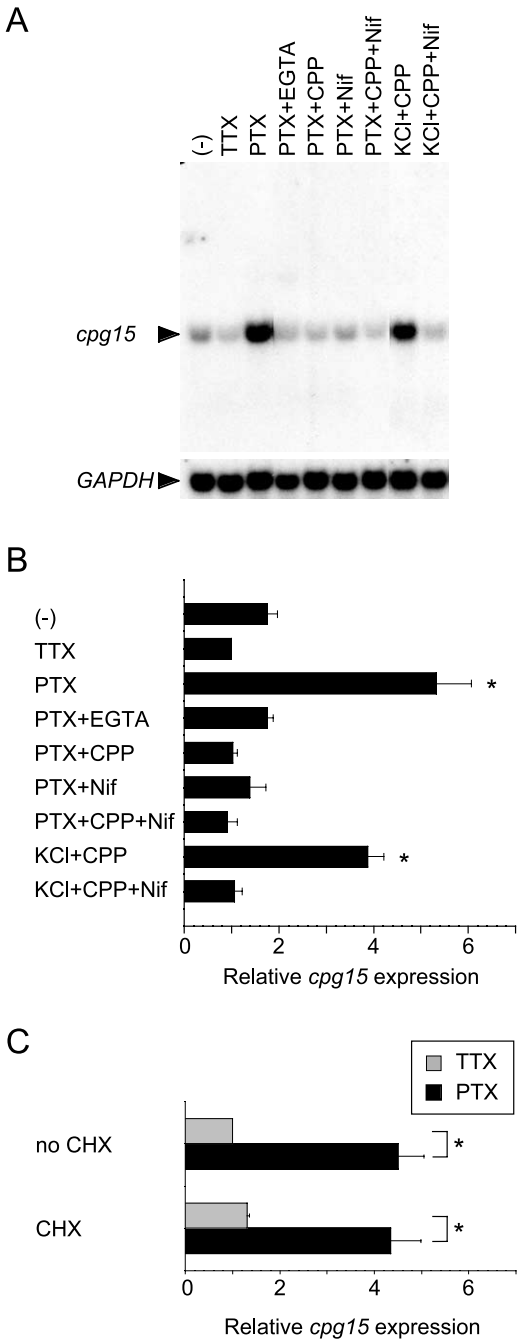

Fig. 1. *cpg15* is an IEG induced by synaptic activity through activation of NMDA receptors and L-type VSCCs. (A) Northern blots of total RNA prepared from cortical cultures treated for 9 h at 14 div. Cultures were untreated (−) or treated with TTX (1 μM), PTX (50 μM), or KCl (50 mM) in the absence or presence of EGTA (2 mM), CPP (10 μM), or nifedipine (Nif, 5 μM) as indicated above. (B) Quantification of *cpg15* mRNA levels shown in A normalized to a *GAPDH* loading control. *cpg15* expression in TTX-treated cultures was designated 1. Cultures treated with PTX or KCl and CPP showed significantly higher *cpg15* expression compared to TTX-treated cultures (*$P < 0.01$ versus TTX, ANOVA and SNK *post hoc* test; $n = 3$). PTX-induced *cpg15* expression was Ca^{2+}-dependent and required NMDA receptors and L-type VSCCs. KCl-induced *cpg15* expression required L-type VSCCs but not NMDA receptors. (C) Activity-dependent *cpg15* expression is protein synthesis independent. Cultures were treated with TTX or PTX in the absence or presence of cycloheximide (CHX). *cpg15* expression was analyzed as in B. PTX-induced *cpg15* expression was not significantly effected by the presence of cycloheximide (*$P < 0.05$, ANOVA and SNK *post hoc* test; $n = 2$), thus defining *cpg15* as an IEG.

tion of activity-independent and activity-dependent expression, consistent with *cpg15* regulation seen in vivo (Corriveau et al., 1999; Lee and Nedivi, 2002).

Elevation of intracellular Ca^{2+} is a key step in the translation of neuronal activity into changes in gene expression (Ghosh et al., 1994; West et al., 2001). The site of Ca^{2+} entry can determine the signaling pathways activated, the genes transcribed, and the biological outcome of their activation (Bading et al., 1993; Ghosh and Greenberg, 1995; Hardingham et al., 2002). The Ca^{2+} chelator EGTA blocked the PTX-induced increase in *cpg15* expression (Fig. 1B), indicating a requirement for extracellular Ca^{2+} in activity-dependent regulation of the *cpg15* gene. To distinguish whether this requirement was associated with a specific site of Ca^{2+} entry, PTX-stimulated cultures were treated with the NMDA receptor antagonist CPP or the L-type VSCC antagonist nifedipine. We found that either CPP or nifedipine alone completely blocked PTX-induced *cpg15* expression, but their effect was not additive (Fig. 1B). These results demonstrate that activity-dependent *cpg15* expression requires Ca^{2+} influx through both the NMDA receptors and L-type VSCCs.

In the case of other activity-regulated genes such as *c-fos* and *BDNF*, activation of L-type VSCCs is sufficient for gene activation (Murphy et al., 1991; Shieh et al., 1998; Tao et al., 1998). To examine if the dual requirement for NMDA receptors and L-type VSCCs activation was specific for synaptic stimulation by PTX, we stimulated cultures by depolarizing cells with KCl in the presence of CPP. This treatment significantly increased *cpg15* expression (Fig. 1B), indicating that NMDA receptors are not required when VSCCs are directly activated by depolarization. *cpg15* induction by KCl and CPP was blocked by nifedipine, indicating that L-type VSCCs are required for activity-dependent *cpg15* expression.

Ca^{2+} influx through NMDA receptors or L-type VSCCs activates signal transduction pathways that can directly induce IEGs without the requirement for protein synthesis. Some IEGs are themselves transcription factors that activate additional downstream genes. To determine if *cpg15* is an IEG or is induced by IEGs in a protein synthesis-dependent manner, cortical cultures were stimulated with PTX for 9 h in the presence of the protein synthesis inhibitor cycloheximide (CHX). *cpg15* induction by PTX was comparable in the presence or absence of CHX (Fig. 1C). CHX blockade of protein synthesis by more than 95% was confirmed by [^{35}S]methionine incorporation (data not shown). This result indicates that *cpg15* is an IEG.

A 1.9-kb promoter fragment is sufficient to confer cpg15's activity-dependent regulation

To identify the region of *cpg15* that directs its activity-dependent transcription, we isolated the mouse *cpg15* gene and mapped its exon–intron structure and promoter region. Genomic Southerns identified a single copy *cpg15* gene (data not shown). The *cpg15* gene contains three exons, encoding amino acids 1–19, 19–67, and 67–142 of CPG15 (summarized in Fig. 2A). To test if the 1.9-kb genomic fragment upstream of the *cpg15* translation start site is sufficient for activity-dependent regulation, we fused this fragment to the promoterless firefly luciferase gene. This reporter plasmid (*cpg15-Luc*) was transfected into cortical cultures, and promoter function was measured by luciferase activity. Transcription driven by the 1.9-kb *cpg15* upstream fragment after 9 h of TTX treatment was significantly higher than that of a promoterless control plasmid (Fig. 2B), indicating that this fragment contains a functional promoter. When *cpg15-Luc* transfected cultures were stimulated for 9 h with PTX, we observed an approximately two-fold increase in *cpg15* promoter activity compared to TTX-treated cultures (Fig. 2B). Luciferase expression driven by the SV40 promoter was unaffected by PTX treatment. These results indicate that the 1.9-kb upstream region of the *cpg15* gene can drive activity-dependent regulation.

We further found that similar to the case for the endogenous *cpg15*, addition of EGTA, CPP, nifedipine, or both CPP and nifedipine all blocked PTX-induced *cpg15* promoter activity (Fig. 2C). Thus, the regulation mediated by the 1.9-kb *cpg15* upstream fragment is qualitatively similar to that seen for the endogenous *cpg15* gene in cortical cultures.

The cpg15 promoter contains multiple potential binding sites for activity-regulated transcription factors

We sequenced the 1.9-kb *cpg15* upstream region to identify potential binding sites for transcription factors known to be regulated by synaptic activity. Two TATA boxes were present at 320 and 354 bp upstream of the translation start site. We infer that the upstream TATA box is the polymerase-binding site because it is conserved between mice and humans. Within the 1.9-kb upstream region, the TFSEARCH program identified three sequences similar to the CREB binding site, CRE, three sequences similar to the AP-1 binding site, TPA-responsive element (TRE), and two sequences similar to the early growth response (EGR) family binding site, EGR responsive element (EGR RE) (Table 1). In addition, we identified by sequence comparison a sequence similar to the CaRF binding site, CaRE1, found in the *BDNF* promoter. Of these sites, two CRE-like sites, one TRE-like site, and the CaRE1-like site were conserved between the mouse and human *cpg15* genes (Table 1). Thus, the *cpg15* promoter contains at least nine potential binding sites for transcription factors that are known to be responsive to neuronal activity (Saffen et al., 1988; Sukhatme et al., 1988; Sheng and Greenberg, 1990; Worley et al., 1990, 1991; Tao et al., 2002).

