A Biological Function for the Neuronal Activity-Dependent Component of *Bdnf* Transcription in the Development of Cortical Inhibition

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SUMMARY

Neuronal activity-regulated gene expression has been suggested to be an important mediator of long-lasting, experience-dependent changes in the nervous system, but the activity-dependent component of gene transcription has never been selectively isolated and tested for its functional significance. Here, we demonstrate that introduction of a subtle knockin mutation into the mouse *Bdnf* gene that blocks the ability of the activity-regulated factor CREB to bind *Bdnf* promoter IV results in an animal in which the sensory experience-dependent induction of *Bdnf* expression is disrupted in the cortex. Neurons from these animals form fewer inhibitory synapses, have fewer spontaneous inhibitory quantal events, and exhibit reduced expression of inhibitory presynaptic markers in the cortex. These results indicate a specific requirement for activity-dependent *Bdnf* expression in the development of inhibition in the cortex and demonstrate that the activation of gene expression in response to experience-driven neuronal activity has important biological consequences in the nervous system.

INTRODUCTION

Experience-dependent changes in the nervous system occur throughout the lifetime of an animal. Sensory experience mediates the structural and functional refinement of developing neuronal circuits (Fox and Wong, 2005), and, in the mature brain, use-dependent modification of neuronal circuits underlies important adaptive functions of the nervous system, including learning, memory, and behavior (Kandel, 2001). The discovery that excitatory neurotransmitters can stimulate new gene transcription by triggering an influx of calcium into postsynaptic neurons (Greenberg et al., 1986; Kornhauser et al., 1990; Morgan et al., 1987) suggested a compelling mechanism by which stimulus-evoked neuronal activity might effect long-lasting changes in the structure and function of the nervous system. In the two decades since, significant advances have been made in elucidating

many of the key activity-responsive transcriptional regulators, the calcium-dependent signaling pathways that couple extracellular stimuli to their activation, and the programs of gene expression that are activated in response to neuronal activity (Lanahan and Worley, 1998; West et al., 2002).

Activity-dependent gene expression occurs when synaptic activation triggers calcium influx at the membrane through ligand- and voltage-gated calcium channels, thereby initiating calcium-dependent signaling cascades that amplify and carry the signal to activate transcription factors in the nucleus. Several searches for activity-regulated genes have identified ~300 genes as being responsive to neuronal activity (Bartel et al., 1989; Lanahan and Worley, 1998; Lin et al., 2008; Nedivi et al., 1993), many of which fall into one of two categories. The first category includes genes that themselves encode for transcriptional regulators, exemplified by the classic immediate-early gene *c-fos* (Greenberg et al., 1986) or the neuronal bHLH/PAS factor *npas4* (Lin et al., 2008). The second category includes genes that encode for neuronally enriched proteins that are believed to function directly at the synapse and play important roles in neural development and plasticity, such as *Bdnf* (Poo, 2001).

There is no doubt that genes whose transcription is regulated by activity are important for the development and function of the brain. However, despite considerable progress in our understanding of the program of neuronal activity-regulated gene expression, direct evidence that the activity-dependent component of transcription per se is specifically important for nervous system development or function has been elusive. This fact is due in part to limitations in our ability to experimentally manipulate the activity-dependent component of transcription independent of the many other genetic programs regulating a given gene's transcription. For instance, the role of an activity-regulated gene is most often investigated through loss-of-function studies that remove the gene entirely, independent of the stimulus state or cellular context. This approach can often lead to an outcome in which the functions of a gene that are dependent on its activity-dependent expression are conflated with, for instance, its trophic functions in supporting cell health or survival. In the case of one of the best-studied activity-regulated genes, brain-derived neurotrophic factor (*Bdnf*), studies in mice that completely lack *Bdnf* have demonstrated that it plays a key role in neuronal survival, differentiation, migration, and dendritic arborization (Ernfors et al., 1994; Jones et al., 1994; Schwartz
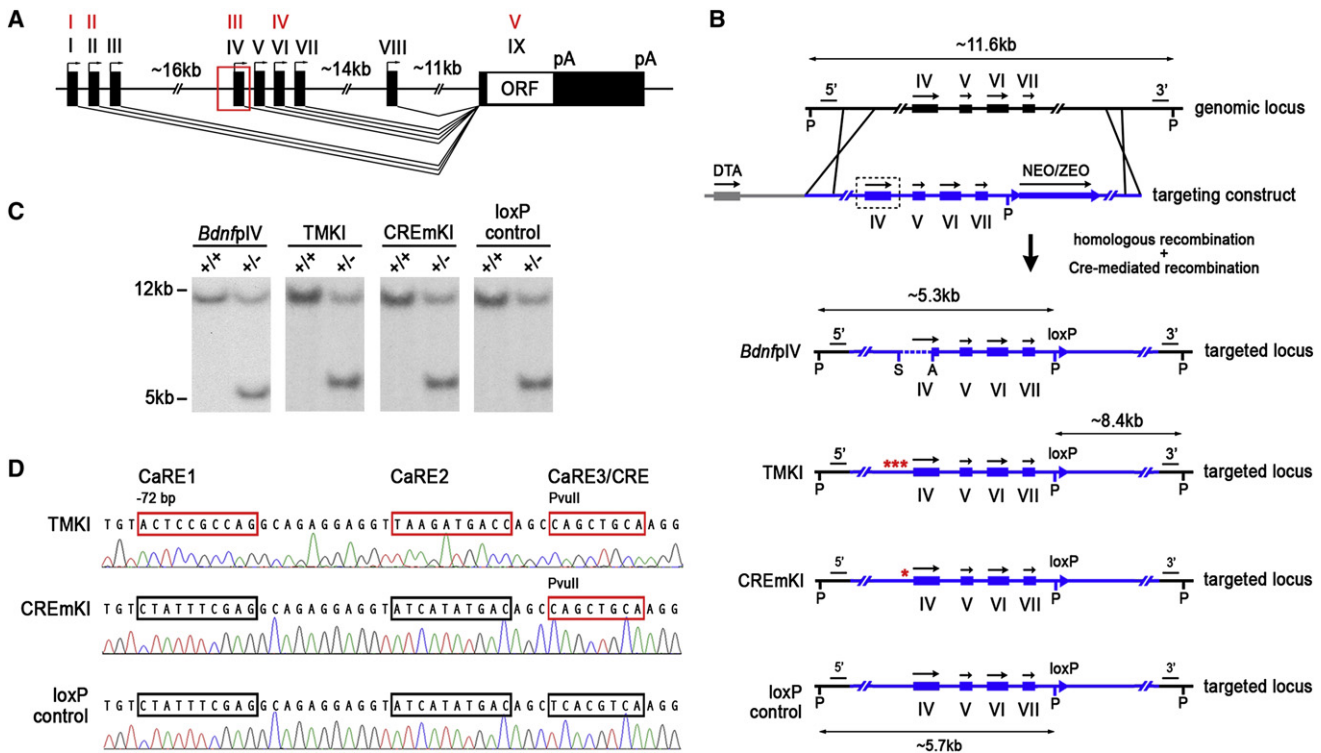
Figure 1. Generation of *Bdnf pIV*, TMKI, CREmKI, and loxP Control Mice

(A) Genomic structure of the rodent *Bdnf* gene adapted from Aid et al. (2007). The discovery of additional exons in the rodent *Bdnf* gene has resulted in changes in exon nomenclature; the four major transcripts now known as I, II, IV, and VI were initially described as I, II, III, and IV (in red), respectively, in Timmusk et al. (1993).

(B) Targeting strategy for the introduction of *pIV*, TMKI, CREmKI, and loxP control mutations into *Bdnf* promoter IV by homologous recombination. P, PciI; A, AgeI; S, SphI; 5′, 3′ Southern probes; NEO/ZEO, neomycin-zeomycin positive selection cassette; DTA, diphtheria toxin negative selection cassette.

(C) Southern blot analysis of PciI-digested genomic DNA from targeted ES cells using the 5′ probe indicates correct targeting of each mutation. *pIV*: WT, 11.6 kb; mut., 5.3 kb; TMKI, CREmKI, loxP control: WT, 11.6 kb; mut., 5.7 kb.

(D) Direct sequencing of PCR products amplified from genomic DNA isolated from TMKI, CREmKI, or loxP control homozygous mice using primers spanning *Bdnf* promoter IV.

et al., 1997). In addition, mice heterozygous for the loss of a *Bdnf* allele show deficits in synaptic development, function, and plasticity, as well as changes in body weight regulation, locomotor activity, and aggression (Abidin et al., 2006, 2008; Carter et al., 2002; Kernie et al., 2000; Korte et al., 1995; Lyons et al., 1999; Patterson et al., 1996). Many of these diverse functions of *Bdnf*, in particular its functions in synaptic development, function, and plasticity, are thought to be regulated at least in part by its neuronal activity-dependent expression (Poo, 2001), but direct evidence for this idea is lacking.

In support of the idea that the many functions of *Bdnf* in the nervous system may be dependent on the tight temporal, spatial, and stimulus-specific regulation of *Bdnf* expression is the complexity of its gene structure. The *Bdnf* gene is comprised of at least eight distinct promoters that initiate transcription of multiple distinct mRNA transcripts, each of which contains an alternative 5′ exon spliced to a common 3′ coding exon that contains the entire open reading frame for the BDNF protein (Aid et al., 2007). Through the use of alternative promoters, splice donors, and polyadenylation sites, at least 18 distinct transcripts can be produced from the *Bdnf* gene (Figure 1A); remarkably, however, each of these *Bdnf* mRNAs encodes an identical BDNF protein.