CREB and EGR family members bind to sequences in the cpg15 promoter

To test if the sequences in the *cpg15* promoter similar to CRE, TRE, EGR RE, and CaRE1 bind their respective

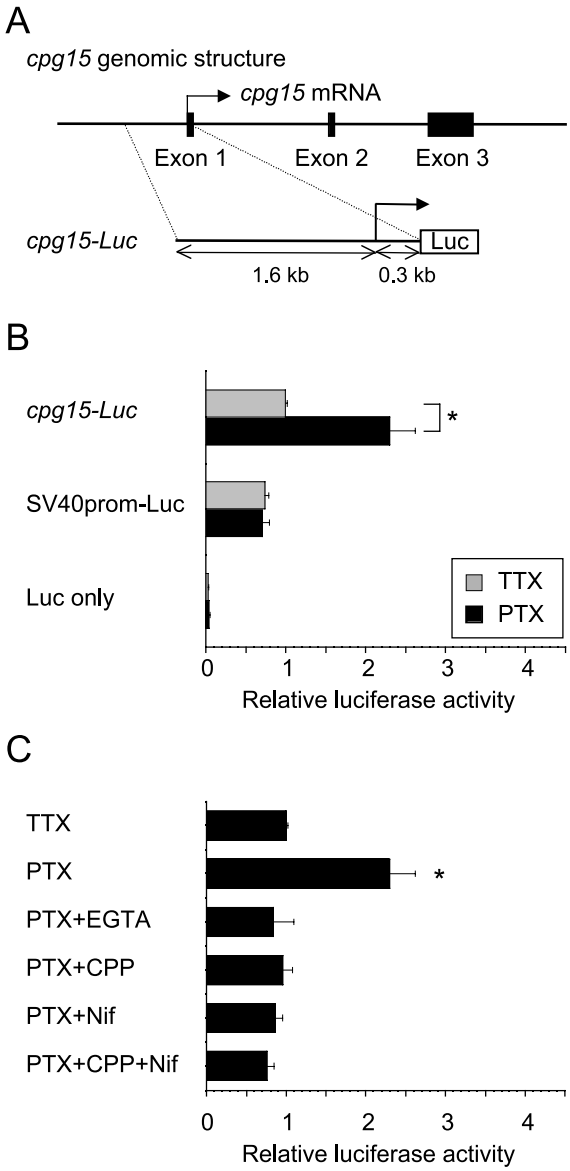

Fig. 2. A 1.9-kb *cpg15* promoter fragment mediates activity-dependent reporter gene expression. (A) Schematic diagram of the *cpg15* genomic structure and the *cpg15-Luc* reporter construct. Closed boxes indicate the three exons of the *cpg15* gene. The *cpg15-Luc* plasmid contains 1.6 kb of *cpg15*'s promoter region and 0.3 kb of its 5′ untranslated region fused to the luciferase reporter gene (Luc). (B) The 1.9-kb *cpg15* upstream fragment drives both activity-independent and activity-dependent transcription. Cortical cultures were transfected with the *cpg15-Luc* reporter plasmid, with the pGL3-promoter vector carrying an SV40 promoter (SV40prom-Luc), or with the promoterless pGL3-basic vector (Luc only) at 6 div. At 14 div, cultures were treated with TTX or PTX for 9 h. Promoter activity measured as firefly luciferase activity was normalized to *Renilla* luciferase activity from the cotransfected plasmid pRL-TK. Luciferase activities shown are relative to *cpg15-Luc* transfected cells treated with TTX. Luciferase activity in the presence of the *cpg15* promoter was higher than in its absence. PTX treatment further increased the luciferase activity from *cpg15-Luc* as compared to TTX treated cells (*$P < 0.01$, ANOVA and SNK *post hoc* test; $n = 6$). (C) PTX-induced *cpg15* promoter activity requires Ca^{2+} influx through NMDA receptors and L-type VSCCs. Cortical cultures transfected with *cpg15-Luc* were stimulated with PTX for 9 h in the absence or presence of the indicated pharmacological agents. The PTX-induced increase in luciferase activity driven by the *cpg15* promoter was blocked by EGTA, CPP, and nifedipine (*$P < 0.01$ versus TTX, ANOVA, and SNK *post hoc* test; $n = 6$).

transcription factors, we performed EMSAs. Double-stranded oligonucleotides corresponding to each candidate site were labeled with ^{32}P, incubated with nuclear extracts from adult mouse brain, and subsequently separated on a nondenaturing polyacrylamide gel. We compared the DNA/protein complexes detected using the candidate binding sites in the *cpg15* promoter, with those detected using the CRE, TRE, EGR RE consensus sequences or the *BDNF* CaRE1. Protein binding was considered specific if it could be competed away with excess unlabeled oligonucleotides of the wild-type consensus sequence but not with a mutated version of this sequence. Consensus sequences of CRE, TRE, EGR RE, and *BDNF* CaRE1 all showed specific binding to factors in the mouse brain extracts, showing that factors specific for these sites are present in brain nuclear extracts (Fig. 3 and data not shown). Only four of the nine potential sites from the *cpg15* promoter formed DNA/protein complexes with similar mobility and specificity as those seen with the consensus sequence of their corresponding transcription factors (Table 1). These were the three CREs at −1.56 kb, −0.84 kb, and +0.04 kb, and the −1.58 kb EGR RE.

The three CREs in the *cpg15* promoter each formed two DNA/protein complexes, with similar mobility and specificity as those seen using the CRE consensus sequence (Fig. 3A). The intensity of each DNA/protein band varied between the sites, suggesting differences in the binding affinities of the three sites. The intensity ratio of the high-mobility and low-mobility bands also differed between each site, indicating that although all three sites bind the same size factors, each site may have a different binding preference for these factors. For example, the −1.56-kb CRE has slightly higher affinity for the factor forming the more slowly migrating band, whereas the +0.04-kb CRE has higher affinity for the factor forming the faster migrating band. CRE can bind at least three CREB family members:

Table 1
In vitro binding activity of the potential binding sites in the *cpg15* promoter to factors in brain nuclear extracts

Transcription factor	Position (kb)[a]	DNA sequence	In vitro binding[b]	Conservation in human[c]
CREB	−1.56	TGACATCA	+	+*
	−0.84	TGACTTCA	+	−
	+0.04	CCACGTCA	+	+
AP-1	−1.34	TGACTGT	−	−
	−0.10	TCAGTCA	−	−
	−0.05	TGATTAA	−	+
EGR	−1.59	CGCCCCCGC	−	−
	−1.58	CGCCCACCC	+	−
CaRF	−0.62	CTATTTCTGG	−	+*

[a] Position relative to transcription start site.

[b] Binding to factors in nuclear extract of adult mouse brain determined by EMSA. (+) or (−) indicate presence or absence, respectively, of specific bands with similar mobility as those detected using the consensus binding site of the corresponding transcription factor.

[c] Conservation of sites in the human *cpg15* gene. An asterisk indicates one base substitution.

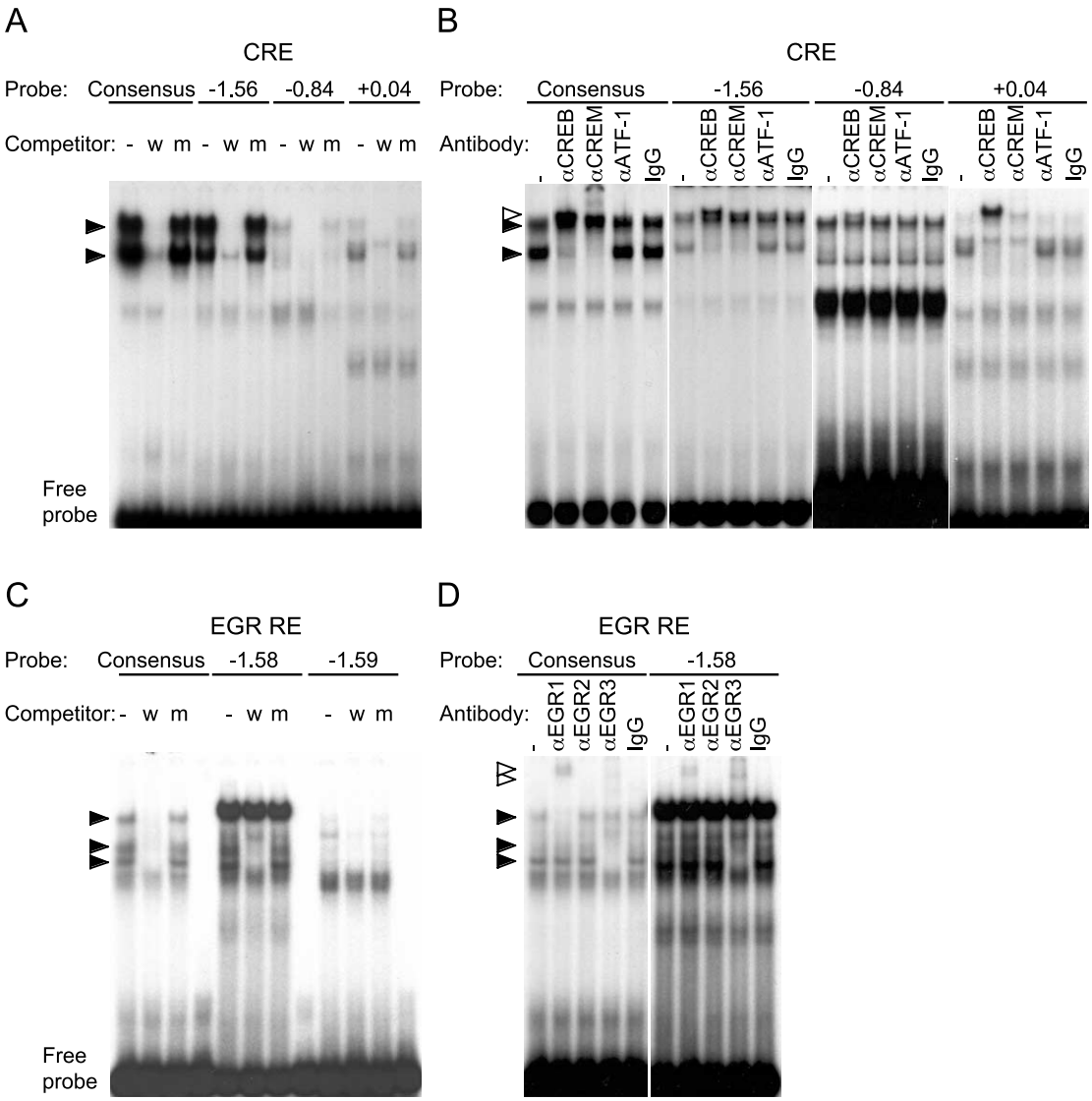

Fig. 3. Three *cpg15* CREs bind CREB, CREM, and an unknown factor in brain nuclear extracts. The −1.58-kb EGR RE binds EGR1, EGR3, and an unknown factor. (A) In EMSAs, ^{32}P-labeled oligonucleotides containing the CRE consensus or each of the CRE-like sequences in the *cpg15* promoter (at −1.56 kb, −0.84 kb, and +0.04 kb) bind two factors from mouse brain nuclear extracts and form DNA/protein complexes with similar electrophoretic mobility (marked by closed arrowheads). The binding is specific, as it can be competed away with excess unlabeled consensus CRE oligonucleotide (w), but not with the same oligonucleotide containing two point mutations (m). (B) EMSAs were done for the consensus and *cpg15* CREs in the presence of antibodies against CREB, CREM, ATF-1, or control IgG. Anti-CREB antibody caused a supershift of the higher mobility DNA/protein complex seen with all four oligonucleotides (open arrowheads). A similar supershift was caused by anti-CREM antibody, but not by antibodies against ATF-1 or control IgG. (C) EMSAs using EGR RE consensus or each of the *cpg15* EGR REs show that only the consensus and −1.58-kb EGR RE bind factors in mice brain nuclear extracts. Binding to these two factors is specific as seen by competition with wild-type and not mutant EGR RE oligonucleotide (closed arrowheads). A third specific band observed with EGR RE consensus could not be resolved with the −1.58-kb EGR RE site due to a comigrating nonspecific band. (D) EMSAs were done for the consensus and the −1.58-kb EGR RE site in the presence of antibodies against EGR1, EGR2, EGR3, or control IgG. Anti-EGR1 antibody supershifted the slowest migrating DNA/protein complex, whereas anti-EGR3 antibody supershifted the fastest migrating complex (open arrowheads). Although the slowest migrating band was not resolved with the −1.58-kb EGR RE site, addition of anti-EGR1 antibody resulted in a supershift similar to that seen with the consensus EGR RE.

CREB, CRE modulatory factor (CREM), and activating transcription factor-1 (ATF-1) (Shaywitz and Greenberg, 1999). To determine if any of these factors are components of the bands detected with the *cpg15* CREs, antibodies to each CREB family member were added to the EMSAs, and the effect on complex mobility was assessed. In the presence of the anti-CREB antibody, there was a supershift of the faster migrating DNA/protein complex formed with the CRE consensus, as well as with the *cpg15* CREs (Fig. 3B). This suggests that all three sites bind CREB. The anti-CREM antibody caused a supershift of the same band as the anti-CREB antibody, suggesting that these sites also bind

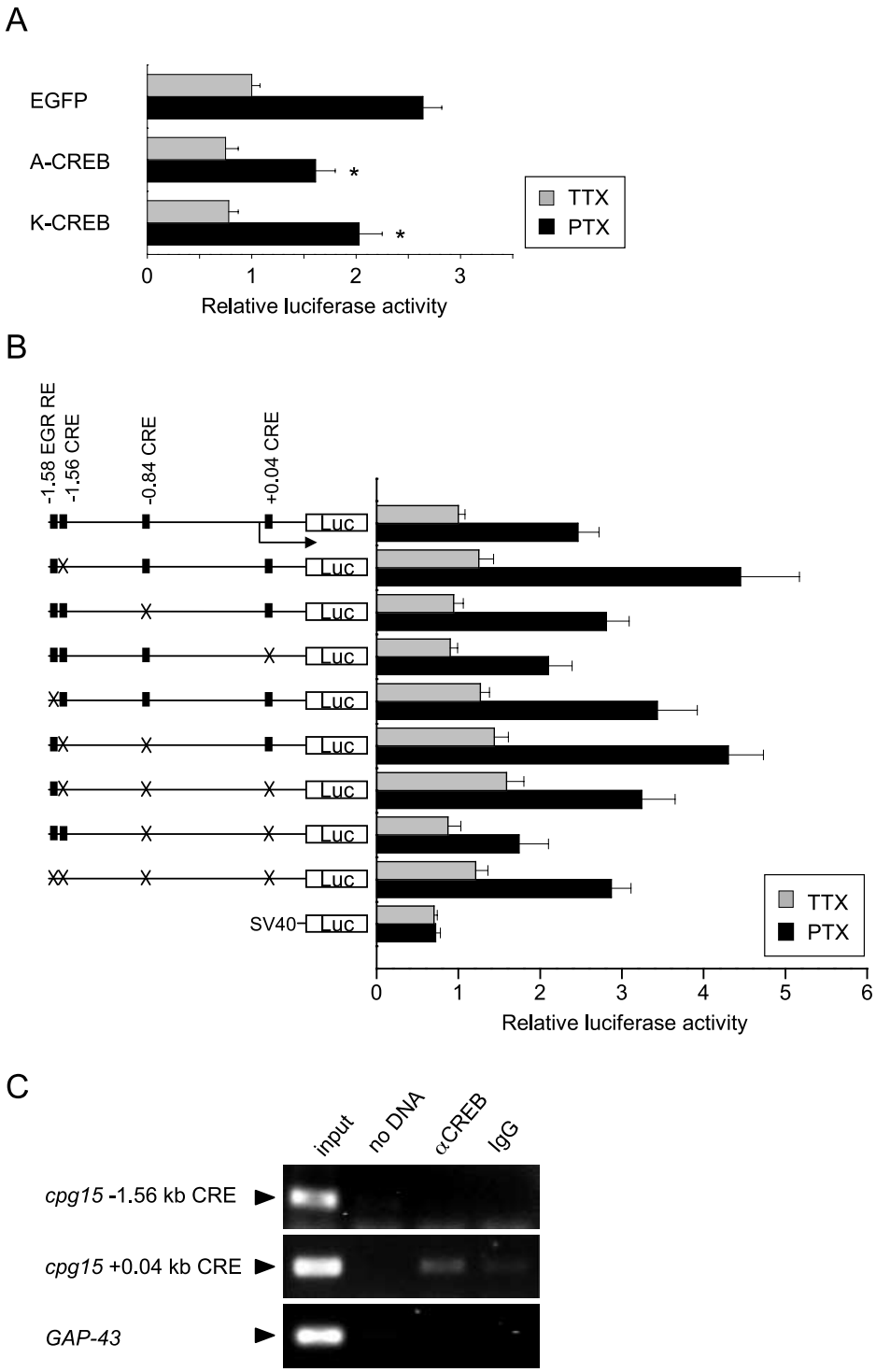

Fig. 4. CREB is involved in activity-dependent regulation of the *cpg15* promoter. (A) Dominant negative CREB mutants block activity-dependent transcription driven by the *cpg15* promoter. Cortical cultures were cotransfected with *cpg15-Luc* and A-CREB or K-CREB dominant negative CREB expression plasmids or a control EGFP expression plasmid. Cultures were treated with TTX or PTX for 9 h, and *cpg15* promoter activity was determined by luciferase assay as in Fig. 2. Luciferase activities shown are relative to TTX-treated cells transfected with *cpg15-Luc* and the EGFP expression plasmid. A-CREB and K-CREB both significantly reduced activity-dependent luciferase expression driven by the *cpg15* promoter (*$P < 0.01$, ANOVA and SNK *post hoc* test; $n = 10–11$). (B) *cpg15* CREs play a role in activity-dependent transcription driven by the *cpg15* promoter. Cortical cultures were transfected with the *cpg15-Luc* plasmid carrying the wild-type *cpg15* promoter (top) or the same plasmid with individual point mutations in the −1.56-kb CRE, −0.84-kb CRE, +0.04-kb CRE, and −1.58-kb EGR RE, or with a combination of point mutations. The luciferase reporter fused to the SV40 promoter served as a control (SV40, bottom). Schematic diagrams of these reporter plasmids are shown on the left. Intact binding sites are indicated by closed boxes, and mutated sites are marked with an X. Transfected cells were treated with TTX or PTX for 9 h, and *cpg15* promoter activity assayed as described for Fig. 2. The effect of mutating each site was analyzed using a two-factor ANOVA with combined data from single and multiple mutations. Mutation of the −1.56-kb CRE significantly increased *cpg15* promoter activity in PTX-treated cultures ($P < 0.001$, $n = 10–15$), whereas mutation of the +0.04-kb CRE significantly

CREM. Anti-ATF-1 antibody and control IgG had no effect on either of the bands. The slower migrating band detected with the four probes was not affected by any of the antibodies against known CREB family members, suggesting that it binds a more distantly related factor.