The functional significance of the transcriptional organization of the *Bdnf* gene remains cryptic, but one attractive hypothesis is that the production of many mRNAs that all encode the same protein product provides for multiple layers of regulation of BDNF expression, for instance, at the level of alternative promoter usage, differential mRNA stability and translatability, or differential subcellular localization of either the mRNA message(s) or protein. In support of this idea, it is well known that the various *Bdnf* promoters are differentially responsive to neuronal activity; in particular, in the cortex, promoter IV-dependent *Bdnf* transcription accounts for the majority of neuronal activity-induced *Bdnf* expression (Tao et al., 1998; Timmusk et al., 1993, 1994). This observation prompted us to ask whether we could take advantage of our understanding of the activity-dependent regulation of the *Bdnf* gene to generate an animal model that allows us to ask which, if any, of the physiological functions of *Bdnf* in the nervous system are specifically controlled by the neuronal activity-regulated component of its expression.

Our lab and others have extensively investigated the activity-dependent activation of *Bdnf* promoter IV as a model for understanding the mechanisms mediating calcium-induced neuronal gene expression. Experiments using a plasmid-based reporter

to recapitulate promoter IV activity revealed that a cAMP/Ca^{2+}-response element-like element (CaRE3/CRE) proximal to the exon IV transcription start site is important for the ability of membrane depolarization to induce promoter IV activation (Shieh et al., 1998; Tao et al., 1998). In vitro studies indicate that this element is bound by the transcription factor CREB, a calcium-regulated factor that has important functions in controlling many adaptive neuronal responses. CREB bound at promoter IV becomes phosphorylated by calcium-regulated kinase cascades in response to neuronal activity and subsequently recruits components of the basal transcriptional machinery to *Bdnf* promoter IV to activate gene expression (Lonze and Ginty, 2002; West et al., 2001).

Here, we report that mutation of the CaRE3/CRE (CREm) at endogenous *Bdnf* promoter IV by gene targeting results in an animal in which the neuronal activity-dependent component of *Bdnf* transcription in the cortex is specifically disrupted. CREm knockin mice exhibit a reduction in the number of inhibitory synapses formed by cortical neurons in culture, a reduction in spontaneous inhibitory quantal transmission measured in acute brain slices, and a reduction in the level of inhibitory presynaptic markers in the cortex. These findings provide a clear demonstration of a biological role for activity-dependent gene expression in the regulation of cortical inhibition and also indicate a requirement for promoter IV-derived *Bdnf* expression in this process. Interestingly, our findings point to a previously unappreciated role for CREB in regulating inhibitory synapse development and demonstrate the utility of mice bearing subtle mutations in gene regulatory regions for uncovering novel, target-specific functions for a transcription factor that may not be revealed by traditional loss-of-function studies.

RESULTS

Generation of a Mouse Model with Impaired Activity-Dependent *Bdnf* Expression

We chose to focus on the *Bdnf* gene as a model for understanding the functional significance of neuronal activity-regulated gene expression in the brain for several reasons. First, a large body of work has demonstrated a critical role for *Bdnf* in many diverse processes in nervous system development and function (Poo, 2001), many of which are regulated by neuronal activity. Second, perhaps more than any other activity-regulated gene, the mechanisms by which calcium influx induces *Bdnf* transcription have been extensively studied, lending insight into the type of genetic lesion that could potentially block activity-dependent expression of the gene (West et al., 2001). In the cortex, for example, promoter IV-derived *Bdnf* transcripts comprise the majority of neuronal activity-induced *Bdnf* expression (Tao et al., 1998; Timmusk et al., 1993, 1994). In addition, unlike many other prototypical immediate-early genes such as *c-fos*, the induction of *Bdnf* exon IV-containing transcripts is selectively activated by the influx of extracellular calcium through ligand- and voltage-gated calcium channels; growth factor stimulation, for instance, does not induce robust promoter IV activation. Thus, we reasoned that disruption of exon IV-containing *Bdnf* transcripts might be sufficient to specifically attenuate activity-dependent expression of *Bdnf* in the cortex.

Previous studies investigating the mechanisms of *Bdnf* promoter IV activation have demonstrated that an evolutionarily conserved 170 bp fragment proximal to the exon IV transcriptional start site is sufficient to confer Ca^{2+}-selective responsiveness to a plasmid-based reporter gene when transfected into neurons in vitro. Mutagenesis analysis revealed the existence of three Ca^{2+}-responsive elements (CaREs), CaRE1, CaRE2, and CaRE3/CRE (Figure 1D), each of which, when individually mutated, was sufficient to attenuate activity-dependent induction of the promoter IV reporter (Tao et al., 1998). At the time we began this study, the prevailing view was that CaRE1 is bound by the calcium-responsive transcription factor, CaRF (Tao et al., 2002); CaRE2 contains an E box element and is bound by the bHLH transcription factors, USF1 and USF2 (Chen et al., 2003b); and CaRE3 is a cAMP/Ca^{2+}-response element (CRE)-like element that is bound by CREB (Tao et al., 1998).

Based on the available information at the time, we used homologous recombination in embryonic stem cells to generate three independent lines of mutant mice in which endogenous *Bdnf* promoter IV is genetically disrupted (Figure 1B). First, we generated a deletion in *Bdnf* promoter IV ($pIV^{-/-}$) that removes the previously mapped 170 bp proximal promoter fragment, as well as the transcriptional start site, to completely abrogate transcription from promoter IV. Second, we introduced point mutations into the CREB-binding site CaRE3/CRE element of *Bdnf* promoter IV (CREmKI, Figures 1B and 1D), which was predicted to block promoter IV induction based on the plasmid reporter assay. Finally, since it is unknown whether mutation of an individual transcriptional control element can independently affect promoter activity in the context of endogenous chromatin, we also generated a third line in which we introduced point mutations into each of the CaRE1, CaRE2, and CaRE3/CRE elements of *Bdnf* promoter IV in combination (triple-site mutant, TMKI, Figures 1B and 1D). We reasoned that a combination of mutations might function more effectively to block promoter IV activation than mutation of the CaRE3/CRE alone, since previous studies at the *c-fos* promoter suggest that the concerted action of multiple transcriptional elements is required for gene regulation (Robertson et al., 1995).

For each mutant line, the gene targeting strategy to introduce the mutation into *Bdnf* promoter IV in embryonic stem cells necessitates the introduction of a 34 bp loxP sequence in the vicinity of the mutation as a by-product of the selection procedure after homologous recombination. To control for the possibility that the presence of the loxP sequence could affect gene expression from the *Bdnf* locus in unexpected ways, we generated a fourth mouse line in which promoter IV is left unaltered but that differs from a wild-type mouse only by the presence of the loxP sequence in an unconserved region of the large intron between exons VII and VIII (loxP control). Since the loxP site in all three mutants, $pIV^{-/-}$, CREmKI, and TMKI, is engineered into this identical location (Figure 1B), any differences seen in the mutant lines as compared to the loxP control must therefore stem from their respective mutations at *Bdnf* promoter IV. Southern blot analysis of genomic DNA isolated from the targeted ES cells, as well as from heterozygous and homozygous mutant mice for all four mutant mouse lines, $pIV^{-/-}$, CREmKI, TMKI, and loxP control, resulted in the predicted mutant allele structure after
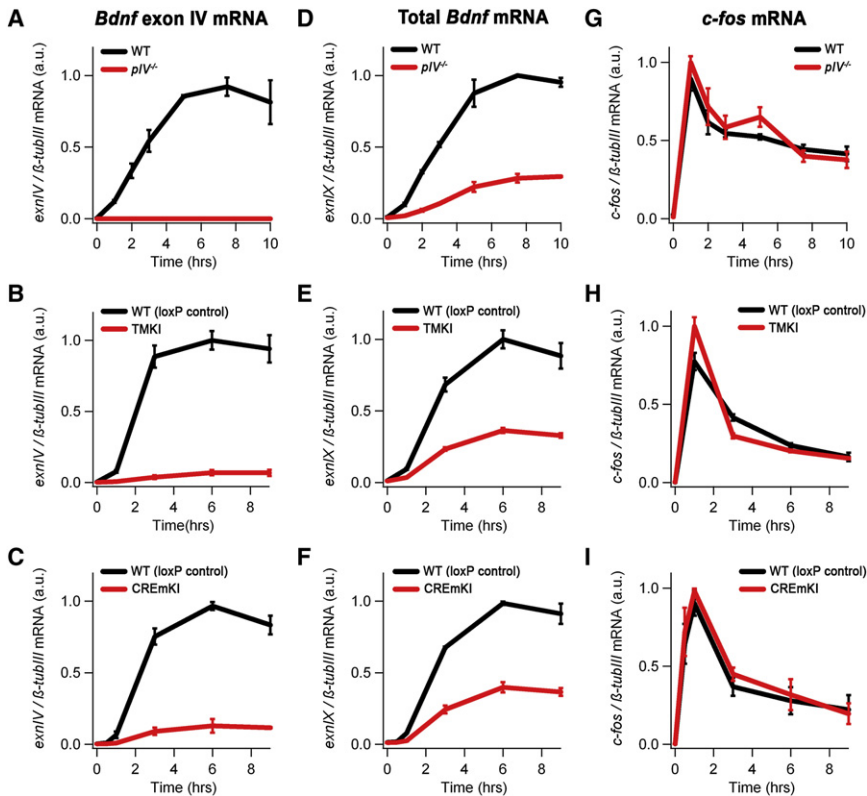
Figure 2. Activity-Dependent *Bdnf* Transcription in $pIV^{-/-}$, TMKI, and CREmKI Cortical Neurons Is Impaired in Response to Membrane Depolarization

Levels of *Bdnf* exon IV (A–C), exon IX (coding exon) (D–F), and *c-fos* (G–I) mRNA in 5 DIV cortical neurons prepared from $pIV^{-/-}$ and wild-type littermates (A, D, and G), homozygous TMKI and loxP control littermates (B, E, and H), or homozygous CREmKI and loxP control littermates (C, F, and I) that were either mock-treated or membrane depolarized with high extracellular potassium for the indicated amounts of time. Differences in *Bdnf* exon IV and total *Bdnf* mRNA expression are statistically significant for $pIV^{-/-}$, TMKI, or CREmKI versus their respective controls (p < 0.01, repeated-measures ANOVA; p < 0.01, pairwise comparisons at each time point 1 hr or longer, Bonferroni-Dunn post hoc test). Data are mean ± SEM from n = 3 ($pIV^{-/-}$ and CREmKI) or n = 2 (TMKI) independent experiments in which each sample was measured in triplicate.

recombination (Figures 1C and S1 [available online], data not shown). The CREmKI, TMKI, and loxP control mutations were further verified by direct sequencing of the genomic DNA from homozygous mutants (Figure 1D).