EMSAs using the *cpg15* −1.58-kb EGR RE showed two DNA/protein complexes with similar mobility and specificity as the two rapidly migrating DNA/protein complexes seen with the EGR RE consensus (Fig. 3C), indicating that the −1.58 kb site in the *cpg15* promoter region binds an EGR family member or related factors. The EGR family has four members: EGR1/*zif268*/NGFI-A/krox24, EGR2/krox20, EGR3/PILOT, and EGR4/NGFI-C (O'Donovan et al., 1999). Addition of the anti-EGR1 antibody caused a supershift of the most slowly migrating DNA/protein complex detected using the consensus sequence (Fig. 3D). This slowest migrating band could not be detected in an EMSA using the −1.58-kb EGR RE due to a nonspecific comigrating band. However, addition of the anti-EGR1 antibody to the EMSA with −1.58 kb EGR RE showed a supershifted band identical to that seen with the consensus EGR RE, suggesting that EGR1 binds to the −1.58-kb EGR RE. The anti-EGR3 antibody supershifted the most slowly migrating DNA/protein complex detected using the EGR RE consensus and the −1.58-kb EGR RE, suggesting that EGR3 also binds to these sites. Addition of anti-EGR2 antibody and control IgG had no effect on any of the bands detected using both probes. The middle band detected using EGR RE consensus and −1.58-kb EGR RE was not affected by the three antibodies tested.

In summary, all three CREs in the *cpg15* promoter bind both CREB and CREM, whereas the −1.58-kb EGR RE binds EGR1 and EGR3. Of all the potential sites in the *cpg15* promoter, these sites emerged as the most likely to be involved in activity-dependent transcriptional regulation of the *cpg15* gene.

CREB mediates activity-dependent transcription of cpg15

Given CREB's importance as a transcription factor essential for synaptic plasticity, and our finding that three CRE sites in the *cpg15* promoter bind CREB in vitro, we further investigated CREB's role in activity-dependent *cpg15* transcription. Two different dominant negative CREB mutants were each coexpressed with *cpg15-Luc* in cortical cultures, and their effect on *cpg15* promoter-driven luciferase expression was assayed. A-CREB and K-CREB are mutant versions of CREB that form high-affinity heterodimers with endogenous CREB family members and inhibit their binding to CRE sites (Walton et al., 1992; Ahn et al., 1998). When coexpressed with *cpg15-Luc*, both A-CREB and K-CREB significantly reduced the levels of activity-dependent luciferase expression in PTX-treated cells (Fig. 4A). To confirm that the decreased transcription was not a result of nonspecific toxic effects of the dominant negative CREB versions, their effect on the SV40 promoter-driven transcription was also assayed. Luciferase expression driven by the SV40 promoter was unaffected by coexpression of A-CREB or K-CREB (data not shown), indicating that the effect of dominant negative CREB was specific to the *cpg15* promoter. These results strongly implicate CREB family members in the activity-dependent regulation of *cpg15*.

The cpg15 CREs mediate both positive and negative regulation of activity-dependent transcription

To test if the CREs or the EGR RE in the *cpg15* promoter mediate *cpg15*'s response to CREB or EGR, we introduced point mutations that abolish CREB or EGR binding in each of the CREs and EGR RE within the *cpg15-Luc* reporter individually or in combination. The effect of these mutations on transcriptional regulation of the luciferase reporter gene was assessed in cortical cultures treated with TTX or PTX. Unexpectedly, we found that mutation of the −1.56-kb CRE significantly increased *cpg15* promoter activity in PTX-treated cultures (Fig. 4B), indicating that this site negatively regulates *cpg15* expression. Mutation of the −0.84-kb CRE had no detectable effect on *cpg15* promoter activity, possibly correlated with the low binding affinity of this site for CREB (see Fig. 3A). Mutation of the +0.04-kb CRE significantly decreased *cpg15* promoter activity. Mutation of the −1.58-kb EGR binding site resulted in a modest increase in *cpg15* promoter activity that was not statistically significant. Thus, the CRE sites in the *cpg15* promoter regulate transcription in both positive and negative ways. Combined mutation of all three CREs and EGR RE showed luciferase expression levels similar to those of the wild-type *cpg15-Luc* reporter, probably due to the positive and negative effects of the different mutations canceling each other out.

CREB binds the endogenous cpg15 promoter in vivo

To examine if CREB binds the endogenous *cpg15* promoter in vivo, we performed a ChIP assay. We cross-linked DNA-bound proteins to chromatin in cortical cultures, sheared the chromatin by sonication, and then immunoprecipitated the chromatin with a specific antibody against

decreased its activity ($P < 0.01$, $n = 10–15$). (C) Endogenous CREB binds to the *cpg15* promoter in vivo. DNA-binding proteins in cortical cultures were cross-linked to chromatin with formaldehyde, sonicated, and then subjected to immunoprecipitation with anti-CREB antibody or control IgG. After reversing the cross-links, presence of promoter fragments in the immunoprecipitates was examined by PCR with primers covering the *cpg15* −1.56-kb CRE, *cpg15* +0.04-kb CRE, or the *GAP-43* promoter as a negative control. Input chromatin (0.5%) was used as a positive control for the PCR reaction. The +0.04-kb CRE *cpg15* promoter region was present in the chromatin immunoprecipitated by the anti-CREB antibody, indicating that it binds CREB in vivo.

CREB. The presence of promoter fragments coimmunoprecipitating with the CREB protein was then assayed using PCR. We detected the +0.04-kb CRE *cpg15* promoter region in the CREB immunoprecipitate (Fig. 4C), but could not detect the −1.56-kb CRE *cpg15* promoter region. The *GAP-43* promoter, a gene expressed in neurons but not regulated by activity, was not detected in the CREB immunoprecipitate, showing that immunoprecipitation of the *cpg15* promoter was specific. To control for antibody specificity, we performed the immunoprecipitation with a control IgG. Neither the *cpg15* promoter nor the *GAP-43* promoter was detected in the immunoprecipitate (Fig. 4C). These data show that CREB binds to the *cpg15* promoter in vivo and strongly suggest that CREB regulates transcription of *cpg15* through the +0.04-kb CRE site.

Activity-dependent cpg15 expression requires both the CaMK and MAPK pathways

Because activity-dependent *cpg15* expression is regulated by Ca^{2+} influx through NMDA receptors and L-type VSCCs, we investigated whether kinase pathways activated by these modes of Ca^{2+} entry may be involved. To test whether the CaMK and MAPK pathways play a role in *cpg15*'s activity-dependent regulation, we analyzed PTX-induced *cpg15* expression in cultures treated with kinase inhibitors. Treatment with the CaMK inhibitor KN93, or the MAP kinase kinase (MEK) inhibitor U0126, but not with their inactive analogs KN92 or U0124, significantly reduced PTX-induced *cpg15* expression (Fig. 5A), suggesting that both the CaMK and MAPK pathways are involved in activity-dependent *cpg15* expression.

KN93 is an inhibitor of all the CaMKs, including CaMKII and CaMKIV. Although both CaMKII and CaMKIV have been associated with transcriptional activation and synaptic plasticity, it is generally thought that CaMKII acts locally near the synapse, whereas CaMKIV is more effective in transcription factor activation because of its nuclear localization (reviewed in Soderling, 2000). We therefore tested whether CaMKIV may be involved in *cpg15*'s transcriptional activation by cotransfecting a dominant negative form of CaMKIV (dnCaMKIV) together with the *cpg15-Luc* reporter plasmid. The transfected dnCaMKIV did not significantly affect *cpg15* promoter activity (Fig. 5B), although its expression and function could be confirmed in HEK293T cells (data not shown).

To verify these unexpected results and to test whether CaMKIV was relevant to *cpg15*'s in vivo regulation, we examined activity-dependent *cpg15* expression in transgenic mice with forebrain-specific expression of dnCaMKIV (Kang et al., 2001). Wild-type and dnCaMKIV transgenic mice were injected with the glutamate analog kainate or with PBS as a control. Six hours later, the mice were sacrificed, the cerebral cortices were removed, and total RNA was extracted. Using Northern blot analysis, *cpg15* expression in response to kainate-induced seizure was compared between groups of wild-type and dnCaMKIV transgenic mice (Fig. 5C and D). *cpg15* expression levels in PBS injected controls were comparable in wild-type and dnCaMKIV transgenic mice. Kainate-injected mice showed significantly higher *cpg15* expression in both wild-type and dnCaMKIV transgenic mice. Increases in *cpg15* expression by kainate ranged from 1.4- to 3.3-fold in wild-type and 1.5- to 4.1-fold in dnCaMKIV transgenics as compared to PBS-injected controls. On average, dnCaMKIV transgenic mice showed no significant difference in fold *cpg15* induction as compared to wild-type mice. Together, these results suggest that CaMKIV is not a major regulator of activity dependent *cpg15* expression.

cpg15 expression is induced by cAMP, but PKA activation is not sufficient for this induction

To test involvement of the PKA pathway in *cpg15* regulation, we examined whether the adenylate cyclase activator forskolin could induce *cpg15* expression. Forskolin treatment for 9 h significantly increased *cpg15* mRNA levels similar to those induced by PTX (Fig. 6A), indicating that *cpg15* is induced by activation of the cAMP pathways. When compared to application of PTX or forskolin alone, combined application of PTX and forskolin resulted in a further increase in *cpg15* expression, although this increase was not statistically significant. Since there is a low level of *cpg15* induction in untreated cultures, likely due to spontaneous activity (see Fig. 1B), we stimulated the cultures with forskolin in the presence of TTX. Unexpectedly, TTX blocked forskolin-induced *cpg15* expression (Fig. 6A), indicating that electrical activity is required for *cpg15* induction by forskolin. To ascertain if Ca^{2+} channels were involved, we stimulated the cultures with forskolin in the presence of CPP and nifedipine. Together, CPP and nifedipine blocked forskolin-induced *cpg15* expression, indicating that NMDA receptors and L-type VSCCs are required for forskolin-induced *cpg15* expression, as for PTX induction.