Mutation of the CREB-Binding Site in *Bdnf* Promoter IV Impairs Neuronal Activity-Regulated *Bdnf* Transcription

To test whether the introduction of $pIV^{-/-}$, CREmKI, and TMKI mutations into *Bdnf* promoter IV blocks neuronal activity-dependent *Bdnf* expression, we prepared dissociated primary cortical cultures from the brains of homozygous mutant and wild-type or loxP control littermates for each mutant line. Following maintenance in vitro for 5 days (5 DIV), neurons were depolarized with high extracellular potassium chloride for varying amounts of time, RNA was extracted, and the levels of *Bdnf* mRNA transcripts were measured by quantitative real-time PCR (qPCR). As expected, deletion of *Bdnf* promoter IV results in a complete loss of exon IV-containing *Bdnf* mRNAs (Figure 2A). Furthermore, mutation of either all three CaREs in combination or of the CaRE3/CRE alone is sufficient to cause an ~90% reduction in the level of *Bdnf* promoter IV induction (Figures 2B and 2C). This failure of *Bdnf* promoter IV activation does not reflect a general defect in calcium-dependent signaling in these mutants because the induction of other immediate-early genes, including *c-fos*, *activity-regulated cytoskeletal-associated protein* (*Arc*), and the other major activity-regulated *Bdnf* transcript exon I, is unaffected in $pIV^{-/-}$, CREmKI, or TMKI neurons (Figures 2G–2I and S2; data not shown). The impairments in promoter IV activation result in an ~70% or 60% reduction in the activity-dependent

induction of total *Bdnf* mRNA in $pIV^{-/-}$ or CREmKI and TMKI cortical neurons, respectively (Figures 2D–2F). This impairment in total *Bdnf* mRNA induction is reflected in an ~50% decrease in BDNF protein levels in $pIV^{-/-}$ or CREmKI cortex (Figure S3, see also Figure 4F). Thus, all three mutations are sufficient to cause a significant impairment in activity-dependent *Bdnf* expression in neurons.

We chose to focus our study on the CREmKI mutant for several reasons. First, the CREmKI mutation represents a subtle change in nucleotide sequence but is sufficient to cause a substantial impairment in the activity-dependent induction of *Bdnf*, comparable to that observed in TMKI neurons. Second, the CREmKI mutant provides several significant advantages over the $pIV^{-/-}$ mutant for selectively evaluating the functional significance of both activity-dependent and promoter IV-dependent *Bdnf* transcription. Whereas the $pIV^{-/-}$ deletion mutant completely removes promoter IV-derived transcript independent of the stimulus state of the neurons, the CREmKI mutant impairs *Bdnf* promoter IV activation specifically in response to neuronal activity-dependent rises in calcium by eliminating binding of the Ca^{2+}-regulated factor CREB to *Bdnf* promoter IV (see below, Figure 8). The CREmKI mutation has an additional advantage in that it maintains the endogenous configuration of the *Bdnf* locus, thereby circumventing potential artifacts that can arise in deletion mutants where the spacing among residual promoter elements and exons is changed. Finally, since previous studies strongly suggest that the CaRE3/CRE element in *Bdnf* promoter IV is bound by CREB (Tao et al., 1998), the CREmKI mutant also has the potential to provide insight into specific functions of CREB-dependent gene regulation in the nervous system.

CREmKI mice are viable, fertile, born in the expected Mendelian ratios, and visually indistinguishable from loxP control littermates. In addition, CREmKI animals have comparably sized brains to loxP control animals, show no gross anatomical

alterations in cortical, cerebellar, or hippocampal structure, no obvious changes in gait, and no obvious defects in cortical layering (see Supplemental Experimental Procedures, Figure S4, and data not shown). These observations suggest that, in contrast to the complete loss of *Bdnf* in $Bdnf^{-/-}$ mutants, the introduction of a subtle knockin mutation into *Bdnf* promoter IV to specifically impair activity-dependent *Bdnf* expression does not have a strong effect on neuronal survival.

To investigate how the CREmKI mutation affects activity-regulated *Bdnf* expression in response to glutamate receptor activation, we treated 12 DIV cortical neurons, which have developed many more synaptic contacts than 5 DIV neurons, with NMDA to stimulate NMDA-type glutamate receptors, a major mediator of excitatory neurotransmission. Similar to our findings under membrane-depolarizing conditions, we observed significant impairments in the activation of *Bdnf* promoter IV and total *Bdnf* expression in CREmKI neurons as compared to loxP control neurons (Figures 3A and 3B) in response to NMDA stimulation. Interestingly, when we examined the expression of the remaining major *Bdnf* transcripts (Figures 1A, 3C, 3D, and 3F) as well as other activity-regulated genes in 12 DIV CREmKI neurons, we were surprised to find that the induction of most immediate-early genes, including *neuronal pentraxin 2* (*NP2*), *Arc*, *c-fos*, as well as *Bdnf* exons I and II, is significantly upregulated in response to stimulation with NMDA (Figures 3C–3E and data not shown). These observations raise the interesting possibility that the general increase in neuronal activity-dependent transcription in 12 DIV CREmKI neurons may be secondary to an increase in their overall level of excitability. These differences may not be obvious after only 5 days in culture when neurons are not extensively synaptically connected (Figures 2G–2I).

Mutation of the CREB-Binding Site in *Bdnf* Promoter IV Impairs Sensory Experience-Driven *Bdnf* Transcription in the Intact Brain

We next examined whether the activity-dependent expression of *Bdnf* in the intact CREmKI brain is affected in response to a physiologically relevant environmental stimulus. Adult mice were maintained in total darkness for 14 days to reduce levels of sensory activity-driven transcripts in the visual cortex. RNA was then harvested from the primary visual cortex of mice that were either maintained in darkness or exposed to light for 90 min, and the levels of *Bdnf* transcripts were measured by qPCR. We found that the nearly 20-fold increase in the levels of exon IV-containing transcripts measured in loxP control visual cortex in response to light exposure is reduced by ~75% in CREmKI visual cortex (Figure 4A). The activity-dependent induction of gene expression measured in the visual cortex is specific to visual experience because it is not observed in the somatosensory cortex of either loxP control or CREmKI brains (Figure S5). Interestingly, we also observed that many other immediate-early genes, including *c-fos*, *Arc*, and *NP2*, are more highly induced in the visual cortex of CREmKI animals in response to light stimulation when compared to loxP controls (Figure 4B and data not shown). Moreover, measurements of activity-dependent gene expression in the cortex of CREmKI and loxP control animals given seizures by administration of the chemoconvulsant kainic acid resulted in similar findings (Figures 4C–4E and S6). The heightened induc-
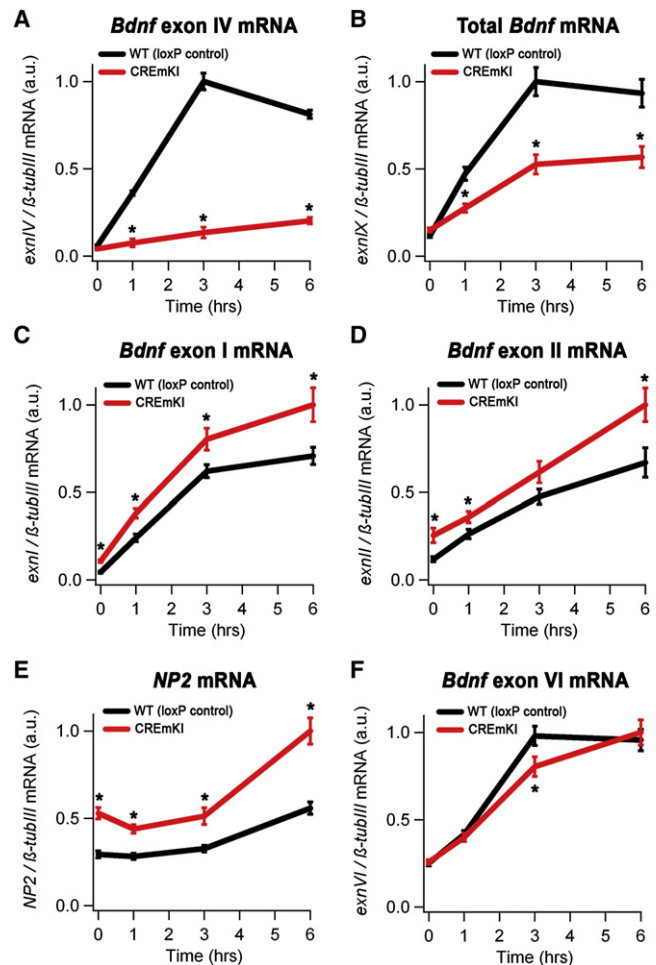
Figure 3. Neuronal Activity-Dependent *Bdnf* Transcription in CREmKI Cortical Neurons Is Impaired in Response to Glutamate Receptor Activation

Levels of *Bdnf* exon IV (A), total *Bdnf* (exon IX) (B), *Bdnf* exon I (C), *Bdnf* exon II (D), *NP2* (E), and *Bdnf* exon VI (F) mRNA in 12 DIV cortical neurons prepared from CREmKI or loxP control littermates that were either mock stimulated or treated with 20 μM NMDA for the indicated amounts of time. *$p < 0.01$, repeated-measures ANOVA, pairwise comparisons at indicated time points, Bonferroni-Dunn post hoc correction. Data are mean ± SEM from n = 2 independent experiments in which each sample was measured in triplicate.

tion of many activity-regulated genes in response to neuronal activity in CREmKI animals compared to controls again suggests the possibility of changes in the excitability of neuronal circuits in the CREmKI brain.