Since forskolin and PTX induce *cpg15* to similar levels, we used an in vitro kinase assay to examine if PKA is activated during PTX stimulation. PTX treatment did not result in PKA activation (Fig. 6B), indicating that induction by PTX does not involve activation of PKA. Forskolin-treated cultures showed significant PKA activation, as expected. Since forskolin-induced *cpg15* expression was blocked by TTX, or CPP and nifedipine, we examined the PKA activity under these conditions. PKA activation was unaffected in the presence of TTX or CPP and nifedipine, indicating that PKA activation is not sufficient for *cpg15* expression. Thus, although *cpg15* is induced by increases in cAMP levels, PKA activation is not sufficient for this induction, but requires concurrent synaptic stimulation mediated by NMDA receptors and L-type VSCCs.

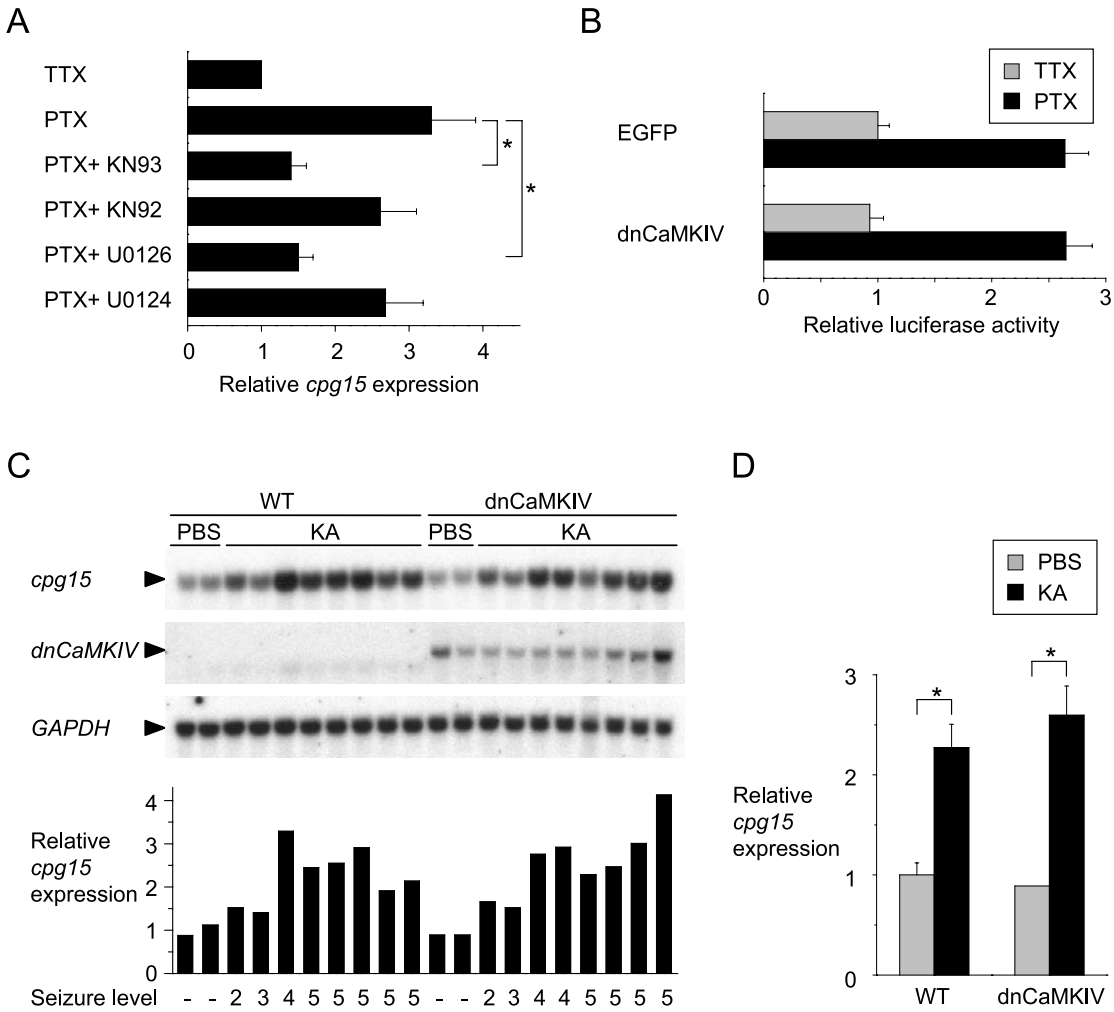

Fig. 5. Activity-dependent *cpg15* expression is mediated by the CaMK and MAPK pathways. (A) PTX-induced *cpg15* expression is dependent on the CaMK and MAPK pathways. Cortical cultures were treated with TTX or PTX in the absence or presence of kinase inhibitors or their inactive analogs: KN93 and KN92 (0.5 μM) for CaMK or U0126 and U0124 (5 μM) for MAPK. *cpg15* expression was analyzed by Northern blot hybridization as in Fig. 1. Both KN93 and U0126 significantly reduced the PTX-induced *cpg15* mRNA expression (*$P < 0.05$, ANOVA and SNK *post hoc* test; $n = 3$–4). (B) Activity-dependent *cpg15* promoter activity is uneffected by dnCaMKIV. Cortical cultures were cotransfected with *cpg15-Luc* and dnCaMKIV or a control EGFP expression plasmid. Cultures were treated with TTX or PTX for 9 h, and promoter activity was assayed as in Fig. 2. Luciferase activities shown are relative to EGFP transfected cells treated with TTX ($n = 8$). (C) Activity-dependent *cpg15* expression is comparable in dnCaMKIV transgenic mice and wild-type controls. Adult wild-type (WT) and dnCaMKIV transgenic mice were injected with PBS or 25 mg/kg kainate (KA), scored for seizure severity (see Experimental Methods) and sacrificed after 6 h. RNA was extracted from the cerebral cortices, and *cpg15*, dnCaMKIV, and GAPDH expression levels were examined by Northern blot analysis as in Fig. 1. Normalized *cpg15* expression relative to PBS-injected wild-type mice is shown for each animal. (D) Quantification of the results shown in C. *cpg15* expression increased significantly by kainate injection in both wild-type and dnCaMKIV transgenic mice (*$P < 0.05$, ANOVA and SNK *post hoc* test; $n = 2$–8). The kainate-induced *cpg15* expression was not significantly different between wild-type and dnCaMKIV transgenic mice.

Discussion

Regulation of cpg15 transcription by Ca^{2+} influx through NMDA receptors and L-type VSCCs

Our results show that activity-dependent *cpg15* expression induced by PTX-evoked synaptic stimulation requires Ca^{2+} influx through both the NMDA receptors and L-type VSCCs. This dual requirement could stem from either parallel or sequential activation of these Ca^{2+} channels (Fig. 7). A dual requirement for NMDA receptors and L-type VSCCs in activation of CREB phosphorylation and *c-fos* expression has been reported for striatal neurons (Rajadhyaksha et al., 1999). In these neurons, a sequential pathway was mapped from AMPA/kainate receptors to NMDA receptors and from NMDA receptors to L-type VSCCs, with the VSCCs being the essential last step necessary for activation of intracellular second messenger pathways. *cpg15* expression induced by depolarization with high potassium requires only the L-type VSCCs, suggesting that such strong stimulation can bypass the need for NMDA receptors by activating L-type VSCCs directly. This is consistent with studies of other activity-regulated genes such as *c-fos* and *BDNF*, where activation of L-type VSCCs is sufficient for

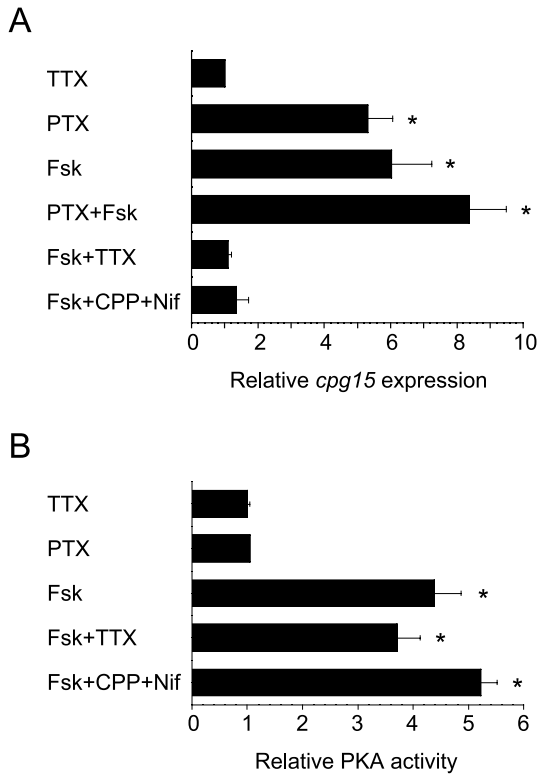

Fig. 6. *cpg15* expression is activated by cAMP, but activation of PKA is not sufficient for *cpg15* induction. (A) *cpg15* induction by forskolin requires concurrent synaptic stimulation. Cortical cultures were treated with TTX, PTX, forskolin (Fsk, 10 μM), CPP or nifedipine for 9 h as indicated on the left. *cpg15* expression was analyzed by Northern blot hybridization as in Fig. 1. Forskolin significantly increased *cpg15* mRNA expression, but not in the presence of TTX or CPP and nifedipine (*$P < 0.01$ versus TTX, ANOVA, and SNK *post hoc* test; $n = 3$). (B) PKA is not activated by PTX stimulation. Cortical cultures were treated as indicated on the left for 10 min. PKA activity was determined by a kinase assay using the specific peptide substrate Kemptide. Forskolin, but not PTX, significantly increased PKA activity (*$P < 0.01$ versus TTX, ANOVA, and SNK *post hoc* test; $n = 3$). PKA activation by forskolin was not blocked by TTX or CPP and nifedipine.

inducing transcription (Murphy et al., 1991; Shieh et al., 1998; Tao et al., 1998) and supports sequential roles for NMDA receptors and L-type VSCCs in activation of *cpg15* transcription in response to synaptic stimulation.