The impairment in activity-dependent *Bdnf* promoter IV transcription in response to light exposure in CREmKI visual cortex results in an ~50% decrease in the induction of total *Bdnf* transcripts driven by sensory stimulation (Figure 4A), as compared to loxP control animals. This reduction in *Bdnf* mRNA is reflected in an ~60% decrease in the induction of BDNF protein levels in visual cortex in response to visual experience (Figure 4F). Importantly, the CREmKI mutation does not appear to affect *Bdnf* expression under conditions of significantly reduced neuronal activity, since levels of both promoter IV-derived and total *Bdnf*
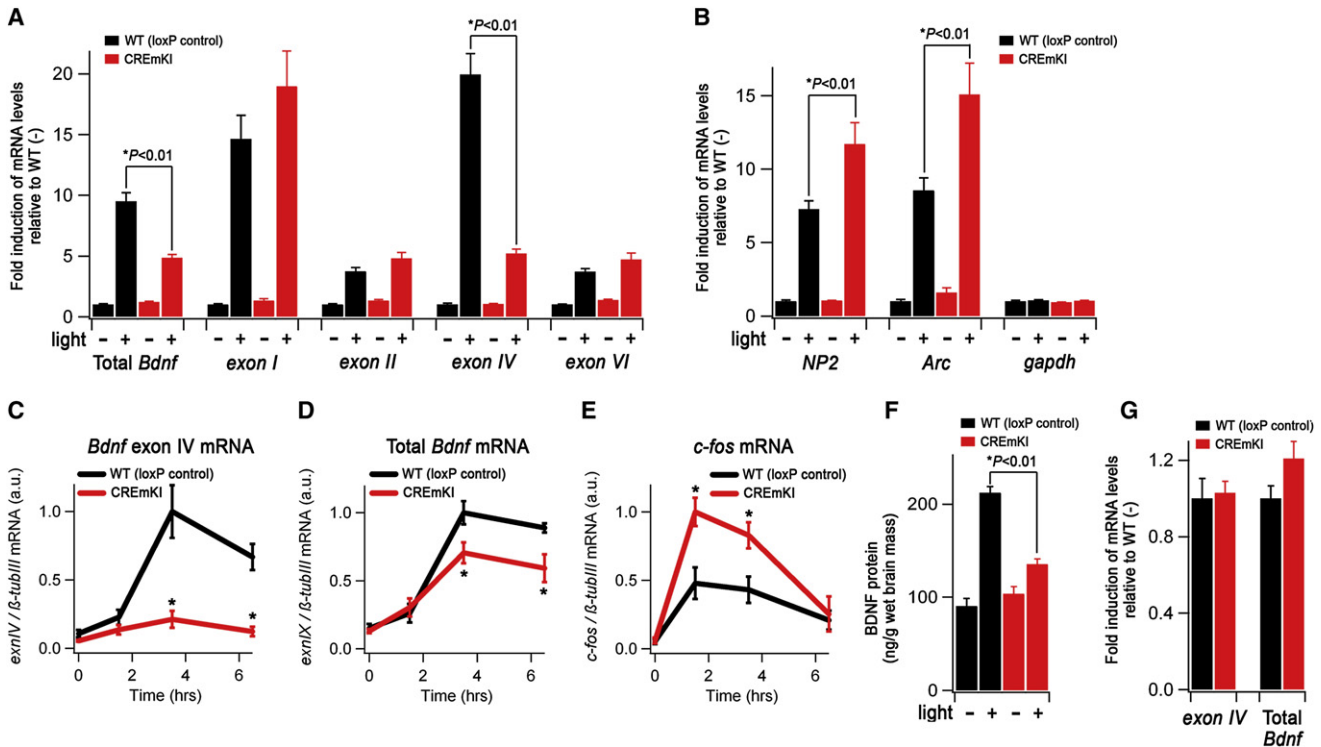
Figure 4. Sensory Experience-Dependent *Bdnf* Expression Is Impaired in the Intact CREmKI Brain

(A and B) Levels of the indicated transcripts in the primary visual cortex of adult CREmKI and loxP control mice reared in darkness for 14 days, after which they were either maintained in darkness (−) or exposed to light for 90 min (+). Within each transcript, mRNA levels are reported as fold induction relative to the loxP control unstimulated (−) condition. In either loxP control or CREmKI visual cortex, light stimulation induced the expression of all mRNAs measured (p < 0.01, two-way ANOVA with Bonferroni Dunn post hoc test), except for *gapdh*, which served as a non-activity-regulated gene control.

(C–E) *Bdnf* exon IV, total *Bdnf*, and *c-fos* mRNA levels in the cortex of CREmKI and loxP control mice injected with either saline (time 0) or kainic acid (KA) for the indicated amounts of time.

(F) Levels of BDNF protein in the primary visual cortex of animals from (A) and (B).

(G) The unstimulated (−) levels of *Bdnf* exon IV and total *Bdnf* in CREmKI and loxP control visual cortex are replotted from (A) for direct comparison (p > 0.05, two-way ANOVA with Bonferroni-Dunn post hoc test).

Data are from n = 5–7 animals per condition for both visual stimulation and KA seizure experiments. Each animal was measured in triplicate, and data are presented as mean ± SEM. Asterisks denote p < 0.01, two-way (visual simulation) or repeated-measures (KA seizure) ANOVA with Bonferroni-Dunn post hoc test between indicated pair.

transcripts are similar in sensory-deprived loxP control and CREmKI visual cortex (Figure 4G, p > 0.05). Taken together, these results provide strong evidence that the neuronal activity-dependent component, but not the basal component, of *Bdnf* transcription is selectively impaired in the intact brains of CREmKI animals in response to a physiologically relevant sensory experience and demonstrate the utility of the CREmKI animal model for examining the functional significance of neuronal activity-regulated gene expression for nervous system development and function.

Neuronal Activity-Dependent *Bdnf* Expression Regulates the Development of Inhibition in the Nervous System

Our observations that the neuronal activity-dependent induction of many immediate-early genes, including non-promoter IV-derived *Bdnf* transcripts, *c-fos*, *NP2*, or *Arc*, is enhanced in mature CREmKI neuronal cultures or in the intact brain of CREmKI

mutants suggested that neuronal connectivity may be altered in CREmKI brains. In addition, a large body of work has independently established both BDNF and neuronal activity as positive regulators of inhibitory synapse development. For example, application of recombinant BDNF or BDNF overexpression promotes the development of inhibition (Huang et al., 1999; Ohba et al., 2005), whereas reduction of neuronal activity using pharmacological blockers or by sensory deprivation retards the maturation of inhibition (Benevento et al., 1995; Hendry and Jones, 1986; Rutherford et al., 1997). A compelling hypothesis that arises from this work suggests that neuronal activity regulates the development of inhibitory synapses through its effects on *Bdnf* gene transcription. The CREmKI mutant allows us to directly test this hypothesis by asking whether the impairment in activity-dependent *Bdnf* expression affects the development of inhibition in the nervous system.

To specifically investigate the effect of the CREmKI mutation on the development of inhibitory synapses, we first prepared
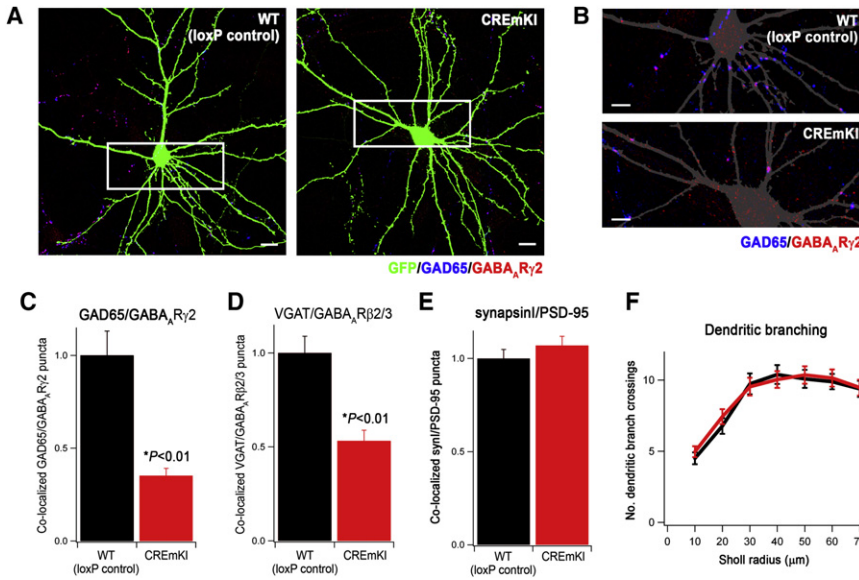
Figure 5. Impaired Activity-Dependent *Bdnf* Expression Reduces the Number of Inhibitory Synapses Formed on CREmKI Neurons in Culture

(A) Representative images of GFP-transfected, E17.5 + 18 DIV CREmKI and loxP control littermate cortical neurons, immunostained with the indicated antibodies to label pre- and postsynaptic inhibitory synapse terminals. Scale bar, 10 μm.

(B) Enlargement of the boxed area in (A) shows details of dendrites. The GFP signal is converted to gray to allow better visualization of the synaptic puncta. Scale bar, 5 μm.