Alternatively, the dual requirement for Ca^{2+} influx through both channels for *cpg15*'s activity-dependent regulation may reflect a dependence on signaling pathways originating from NMDA receptors and L-type VSCCs, because both channels are directly coupled to signal transduction pathways (Deisseroth et al., 1996; Husi et al., 2000; Dolmetsch et al., 2001). L-type VSCCs can activate the MAPK pathway through direct binding of Ca^{2+}-calmodulin, which functions as a local Ca^{2+} sensor at the mouth of the channel (Peterson et al., 1999; Zuhlke et al., 1999; Dolmetsch et al., 2001). The NMDA receptor is part of a multiprotein complex with multiple kinases in the MAPK pathway as well as CaMKII and PKA (Husi et al., 2000). A requirement for convergent signals from multiple signaling pathways that are initiated at specific sites of Ca^{2+} entry is consistent with studies showing that local increases in Ca^{2+} concentration at its site of entry are more important for activation of signaling pathways and gene expression than global Ca^{2+} levels (Deisseroth et al., 1996; Dolmetsch et al., 2001; Hardingham et al., 2001). It is also consistent with our finding that *cpg15*'s activity-dependent regulation requires both the CaMK and MAPK pathways.

Activity-dependent cpg15 expression requires both the CaMK and MAPK pathways but not the activation of PKA

The requirement for both the CaMK and MAPK pathways for *cpg15* induction by synaptic stimulation suggests that the CaMK and MAPK pathways act on multiple transcription factors, some of which are nonoverlapping, that together activate *cpg15* expression. An example of this can be seen in regulation of the *c-fos* gene, where activation of two transcription factors CREB and Elk-1 are important in induction of the *c-fos* in response to Ca^{2+}. For *c-fos*, both CaMK and MAPK act through CREB to activate transcription, but MAPK additionally activates Elk-1 (Xia et al., 1996). Although we found no Elk-1 binding sequence in the 1.9-kb upstream region of *cpg15*, other transcription factors could potentially serve a similar function.

Our finding that CaMKIV is not involved, despite the requirement for CaMKs for *cpg15*'s activity-dependent expression, was surprising in light of the studies showing the importance of CaMKIV in regulating Ca^{2+}-dependent transcription (Bito et al., 1996; Chawla et al., 1998; Hardingham et al., 1999; Ho et al., 2000; Impey et al., 2002). The requirement for CaMKs but not CaMKIV suggests that other CaMKs such as CaMKII are involved. CaMKII is

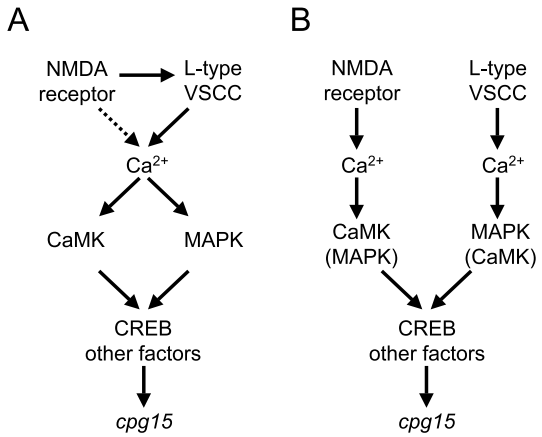

Fig. 7. Two alternative models of activity dependent *cpg15* induction. In the sequential activation model (A), NMDA receptor activation by synaptic activity leads to activation of L-type VSCCs and Ca^{2+} influx through these channels, triggering CaMK and MAPK pathways. In the parallel activation model (B), influx of Ca^{2+} from NMDA receptors and L-type VSCCs each trigger local activation of either the CaMK or the MAPK pathways. In both cases, activation of CaMK and MAPK pathways converge on a set of transcription factors, including CREB, that together activate *cpg15* transcription.

enriched at the synapse and is closely associated with L-type VSCCs and NMDA receptors (Deisseroth et al., 1996; Husi et al., 2000; Dolmetsch et al., 2001). CaMKII may regulate transcription indirectly by activating downstream signaling molecules that translocate to the nucleus (Hook and Means, 2001). Alternatively, nuclear isoforms of CaMKII-α, -δ, and -γ may be involved (Soderling et al., 2001).

Because PTX did not induce PKA activity despite increasing *cpg15* expression, PKA activation is apparently not required for mediating *cpg15*'s response to synaptic activity. However, basal level of PKA may play a permissive role in activity-dependent *cpg15* expression. PKA activity has been shown to be required for nuclear translocation of the MAPK signals necessary for CREB-dependent transcription (Impey et al., 1998). Basal PKA activity may be sufficient for this function in the context of *cpg15* regulation.

Regulation of cpg15 by multiple transcription factors including CREB

Our finding that two different forms of dominant negative CREB significantly reduce activity-dependent *cpg15* expression indicates a role for CREB in *cpg15* regulation. A role for CREB in mediating the activity-dependent induction of *cpg15* is also supported by in vivo studies of *cpg15* expression in barrel cortex of control and CREB α,Δ-knockout mice (C. Harwell, B. Burbach, K. Svoboda, and E. Nedivi, unpublished observations). After eliciting receptive-field plasticity by whisker trimming, *cpg15* expression increases in the barrel corresponding to the spared whisker. In CREB mutant mice, *cpg15* expression is not induced to the same extent as in wild-type littermates, suggesting that CREB is necessary for *cpg15* regulation during receptive field plasticity in the adult cortex.

Our finding that *cpg15* is an IEG indicates that CREB directly regulates *cpg15* by binding to CRE sites in its promoter region. Consistent with this, we found three CREs in the *cpg15* promoter that binds CREB in vitro. Unexpectedly, the *cpg15* CREs had both positive and negative effects on *cpg15* regulation. The +0.04-kb CRE had a positive effect, likely mediated by CREB. This is supported by ChIP assays demonstrating in vivo CREB binding to the +0.04-kb CRE region of the endogenous *cpg15* promoter. The −1.56-kb CRE had a negative effect that could be mediated by CREM, because isoforms of CREM are known to function as inhibitors of CREB-mediated transcription (Foulkes et al., 1991) or by a non-CREB family member. EMSAs detected binding of a non-CREB family member to each of the three *cpg15* CRE sites. The −1.56-kb CRE showed a higher affinity to this factor compared to the +0.04-kb CRE. If this factor is a repressor, its differential binding preference to the two CRE sites could explain the opposite effects of mutating these sites.

Given that transcriptional regulation is combinatorial by nature and commonly involves multiple factors, it is not unusual that the mutation of individual CRE sites in the *cpg15* promoter did not result in a large and cumulative down-regulation of its transcription. In the case of the *c-fos* promoter, combined mutations of the CRE, SRE, and cis-inducible elements (SIE) result in marked reduction of activity-dependent expression (Johnson et al., 1997). However, mutation of each individual site has a small or insignificant effect. It is therefore possible that the effect of mutations in *cpg15*'s CREs or EGR RE would become more significant when combined with mutations of other, as yet unidentified transcription factor binding sites. Our analysis of the *cpg15* promoter and the requirement for multiple kinase pathways suggest that multiple transcription factors, including CREB, regulate *cpg15* expression through multiple promoter elements.

Activation of cpg15 by cAMP pathways

Our results showing that forskolin induces *cpg15* expression indicate that cAMP-dependent pathways can activate *cpg15* transcription. However, activation of PKA was not sufficient for *cpg15* expression and required electrical activity mediated by NMDA receptors and L-type VSCCs. Spontaneous electrical activity in culture may result in low-level activation of MAPK or CaMK pathways that may converge with the PKA pathway to activate *cpg15* expression. Another possibility is that cAMP may stimulate the electrical activity of cultured neurons, possibly through cyclic nucleotide gated channels (Zagotta and Siegelbaum, 1996), resulting in activation of pathways similar to PTX stimulation.

Activation of *cpg15* expression by cAMP pathways suggests that in addition to synaptic stimulation, *cpg15* may be activated by extracellular signals such as neurotransmitters, hormones, or growth factors that activate receptors coupled to adenylate cyclase. The requirement for electrical activity in cAMP mediated activation of *cpg15* suggests that extracellular signals activating adenylate cyclase are only effective on neurons that are simultaneously active. Limiting stimulation of *cpg15* expression by nonsynaptic signals to active neurons may be important for *cpg15*'s role in plasticity.

Similarities and differences in regulation of cpg15 and other effector genes

In comparing the signaling pathways that regulate activity-dependent expression of various effector genes, we can begin to identify common elements that are likely to be generally important for plasticity. Activity-dependent expression of *cpg15* requires both the MAPK and CaMK pathways but not PKA activation. In contrast, *homer 1a* expression is dependent on MAPK but not on CaMK pathways (Sato et al., 2001), whereas *arc* expression is dependent on both PKA and MAPK pathways (Waltereit et al., 2001). From these limited comparisons, MAPK appears to be the common regulator of activity-dependent effector

genes, whereas requirements for CaMK and PKA vary. The contribution of CREB to regulation of some effector genes like *neuronal NO synthase* (nNOS) (Sasaki et al., 2000) and *BDNF* (Shieh et al., 1998; Tao et al., 1998) is large, whereas it is less pronounced in the case of *cpg15* and undetectable for *arc* (Waltereit et al., 2001), indicating that CREB may be involved to a different degree in regulating expression of activity-dependent genes. The requirement for different signaling pathways may also depend on the cell type, as shown for *c-fos* (Johnson et al., 1997). Further analysis of the signaling pathways that regulate different activity-dependent effector genes will be important for understanding how their combinatorial activation is translated into functional and structural changes at the synapse.

Conclusion

cpg15 is an activity-dependent gene regulated by Ca^{2+} influx through NMDA receptors and L-type VSCCs, MAPK and CaMK signal transduction pathways, and CREB. Such genes that are also capable of directly influencing neuronal morphology and synaptic physiology are likely to be key players in activity-regulated transcriptional programs that affect long-term structural and functional synaptic plasticity.

Experimental methods

Analysis of mouse genomic DNA and promoter sequence

A bacterial artificial chromosome library containing C57BL/6 mouse genomic DNA (Incyte Genomics, Palo Alto, CA) was screened with a 0.4-kb rat *cpg15* cDNA fragment corresponding to the coding region. Twelve clones were isolated, digested with multiple restriction enzymes, and screened on Southern blots using oligonucleotides corresponding to different parts of the *cpg15* cDNA. Nine overlapping restriction fragments spanning 20.8 kb of genomic DNA, and containing all *cpg15* exons with 8.9 kb of additional upstream sequence, were subcloned into pBluescript II SK (Stratagene, La Jolla, CA). After restriction mapping of this entire region, exon/intron boundaries and 1.9 kb upstream of the translation start site were sequenced (GenBank accession number AY150584). Database searches identified the homologous human *cpg15* gene in a working draft sequence segment of chromosome 6 (GenBank accession number 12732575). The promoter region was searched for potential transcription factor binding sites using the TFSEARCH program (Heinemeyer et al., 1998; http://www.cbrc.jp/research/db/TFSEARCH.html).

Nuclear extracts

Nuclear extracts were prepared as described (Ausubel et al., 1999) with the following modifications. Adult mouse brains were homogenized in a Dounce homogenizer (A pestle) with 5 volumes of homogenization buffer [15 mM HEPES (pH 7.9), 250 mM sucrose, 60 mM KCl, 10 mM NaCl, 1 mM EDTA] and a protease/phosphatase inhibitor mix (2 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, 1 mM DTT, 1% protease inhibitor cocktail; Sigma, St. Louis, MO). Cells were pelleted by centrifugation at 2,000 rpm for 10 min and then resuspended in 5 volumes of hypotonic solution [10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM EDTA] and protease/phosphatase inhibitor mix. After incubation on ice for 10 min, cells were homogenized by Dounce homogenizer (B pestle). Nuclei were collected by centrifugation at 7,000 rpm for 10 min. The pellet was suspended in 1 bed volume of high-salt solution [20 mM HEPES (pH 7.9), 0.8 M NaCl, 1.5 mM $MgCl_2$, 25% glycerol, 1 mM EDTA] and protease/phosphatase inhibitor mix. After rocking for 30 min at 4°C, the extract was centrifuged at 14,000 rpm for 30 min to remove membrane debris. Protein concentration was determined by the Bradford method (Bio-rad, Hercules, CA).

Electrophoretic mobility shift assay (EMSA)

Consensus and mutated oligonucleotides for CRE, TRE and EGR RE were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). MWG Biotech (High Point, NC) synthesized the following oligonucleotides and their complementary sequence. The binding sequence of each factor is underlined.

−1.56 kb CRE	5′-ACCCACCACTGACATCACCA-GGGGCA-3′
−0.84 kb CRE	5′-CCCTACAGATGACTTCAG-GAGGCCCC-3′
+0.04kb CRE	5′-TGTCATCCCCCACGTCAG-GCCTGCTC-3′
−1.3 kb TRE	5′-AGATGGCTGACTGTGT-GGGCT-3′
−0.1 kb TRE	5′-ATGAAAATCAGTCACTA-CAGA-3′
−0.05 kb TRE	5′-AGATATCTGATTAATTC-CAGA-3′
−1.58 kb EGR RE	5′-CCGCTACCCCGCCCAC-CCACCACTGAC-3′
−1.59 kb EGR RE	5′-GGGAAAGCGCGCCCCCGC-TACCCCGCC-3′
−0.6 kb CaRE1	5′-CACGCAGAGTCTATTTCT-GGGGGCCCCCATGCGAGC-3′
BDNF CaRE1	5′-ACTAGAGTGTCTATTTC-GAGGCAGAGGAGGTATCAT-3′
mut BDNF CaRE1	5-ACTAGAGTGTCTTCCGCCAG-GCAGAGGAGGTATCAT-3′

Annealed, double-stranded oligonucleotides were ^{32}P-labeled by phosphorylation using T4 kinase (New England Biolabs, Beverly, MA). For each reaction, 0.1 ng of labeled oligonucleotide was incubated with 5 μg nuclear extract and 0.5 μg poly(dI-dC) in binding buffer [20 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$, 40 mM KCl, 5 mM DTT, 10% glycerol] for 20

min at room temperature. For competition assays, a 100-fold molar excess of unlabeled double-stranded oligonucleotide was added to each reaction. For super-shift assays, 0.4 μg of antibodies (all from Santa Cruz Biotechnology, Santa Cruz, CA) against CREB1 (mouse monoclonal 24H4B), CREM1 (rabbit polyclonal X-12), ATF-1 (mouse monoclonal C41-5.1), EGR1 (rabbit polyclonal C-19), EGR2 (rabbit polyclonal C-14), EGR3 (rabbit polyclonal C-24), or normal rabbit IgG were added after the binding reaction and further incubated for 30 min at room temperature. Samples were separated on a 6% polyacrylamide gel in 1X TGE buffer (50 mM Tris, 0.5 M glycine, 0.5 mM EDTA). The gel was dried and exposed to film.

Mouse primary cortical cultures

Dissociated cultures of cortical neurons were prepared as described (Banker and Goslin, 1998) and modified as follows. Cerebral cortices from embryonic day 16 C57BL/6 mice were dissected in ice-cold Hanks' buffered salt solution (HBSS, Sigma). Cortices were washed with HBSS and then digested by 10 mg/ml trypsin (Sigma) and 0.5 mg/ml DNase (Sigma) in digestion solution [25 mM HEPES (pH 7.2), 137 mM NaCl, 5 mM KCl, 7 mM Na_2HPO_4] for 10 min at 37°C. After digestion, tissue was washed in HBSS and then triturated with fire-polished Pasteur pipettes in 2 ml of HBSS supplemented with 12 mM $MgSO_4$ and 0.5 mg/ml DNase. Cells were centrifuged for 10 min at 2000 rpm. Cell pellet was resuspended in preequilibrated Neurobasal medium (Life Technologies, Carlsbad, CA) supplemented with B27 (Life Technologies, Carlsbad, CA), 0.5 mM glutamine, and 12.5 μM glutamate. Cells were plated on dishes coated with laminin (Fisher, Pittsburgh, PA) and poly-D-lysine (Fisher, Pittsburgh, PA) at 1×10^6 cells per well on 12-well plates for luciferase assays and PKA assays, at 2.5×10^6 cells per well on 6-well plates for RNA extraction, and at 7.5×10^6 cells per 10 cm dish for chromatin immunoprecipitation. Cultures were maintained in a humidified 37°C incubator with a 5% CO_2, 95% air atmosphere. Half of the medium was replaced every 4–5 days with fresh medium without glutamate.

cpg15 promoter and dominant negative constructs

For construction of wild-type *cpg15-Luc*, a 1.9-kb genomic fragment containing 1.6 kb of the promoter region and 0.3 kb of the 5′ untranslated region of *cpg15* was amplified by PCR (Vent DNA polymerase, New England Biolabs, Beverly, MA) from a *cpg15* genomic template using primers wt-s and wt-as. The PCR product was digested at *Kpn*I/*Bam*HI sites designed into the primer sequence and cloned into *Kpn*I/*Bql*II sites of the pGL3-Basic vector (Promega, Madison, WI). This placed the *cpg15* promoter and 5′ untranslated region upstream of the promoterless firefly luciferase gene. Point mutations were introduced in the *cpg15* promoter region using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) using the primers designated below and wild-type *cpg15-Luc* as template. For multiple mutations, the same procedure was repeated sequentially. All mutations were confirmed by sequencing.