(C–E) Quantification of the average density of GAD65/GABA$_A$Rγ2 ([C], 78–81 cells/condition), VGAT/GABA$_A$Rβ2/3 ([D], 65–66 cells/condition), and synapsinI/PSD-95 ([E], 63–65 cells/condition) coclusters along the dendrites of GFP-transfected CREmKI and loxP control neurons. *p < 0.01, two-way ANOVA with pairwise comparison by Bonferroni-Dunn post hoc test. Data are mean ± SEM from three to four independent experiments.

(F) Quantification of dendritic branch complexity by Sholl analysis plots the number of dendritic branches intersecting concentric circles of increasing radii centered on the cell body. p > 0.05 by repeated-measures ANOVA; data are mean ± SEM from 26–28 cells/condition from two independent experiments.

low-density cortical cultures from CREmKI and loxP control littermates that allowed us to quantify synapse density by immunostaining. Neurons were transfected with a plasmid encoding green fluorescent protein (GFP) at 5 DIV and were fixed and immunostained at 18 DIV with antibodies that recognize the presynaptic inhibitory marker GAD65 and the postsynaptic inhibitory marker GABA$_A$ receptor γ2 subunit (GABA$_A$Rγ2). The number of inhibitory synapses that form on a neuron was quantified as the number of colocalized GAD65 and GABA$_A$Rγ2 puncta that overlap with GFP signal (Figures 5A and 5B).

Using these criteria, we observed that significantly fewer inhibitory synapses form on CREmKI cortical neurons than on loxP control littermate neurons in culture (Figures 5A–5C). A similar result was also seen when inhibitory synapses were quantified using an independent set of markers, the inhibitory presynaptic marker VGAT and the inhibitory postsynaptic marker GABA$_A$ receptor β2/3 subunits (Figure 5D). The decrease in synapse number in CREmKI neurons appears specific to inhibitory synapses because no difference in the number of excitatory synapses was detected when CREmKI neurons were compared to loxP control neurons using the presynaptic marker synapsin I and the excitatory postsynaptic marker PSD95 (Figure 5E). In addition, the reduction in inhibitory synapse number in CREmKI neurons does not appear to be secondary to poor cell health since no significant differences between CREmKI and loxP control littermate neuronal cultures were observed in the number of pyknotic nuclei present (data not shown) or in the extent of dendritic arborization, as quantified by Sholl analysis (Figure 5F). These results demonstrate that the activity-dependent expression of *Bdnf* is important for the development of inhibitory, but not excitatory, synapses in neurons maintained in culture.

Although many of the key steps of synaptogenesis are recapitulated by dissociated neurons as they reestablish their connections in culture (Ziv and Garner, 2004), we also wanted to examine the role of activity-dependent *Bdnf* expression in regulating the development of inhibition in the context of an endogenous neural circuit. Since our previous data indicate that visual experience-dependent *Bdnf* transcription is impaired in CREmKI brains and because previous studies have implicated *Bdnf* in the regulation of cortical inhibition and plasticity in the visual cortex, we chose to measure the number of functional inhibitory synapses that form on neurons in an intact circuit in the primary visual cortex of CREmKI animals. We prepared acute slices containing primary visual cortex (V1) from the brains of P16–P18 CREmKI or loxP control littermates and obtained whole-cell patch-clamp recordings from layer II/III pyramidal neurons in V1 to measure spontaneous miniature inhibitory postsynaptic currents (mIPSCs) (Figure 6A). Pharmacologically isolated mIPSCs were demonstrated to be GABA$_A$R mediated, since they were abolished by treatment with picrotoxin (data not shown). We observed a significant decrease in the frequency of mIPSC events in CREmKI neurons (interevent interval, 742.4 ± 36.8 versus 355.4 ± 14.7 ms, CREmKI and loxP control, respectively; Figures 6B–6C(I)), suggesting that impaired activity-dependent *Bdnf* transcription in CREmKI cortex results in fewer functional inhibitory synapses as compared to loxP control. Interestingly, we also found a significant increase in the amplitude of mIPSCs in CREmKI neurons (28.1 ± 0.7 versus 23.9 ± 0.7 pA, CREmKI and loxP control, respectively; Figures 6B–6C(II)), suggesting that activity-dependent *Bdnf* expression can regulate inhibitory synapse strength. Whereas the change in mIPSC amplitude in CREmKI neurons might reflect the direct action of activity-dependent *Bdnf* on
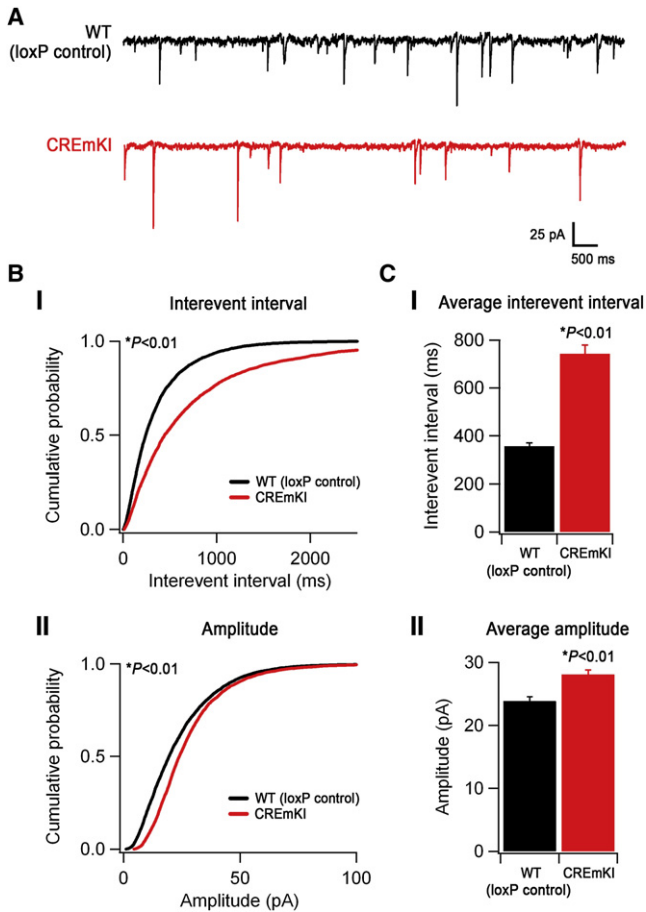
Figure 6. Neuronal Activity-Dependent *Bdnf* Expression Controls the Development of Cortical Inhibition

(A) Representative traces of mIPSCs recorded from layer II/III V1 pyramidal neurons in loxP control and CREmKI acute cortical slices.

(B) Cumulative probability distributions of mIPSC interevent intervals (I) and amplitudes (II) recorded from loxP control and CREmKI neurons. p < 0.01 by either Kolmogorov-Smirnov test or Monte Carlo simulation (see Supplemental Experimental Procedures).

(C) Average interevent interval (I) and amplitude (II) of mIPSCs recorded from loxP control and CREmKI neurons. Data are mean ± SEM, p < 0.01 by Student's t test. Data are from 18–20 cells/genotype recorded from 12 pairs of littermates.

vesicle neurotransmitter content or postsynaptic GABA$_A$ receptor number, it could also occur secondarily as a homeostatic adjustment to the decrease in inhibitory input number. Nevertheless, these findings are consistent with our measurements by immunofluorescence staining in cultured cortical neurons indicating that fewer inhibitory synapses form on CREmKI neurons and strongly support the hypothesis that activity-dependent *Bdnf* transcription plays a critical role in GABAergic synapse development in the nervous system.

Because mIPSCs reflect the spontaneous release of individual neurotransmitter vesicles at presynaptic sites of contact, changes in mIPSC frequency correlate with changes in inhibitory synapse number, but they can also be affected by changes in presynaptic properties, such as the probability of neurotransmit-

ter vesicle release. As an independent measurement of inhibitory synapse number in CREmKI neurons connected together in an endogenous circuit, we immunostained histological sections of visual cortex prepared from P20–P24 CREmKI or loxP control littermates for inhibitory synaptic markers. We observed modest, but significant decreases in the intensity of staining for the inhibitory presynaptic markers GAD65 and VGAT in layer II/III of CREmKI visual cortex as compared to loxP control visual cortex (Figures 7A–7C), consistent with a reduction in inhibitory synapse number in CREmKI neurons. However, immunostaining for the presynaptic marker synapsin I (data not shown) or the excitatory presynaptic marker VGLUT1 (Figures 7A and 7D) is similar in CREmKI and loxP control visual cortex, consistent with our previous observation that excitatory synapse development is unaffected in CREmKI neurons in culture. Taken together, our data demonstrate a role for neuronal activity-dependent *Bdnf* expression in the development of inhibition in the cortex, and suggest that impairments in activity-dependent *Bdnf* transcription lead to a shift in the ratio of excitation to inhibition in the brain that may increase the excitability of the CREmKI brain.

To begin to gain insight into the cellular mechanisms by which activity-dependent *Bdnf* expression regulates inhibitory synapse number, we next asked whether the decrease in inhibition observed in CREmKI brains might be a simple consequence of changes in the number of cortical inhibitory neurons, since total *Bdnf* null mice exhibit a dramatic reduction in the density of parvalbumin-positive and NPY-positive inhibitory neurons in the cortex (Jones et al., 1994). We focused our analysis on these populations of interneurons and employed stereological methods to quantify the number of parvalbumin- or NPY-positive neurons in the visual cortex of CREmKI or loxP littermate control mice. To control for any possible changes in cell density, interneuron counts were normalized to the total number of cell nuclei as quantified by the DNA-binding dye Hoechst 33342. We observed no significant difference in the number of either parvalbumin- or NPY-positive interneurons in CREmKI visual cortex (Figures 7E and 7F), suggesting that reductions in inhibitory synapse number in CREmKI neurons are not secondary to changes in inhibitory neuron number. Since no molecular marker exists that can easily label all known GABAergic cell types, we cannot exclude the possibility that the differentiation of other classes of interneurons is affected in CREmKI mutants. However, since parvalbumin- and NPY-positive interneurons together comprise more than 50% of the inhibitory neurons in the cortex, and since these specific populations of interneurons have been previously shown to be *Bdnf* dependent in the context of the complete loss of *Bdnf*, our data suggest that the activity-dependent component of *Bdnf* expression is important for the formation or maintenance of inhibitory synapses by GABAergic neurons, but not for the survival or differentiation of GABAergic neurons.