Primers used for mutagenesis (mutated bases are shown in lowercase):

wt-s	5′-GGGGTACCTACCCCGCCCAC-CCACCACTGACATCA-3′
wt-as	5′-CGGGATCCATCCTACGTT-TAGTCAAAC-3′
mCRE −1.56	5′-ACCCACCACTGtgATCACCAG-GGGCA-3′
mCRE −0.84	5′-CCCTACAGATGtgTTCAGGAG-GCCCC-3′
mCRE +0.04	5′-TGTCATCCCCCtgGTCAGGCC-TGCTC-3′
mEGR RE −1.58	5′-CTACCCCGCCCtaCCACCAC-TGACATCACC-3′

The pEGFP-C1 expression vector was from Clontech (Palo Alto, CA). A-CREB (Ahn et al., 1998) and K-CREB (Walton et al., 1992) have been previously described. Dominant negative CaMKIV cDNA (Gringhuis et al., 1997) was subcloned into pcDNA3 (Invitrogen, Carlsbad, CA).

Transfections and luciferase assays

Mouse primary cortical cultures at 6 days in vitro (div) were transfected by the calcium phosphate method (Banker and Goslin, 1998). Culture medium was replaced for 1 h with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 mM $MgCl_2$ and 1 mM kynurenic acid. To form the calcium phosphate/DNA precipitate, DNA was incubated for 25 min in 125 mM $CaCl_2$ with 1X HBSS [137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 7 mM glucose, 21 mM HEPES (pH 7.07)]. For each transfection, 0.5 μg of firefly luciferase reporter plasmid and 0.5 μg of internal control vector pRL-TK carrying a *Renilla* luciferase gene driven by the HSV thymidine kinase promoter (Promega, Madison, WI) were used per well. For assays using dominant negative constructs, 3 μg of expression vector containing the appropriate gene were co-transfected with the reporter plasmids. After the precipitate was added to the cells and incubated for 30 min, cells were washed with supplemented DMEM and then replaced in their original medium. Pharmacological treatments were done for 9 h at 14–16 div. After treatment, cells were harvested with 200 μl of passive lysis buffer (Promega, Madison, WI) per well, and 20 μl of lysate was used to measure the luciferase activity using the Dual-luciferase reporter assay system (Promega, Madison, WI). Firefly luciferase activity was normalized to *Renilla* luciferase activity to control for transfection efficiency and sample handling. In each case, standard error of mean (SEM) was calculated for at least three experiments. Statistical significance was determined by analysis of variance

(ANOVA) and Student–Newman–Keuls (SNK) post hoc analysis. Effects of CRE and EGR RE mutations were determined by two-factor factorial ANOVA. Both analyses were done using StatView software (SAS Institute, Cary, NC).

Pharmacology

Primary cortical cultures were cultured for 14–16 div and treated with picrotoxin (PTX, 50 μM; Tocris, Ellisville, MO), forskolin (10 μM; Sigma), or tetrodotoxin (TTX, 1 μM; Calbiochem). Each drug was added directly to the culture medium and cells were harvested 9 h later. In experiments using Ca^{2+} chelators, Ca^{2+} channel blockers, and kinase inhibitors, EGTA (2 mM; Sigma), CPP (10 μM; Tocris), nifedipine (5 μM; Tocris, Ellisville, MO), KN93 (0.5 μM, Calbiochem), KN92 (0.5 μM; Calbiochem), U0126 (5 μM; Calbiochem), or U0124 (5 μM; Calbiochem) were added directly to the culture medium 30 min prior to stimulation. To stimulate the L-type VSCCs, cultures were treated with 50 mM KCl in the presence of CPP. In experiments using cycloheximide (20 μg/ml; Sigma), it was added 30 min prior to addition of PTX or TTX.

Animal manipulations and tissue isolation

All animal work was approved by the Massachusetts Institute of Technology Committee on Animal Care and conforms to NIH guidelines for the use and care of vertebrate animals. Adult wild-type and dnCaMKIV transgenic mice (Kang et al., 2001) were injected intraperitoneally with either kainate (25 mg/kg) in PBS or with an equivalent volume of PBS. Animals were scored for seizure levels according to the following behavioral criteria (according to Ben-Ari, 1985): scale 1: "staring" spells; scale 2: head nodding or brief limbic motor seizures; scale 3: recurrent limbic motor seizures; scale 4: complex and prolonged seizures with reduction in interictal pause; and scale 5: continuous convulsions. Animals were scored blind to genotype and then sacrificed 6 h after injection. Cerebral cortices were collected, frozen in liquid nitrogen, and kept at −80°C until RNA extraction. For Northern blot analysis an equal number of animals with matching seizure severity were selected from each group.

Northern blot hybridization

Total RNA was extracted from primary cortical cultures and cerebral cortices using TRIZOL reagent (Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. Northern blot hybridization was done as described (Sambrook et al., 1989). Total RNA from cortical cultures (10 μg) or from cerebral cortices (20 μg) was separated on 1% agarose gel containing formaldehyde, transferred to a nylon membrane, and hybridized with ^{32}P-labeled probes using stringent conditions. Probes were synthesized using the High Prime labeling kit (Roche, Indianapolis, IN) from the 1.6-kb mouse *cpg15* cDNA fragment, the 316-bp mouse *GAPDH* cDNA fragment excised from pTRI-GAPDH-mouse (Ambion, Austin, TX), or the 0.85-kb *Bam*HI/I fragment of SV40 polyadenylation region excised from pMSG (Amersham Pharmacia, Piscataway, NJ) to detect the dnCaMKIV transgene. The blot was hybridized first with the *cpg15* probe and then reprobed for *GAPDH*, scanned, and then the radioactivity of each band was quantified by Bio Image Analyzer BAS2500 (Fuji Film, Tokyo, Japan). The *cpg15* signal was divided by *GAPDH* signal to normalize for RNA loading. Mean and SEM were calculated from the normalized *cpg15* signal of two to three experiments. Statistical significance was determined by ANOVA and SNK post hoc analysis using StatView software (SAS Institute, Cary, NC).

Chromatin immunoprecipitation (ChIP) assay

ChIPs were done as described (Takahashi et al., 2000) with the following modifications. Primary cortical neurons (3×10^7 cells) at 4 div were cross-linked and lysed as described and sonicated for a total of 300 s (30 s × 10 pulses) to shear DNA to 200–1,000 bp. Cellular debris was removed by centrifugation at 14,000 rpm for 10 min. Sonicated chromatin from -1.2×10^7 cells was incubated with 4 μg of rabbit polyclonal-anti-CREB-1 antibody (C-21, Santa Cruz Biotechnology, Santa Cruz, CA) or control IgG in the presence of 1% Triton X-100, 0.1% deoxycholic acid, 0.5% protease inhibitor cocktail (Sigma), and 1 mM phenylmethylsulfonylfluoride at 4°C overnight. Protein A agarose (10-μl packed volume) pretreated with salmon sperm DNA and BSA were added and incubated for 3 h at 4°C to precipitate the immunocomplex. Samples were then washed, eluted, and treated with RNase A and Proteinase K and cross-links were reversed as described. DNA was purified by phenol/chloroform extraction and ethanol precipitation and resuspended in 60 μl of distilled water. Four microliters of this DNA was taken for 35 cycles of PCR (94°C 30 s, 60°C 30 s, 72°C 1 min per cycle). PCR primer sequences for *cpg15* −1.56 kb CRE were 5′-TCTGGGAGCCTCTGAACACT-3′ and 5′-CCTGCCCCTGGTGATGTC-3′; for *cpg15* +0.04 kb CRE were 5′-GAGATTTCGTTGAGATCGCAC-3′ and 5′-AGAGCTGGGAATGGTTCACT-3′; for the *GAP-43* promoter were 5′-CATATGTGTGCAGTTAGGTTAT-3′ and 5′-CCCAGTACCACAAGAAGGAA-3′. PCR products were resolved on 2% agarose and visualized by ethidium bromide staining.

PKA assay

PKA activity was assayed as described with minor modifications (Carter, 2002). Briefly, cultures were stimulated for 10 min and then each well of a 12-well dish was harvested in 100 μl of lysis buffer [50 mM HEPES (pH 7.4), 100 mM NaCl, 10 mM NaF, 2 mM Na_3VO_4, 4 mM

EDTA, 4 mM EGTA, 1% Triton X-100, 20 mM DTT, 1 mM 3-isobutylmethylxanthine, 1% protease inhibitor cocktail (Sigma), 2 mM PMSF]. After incubation on ice for 10 min, the lysate was centrifuged at 14,000 rpm for 10 min, and supernatant was collected. Cell lysate (10 μl) was added to 10 μl of reaction buffer to final concentrations of 50 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$, 30 μM Kemptide (Sigma), 5 μM ^{32}P-labeled γ-ATP (2.2×10^4 dpm/pmol, Amersham Pharmacia, Piscataway, NJ), with or without 10 μM cAMP. After a 5-min incubation at 30°C, 15 μl of the reaction was spotted onto phosphocellulose filters (P81, Whatman, Clifton, NJ), and these were washed five times in 75 mM phosphoric acid and once in ethanol. Filters were air-dried and radioactivity was measured with a liquid scintillation counter. Statistical significance was determined by ANOVA and SNK post hoc analysis using StatView software (SAS Institute, Cary, NC).

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