Loss of CREB Binding Leads to Disassembly of the Transcriptional Activating Complex at *Bdnf* Promoter IV

As the loss of activity-dependent *Bdnf* expression in CREmKI animals is due to the specific mutation of the CaRE3/CRE in *Bdnf* promoter IV, a sequence that is bound by the transcription factor CREB, our results also potentially point to a role for CREB in inhibitory synapse development that has not been previously
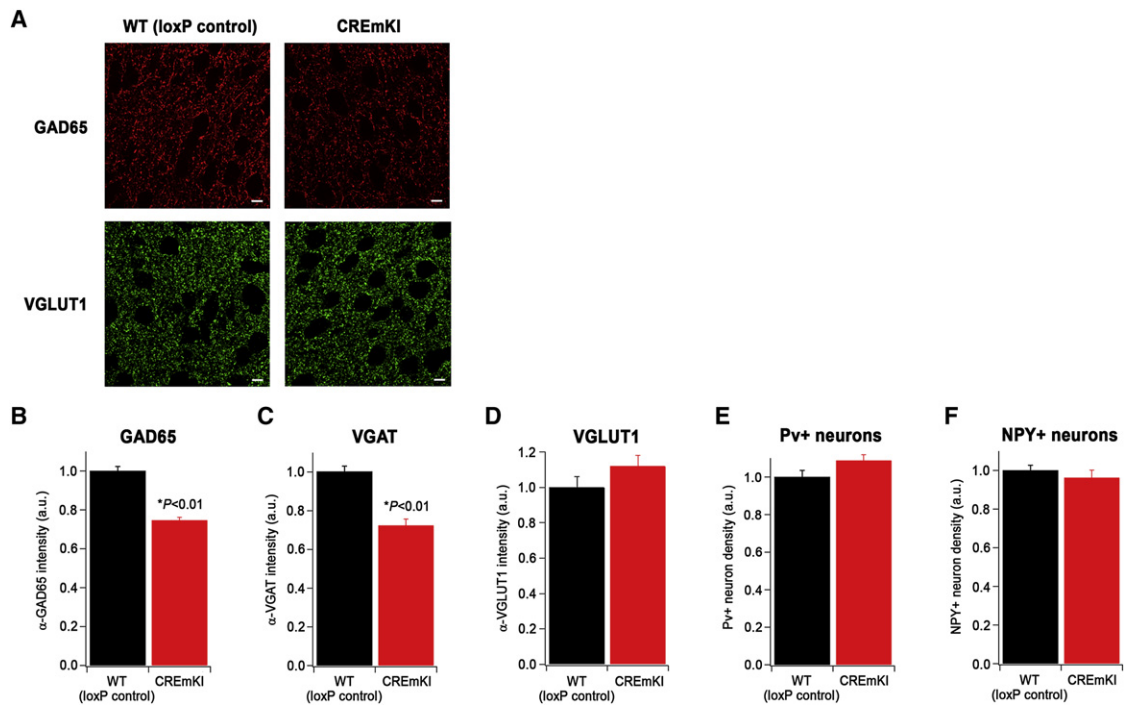
Figure 7. Reduced Immunoreactivity for Inhibitory Synaptic Markers in CREmKI Cortex

(A) Representative images of immunostaining with antibodies recognizing GAD65 (red), which is enriched in inhibitory presynaptic terminals, and VGLUT1 (green), which is enriched at excitatory presynaptic terminals, in layer II/III of CREmKI and loxP control primary visual cortex. Scale bar, 5 μm.

(B–D) Quantification of the average intensity of α-GAD65 ([B], 74–77 fields/genotype), α-VGAT ([C], 48 fields/genotype), and α-VGLUT1 ([D], 48 fields/genotype) immunostaining in CREmKI and loxP control littermates. *p < 0.01, two-way ANOVA with pairwise comparison by Bonferroni-Dunn post hoc test. Data are mean ± SEM from three to four pairs of littermates.

(E) and (F) Quantification of the number of parvalbumin- (E) and NPY- (F) positive inhibitory neurons in the primary visual cortex of CREmKI and loxP control littermates, normalized to the number of total nuclei. p > 0.05 for either parvalbumin-positive or NPY-positive neurons, two-way ANOVA. Data are mean ± SEM collected from n = 24 hemispheres from three pairs of littermates.

described. CREB is a well-established regulator of calcium-dependent gene expression that has important functions in nervous system development and function, including survival, synaptic plasticity, and learning and memory (Flavell and Greenberg, 2008; West et al., 2002). The central role of CREB in neuronal survival and maintenance has hampered the study of CREB function in mature neurons; for instance, postnatal disruption of CREB in the forebrain leads to widespread neurodegeneration (Mantamadiotis et al., 2002), preventing the study of other CREB functions in these neurons. However, given that CREB regulates a transcriptional program comprising hundreds, if not thousands of genes (Impey et al., 2004), only a portion of which are involved in survival, many additional functions of CREB in the nervous system undoubtedly remain to be discovered. Our findings with CREmKI mice suggest that one of these additional functions of CREB is the control of inhibitory synapse development via the calcium-dependent regulation of *Bdnf* expression.

To explore this idea, we used chromatin immunoprecipitation (ChIP) to directly assess the effect of the CREmKI mutation on the binding of CREB to endogenous *Bdnf* promoter IV in the brain. Forebrains from CREmKI and loxP littermate control mice were treated with formaldehyde to covalently crosslink endogenous protein-DNA complexes, which were then mechan-

ically sheared and immunoprecipitated with an antibody against CREB. The specificity of the anti-CREB antibody for use in ChIP experiments has been previously validated in neurons that lack CREB protein (Riccio et al., 2006). qPCR measurements indicated that whereas *Bdnf* promoter IV-containing DNA fragments are enriched in CREB immunoprecipitates prepared from loxP control brains as compared to control immunoprecipitates from loxP control brains, CREB immunoprecipitates from littermate CREmKI brains do not contain detectable levels of *Bdnf* promoter IV gDNA (Figure 8B). The failure to detect CREB binding at promoter IV in CREmKI brains is not due to differences in the level of CREB expression (Figure 8A) or the ability to immunoprecipitate CREB under crosslinking conditions between CREmKI and loxP control brains (Figure 8A), and we detected robust CREB binding to other known CRE-containing CREB target promoters, such as *c-fos* or *Nr4a1*, in CREmKI brains (Figure 8B and data not shown). In contrast, we detected no binding of CREB in either loxP control or CREmKI brains to negative control regions in *Bdnf* intron VII, in the *Bdnf* 3′ untranslated region (UTR), or within the *Nr4a1* gene (Figure 8B and data not shown). Taken together, these data provide direct demonstration that *Bdnf* promoter IV is an endogenous target gene of CREB in the brain and demonstrate that mutation of the
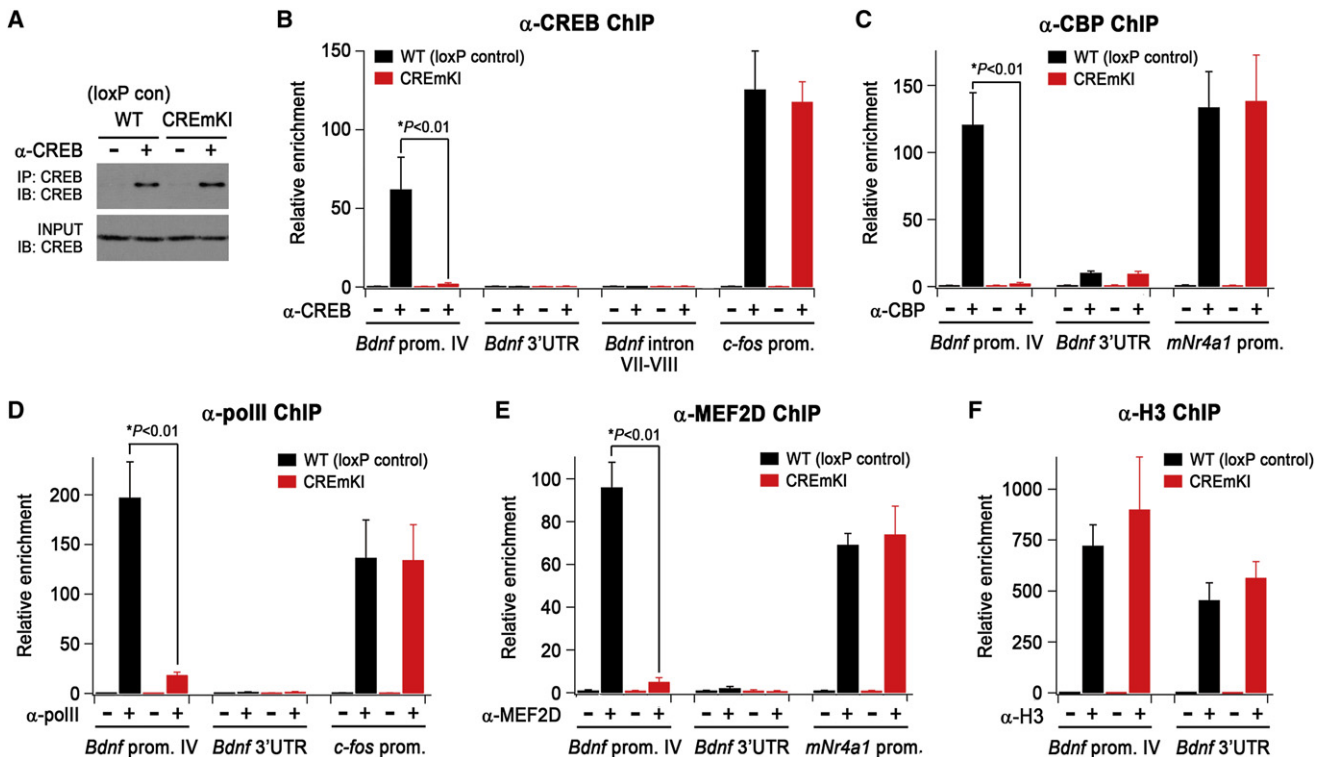
Figure 8. Loss of CREB Binding at *Bdnf* Promoter IV in CREmKI Brains Leads to the Disassembly of the Transcriptional Activating Complex at *Bdnf* Promoter IV

(A) α-CREB immunoprecipitates were isolated from the cross-linked forebrains of CREmKI and loxP control littermates under conditions identical to those used for chromatin immunoprecipitation (ChIP) and analyzed by western blotting with an independent antibody against CREB.

(B)–(F) qPCR measurement of the levels of the indicated genomic DNA regions in α-CREB (B), α-CBP (C), α-polymerase II (D), α-MEF2D (E), and α-histone H3 (F) chromatin immunoprecipitates prepared from the forebrains of CREmKI and loxP control littermates. *p < 0.01, two-way ANOVA with pairwise comparison by Bonferroni-Dunn post hoc test. Data are mean ± SEM from n = 4 (α-CREB), n = 3 (α-MEF2D, α-CBP), or n = 2 (α-pol II, α-H3) independent experiments in which each sample was measured in triplicate.

promoter IV CaRE3/CRE element abrogates CREB binding to *Bdnf* promoter IV in the intact brain. Together with our earlier experiments demonstrating that *Bdnf* promoter IV transcription is induced upon activity-dependent CREB phosphorylation (Tao et al., 1998), these findings strongly suggest that the impairment in neuronal-activity dependent induction of *Bdnf* expression in CREmKI mice is due to a loss of CREB-binding and regulation at promoter IV.

Since our initial characterization of CaRF (Tao et al., 2002), USF1/2 (Chen et al., 2003b), and CREB (Tao et al., 1998) as important regulators of *Bdnf* promoter IV activation, ongoing work in our lab and others has described additional components of the transcriptional regulatory complex at promoter IV, including the transcription factors BHLHB2 (Jiang et al., 2008), NPAS4 (Lin et al., 2008), and MEF2 (S.W. Flavell, T.K. Kim, and M.E.G., unpublished data); the methyl-DNA binding protein MeCP2 (Chen et al., 2003a; Martinowich et al., 2003); and the transcriptional coactivator CBP (West et al., 2001). These factors are believed to function together with CREB to confer calcium- and neural cell type-specific regulation of *Bdnf* transcription, but little is understood about how they cooperate with one another to regulate transcription or how they affect the recruitment

of one another to *Bdnf* promoter IV. We took advantage of the CREmKI mutant to assess the contribution of CREB to the assembly of various components of the transcriptional activating complex at *Bdnf* promoter IV.

We first examined whether loss of CREB binding affects the recruitment of the transcriptional coactivator CBP to *Bdnf* promoter IV. Phosphorylation of CREB at Ser133 in response to rises in intracellular calcium recruits the binding of CBP, which functions as a transcriptional coactivator both through its intrinsic histone acetyltransferase activity and through its stabilization of components of the preinitiation complex at promoters (Hong et al., 2005; Lonze and Ginty, 2002). Although CBP has been best studied in the context of coregulation with CREB, it is a general transcriptional cofactor that cooperates with a wide variety of other transcription factors, including MEF2 (Sartorelli et al., 1997). Performing ChIP with an antibody directed against CBP, we observed significant binding of CBP to *Bdnf* promoter IV in loxP control brains that was almost completely absent in CREmKI littermate brains (Figure 8C). By contrast, in both CREmKI and loxP control brains, we detected strong binding of CBP to the *mNr4a1* promoter and relatively little binding of CBP in the *Bdnf* 3′ UTR negative control region (Figure 8C).

These data indicate that loss of CREB binding to *Bdnf* promoter IV disrupts the binding of the general transcriptional coactivator CBP to the promoter as well. To determine how loss of CBP binding to *Bdnf* promoter IV affects the recruitment of the basal transcriptional machinery, we also performed ChIP with an antibody (monoclonal 8WG16) directed against the hypophosphorylated state of the largest subunit of RNA polymerase II, which is specifically enriched at transcriptional start sites (Brodsky et al., 2005). In the absence of CREB binding to promoter IV in CREmKI brains, we also observed a significant reduction in the level of Pol II docked at the promoter (Figure 8D), consistent with the impairment in activity-dependent transcription from *Bdnf* promoter IV (Figures 2C, 3A, 4A). Pol II binding at the *c-fos* promoter was not affected in CREmKI brains (Figure 8D), indicating that the loss of polymerase binding in CREmKI brains is specific to promoter IV. Together, these results indicate that the loss of binding of a single sequence-specific factor, CREB, to *Bdnf* promoter IV is sufficient to disrupt the assembly of the general transcriptional activation machinery used by many transcription factors at *Bdnf* promoter IV.

Finally, we asked whether loss of CREB binding to *Bdnf* promoter IV affects the recruitment of other sequence-specific transcription factors that are believed to cooperate with CREB to mediate calcium-specific gene expression. We focused on the calcium-regulated factor MEF2 because ongoing studies in our lab have extensively validated the antibody directed against MEF2D for use in ChIP (S.W. Flavell, T.K. Kim, and M.E.G., unpublished data). Using this antibody, we observed strong binding of MEF2D to *Bdnf* promoter IV in loxP control brains that is significantly reduced in CREmKI littermate brains (Figure 8E). In contrast, we detected enrichment of the *Nr4a1* promoter, a well-characterized target of MEF2 transcriptional regulation, in anti-MEF2D immunoprecipitates from both CREmKI and loxP control brains (Figure 8E), and little MEF2D binding to negative control regions in the *Bdnf* and *Nr4a1* genes in either CREmKI or loxP control brains (Figure 8E and data not shown). Thus, the loss of CREB binding to *Bdnf* promoter IV disrupts the binding of MEF2D to *Bdnf* promoter IV as well. Our failure to observe the binding of many components of the transcriptional activating complex—CBP, polII, and MEF2D—to *Bdnf* promoter IV in CREmKI brains does not reflect a general inability to immunoprecipitate promoter IV-containing gDNA fragments from CREmKI brains because promoter IV fragments are found to be equally enriched in CREmKI and loxP control chromatin immunoprecipitates collected with an antibody that recognizes histone H3, a core structural component of chromatin (Figure 8F). Taken together, our findings indicate that loss of CREB binding to *Bdnf* promoter IV disrupts the multifactor transcriptional activating complex at promoter IV and suggest a new function for CREB in nucleating the assembly of transcriptional complexes at its target promoters.

DISCUSSION

Despite more than two decades of investigation into the program of gene expression that is regulated by neuronal activity in the brain, a significant gap persists in our understanding of the biological significance of the activity-dependent component of gene transcription. In this study, the introduction of a subtle mutation into the endogenous *Bdnf* gene in mice to disrupt the ability of CREB to bind *Bdnf* promoter IV represents a powerful approach for selectively evaluating the functional significance of activity-dependent *Bdnf* transcription. We show that disruption of the ability of CREB to regulate *Bdnf* promoter IV results in an animal in which the activity-dependent transcription of *Bdnf* is impaired in response to sensory experience-driven synaptic activation in the intact brain. Using this animal, we find that activity-dependent *Bdnf* expression is required for the appropriate development of inhibition in the cortex, but does not appear to affect the survival or differentiation of GABAergic neurons. These results demonstrate the biological importance of activity-regulated gene transcription for the establishment of appropriate connectivity in the nervous system. In addition, our findings also indicate a requirement for promoter IV-derived *Bdnf* in the development of inhibition in the cortex and thus provide insight into the functional significance of the complex, multipromoter organization of the *Bdnf* gene.

Most previous studies examining the role of activity-regulated gene transcription in the nervous system have taken the approach of disrupting either genes whose transcription, or transcription factors whose function, is regulated by neuronal activity. For example, the observation of defects in synaptic plasticity and learning in hypomorphic mutants of the transcription factor CREB (Balschun et al., 2003; Bourtchuladze et al., 1994; Gass et al., 1998) has been taken as evidence of the importance of neuronal activity-dependent transcription in these adaptive processes, since CREB is a well-established regulator of calcium-dependent gene expression. Experiments with this design, however, cannot easily dissociate the functions of a gene that are due specifically to its regulation by neuronal activity from the many other influences on its action. In the case of CREB, for instance, interpretation of its role in synaptic and behavioral plasticity is complicated by its essential role in neuronal survival and maintenance (Mantamadiotis et al., 2002; Rudolph et al., 1998). The current study is distinguished by our ability to make use of the substantial existing mechanistic understanding of *Bdnf* transcription to specifically disrupt neuronal activity-dependent *Bdnf* transcription in vivo, via the genetic mutation of the CREB-binding site in *Bdnf* promoter IV. This objective can be achieved because, although CREB can be activated by multiple signaling pathways including those mediated by rises in cyclic AMP, exon IV-containing *Bdnf* transcripts are induced selectively in response to the influx of extracellular calcium. Furthermore, since promoter IV-derived *Bdnf* transcripts comprise the majority of activity-induced *Bdnf* transcripts expressed in the cortex, the disruption of activity-regulated promoter IV activity results in a substantial impairment in overall neuronal activity-dependent *Bdnf* transcription in the cortex.

Several phenotypic aspects of the CREmKI mutant animal also suggest that the changes in the development and function of its nervous system specifically reflect defects in the neuronal activity-regulated expression of *Bdnf*. For instance, in contrast to total *Bdnf*$^{-/-}$ knockout mice, which typically die in the first few days of life (Ernfors et al., 1994; Jones et al., 1994), CREmKI mutants are viable, fertile, and appear indistinguishable from control littermates. In addition, at a gross anatomical level, CREmKI

brains do not appear significantly different from wild-type or loxP control littermate brains (Figure S4A), and cortical layering in CREmKI brains appears normal (Figure S4B), suggesting that impairment of activity-dependent promoter IV expression does not have strong effects on neuronal survival. Furthermore, whereas total $Bdnf^{-/-}$ null animals have a dramatic reduction in the number of inhibitory neurons in the cortex (Jones et al., 1994), we do not observe a change in inhibitory neuron number in CREmKI cortex (Figures 7E and 7F), again indicating that not all *Bdnf*-dependent phenotypes are affected by deficits in activity-dependent promoter IV activation. Like in $Bdnf^{+/-}$ heterozygous mutants, the level of cortical BDNF protein in CREmKI animals reared in standard housing conditions is ~50% of controls (Figure S3), and, like CREmKI animals, $Bdnf^{+/-}$ heterozygotes also exhibit deficits in the development of inhibition in the visual cortex (Abidin et al., 2008). However, a simple 50% reduction in BDNF protein level cannot account for the phenotype of the CREmKI animal because, unlike $Bdnf^{+/-}$ heterozygous mutants that develop obesity by 3 months of age (Kernie et al., 2000; Lyons et al., 1999), preliminary evaluation suggests that CREmKI mutants do not exhibit changes in body weight regulation (Figure S7). Taken together, these observations are consistent with the idea that the defects in cortical inhibition seen in the CREmKI mutant reflect a requirement for *Bdnf* function that depends specifically on the activity-dependent expression of the gene. It is important to note that, since activity-dependent *Bdnf* expression is significantly attenuated, but not completely eliminated in the CREmKI animal, we cannot exclude the possibility that many of the well-characterized phenotypes of $Bdnf^{-/-}$ and $Bdnf^{+/-}$ mice that are not apparent in the CREmKI animal may ultimately prove to also be dependent on activity-dependent *Bdnf* transcription.

The finding that activity-dependent *Bdnf* expression is important for the development of GABAergic synapses in the visual cortex indicates an important role for activity-regulated gene transcription in the control of cortical excitability. The establishment and maintenance of appropriate excitatory-inhibitory balance is critical to normal brain physiology and function; defects in excitatory-inhibitory balance can lead to the emergence of seizures and aberrant critical periods for cortical plasticity (Hensch and Fagiolini, 2005; Sun, 2007). Previous studies have demonstrated that a reduction in excitatory drive caused by sensory deprivation leads to a decrease in GABA immunoreactivity in the visual cortex (Benevento et al., 1995; Hendry and Jones, 1986), suggesting the hypothesis that the level of neuronal activity can adjust the strength of cortical inhibition to maintain appropriate firing rates in cortical circuits. Since BDNF levels can serve as a molecular "sensor" of global levels of neuronal activity, it has been suggested that the induction of *Bdnf* expression in response to increases in the level of neuronal activity may act to dampen cortical excitability by promoting the development and/or strengthening of inhibitory synapses in local circuits (Genoud et al., 2004; Rutherford et al., 1997). However, direct evidence that the neuronal activity-dependent regulation of *Bdnf* expression controls the level of excitability of cortical circuits has been lacking. Our finding that the disruption of neuronal activity-regulated *Bdnf* expression impairs the development of inhibitory synapses provides strong evidence in

support of this hypothesis. Since levels of *Bdnf* no longer function as an accurate sensor of neuronal activity in CREmKI brains, cortical circuits appear to form as if under conditions of chronic sensory activity deprivation and fail to develop the appropriate level of inhibition. Important future experiments include pinpointing the source(s) of activity-dependent BDNF expression and release, the cellular target(s) on which it acts, and understanding whether activity-regulated BDNF functions equivalently at all inhibitory inputs or may have local effects near specific or active synapses.

In addition to demonstrating the biological significance of activity-regulated *Bdnf* transcription for brain development and function, our findings provide insight into the action of CREB in the nervous system. We show that loss of the ability of CREB to bind and regulate *Bdnf* promoter IV in CREmKI brains results in defects in the development of cortical inhibition, implicating CREB, or possibly its closely related family members CREM and ATF-1, as having a previously unappreciated function in the development of inhibition in the nervous system. Our results with the CREmKI animal demonstrate that assessing the function of transcriptional regulators via the mutation of their binding sites in their target promoters is a powerful approach that can deconvolve the many functions of the factor (Xiang et al., 2006). Additionally, in the case of a transcriptional regulator like CREB that belongs to a multifactor family, the approach of mutating the factor binding site in target promoters of interest avoids a potential complication of gene knockout studies, in which compensation of one family member for another's function is common.

Our results also provide insight into the role of CREB in the formation of transcriptional regulatory complexes at gene promoters. We find that loss of CREB binding in CREmKI brains results in disruption of core components of the transcriptional activating complex at *Bdnf* promoter IV, including CBP and PolII, as well as loss of another sequence-specific transcription factor, MEF2, that cooperates with CREB to regulate promoter IV activity (S.W. Flavell, T.K. Kim, and M.E.G, unpublished data). These results suggest a central function for CREB in nucleating the assembly of the multifactor transcriptional regulatory complex that mediates calcium-dependent exon IV transcription. This idea is supported by our observation that additional mutation of the CaRE1 and CaRE2 sites in addition to the CaRE3/CRE site does not impair activity-dependent promoter IV induction significantly more than mutation of the CREB-binding site alone. Although there is evidence that CREB, both directly and through its association with CBP, can bind and stabilize components of the preinitiation complex (Lonze and Ginty, 2002), whether CREB recruits other sequence-specific transcriptional activators to its target promoters was less clear. One possibility is that the coactivator CBP has additional functions as a molecular scaffold to bridge the interactions among the many factors in a transcriptional complex, such that recruitment of CBP by activated CREB leads to the stabilization of additional sequence-specific factors at the promoter. Alternatively, CREB may interact directly with other sequence-specific factors to recruit them to promoter IV, as has recently been shown for the transcriptional regulator MeCP2 (Chahrour et al., 2008). Further characterization of the multifactor transcriptional complex, as well as putative changes in chromatin state, at *Bdnf* promoter IV in CREmKI neurons may yield new

insight into the general mechanisms by which CREB coordinates the stimulus-specific activation of its target promoters.

Taken together, our results demonstrate that the neuronal activity-dependent component of gene expression is an important functional mediator of the effects of neuronal activity in reorganizing synaptic connectivity in the nervous system. It is remarkable that the impairment in the ability of neuronal activity to trigger the expression of just one of eight promoters of a single gene, *Bdnf*, results in significant defects in the development of cortical inhibition, highlighting the importance of the exquisite level of transcriptional control over *Bdnf* expression in the nervous system. Our finding that activity-regulated *Bdnf* expression is an important mediator of the effect of neuronal activity on the development of cortical inhibition suggests that, more generally, a major function of the program of neuronal activity-regulated gene expression may be to regulate the level of excitability of cortical circuits. Aberrant excitatory-inhibitory balance in the cortex can have significant consequences for the nervous system, including the development of seizures and abnormal critical periods for cortical plasticity, two common features of mental retardation and neurodevelopmental and autism spectrum disorders (Hensch and Fagiolini, 2005; Rubenstein and Merzenich, 2003). These observations highlight the importance of continued investigation into the full program of neuronal activity-regulated genes, and the synapse-to-nucleus signaling pathways that regulate their expression, for understanding how experience shapes the nervous system and how defects in this process may lead to disorders of cognition.

EXPERIMENTAL PROCEDURES

See Supplemental Experimental Procedures for details of animal husbandry and colony management, neuronal cell culture, immunocytochemistry, immunohistochemistry, image analysis, and electrophysiology.

Generation of *Bdnf pIV*$^{-/-}$, TMKI, CREmKI, and loxP Control Mice
See Supplemental Experimental Procedures for description of gene targeting. The *Bdnf pIV* deletion spans basepairs −160 to +281 relative to the TSS. All experiments were performed with animals in a sv129/C57BL/6 mixed genetic background, with the mutation backcrossed into the C57BL/6 background between four and six generations.

Visual Stimulation and Seizure Induction
Eight- to twelve-week-old adult mice reared in a 12 hr:12 hr light:dark cycle were transferred into constant darkness for 14 days. Animals in the stimulated (+) condition were exposed to light for 90 min. Seizures were induced in 8- to 12-week-old adult mice by intraperitoneal injection of kainic acid (20 mg/kg). At the indicated times postinjection, mice were killed and the cortex isolated. See Supplemental Experimental Procedures for details.

Gene Expression Analysis
cDNA was synthesized by reverse transcription of total RNA using oligo-dT priming, and transcript levels were measured by quantitative real-time PCR using SYBR Green detection. BDNF protein levels were quantified by two-site ELISA. See Supplemental Experimental Procedures for details.

Chromatin Immunoprecipitation
For anti-CREB or anti-MEF2D ChIP, the antibody was preincubated with either the antigen peptide against which the antibody was raised (negative control IP) or an irrelevant peptide (experimental IP) prior to use in immunoprecipitation. For all other factors, normal mouse or rabbit IgG was used for the negative control IP. See Supplemental Experimental Procedures for details.

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