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Supplemental Data

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

Elizabeth J. Hong, Alejandra E. McCord, and Michael E. Greenberg

Supplemental Experimental Procedures

Generation of Bdnf pIV−/−, TMKI, CREmKI, and loxP control mice

The targeting vector was constructed from a 129s6/SvEvTAC mouse genomic fragment PCR-amplified from clone 487F21 of BAC library RPCI-22 (BACPAC Resources Center, CHORI). The 6-kb genomic fragment, spanning exons IV through VII of the Bdnf locus, was amplified with primers 5’-agtcaggttacggtggcaaggattcagttccac-3’ and 5’-agtcagctcagacttagccccgagtcaggtcc-3’ and cloned using restriction sites XhoI and KpnI into pBluescript II. A diphtheria toxin negative selection cassette (PGK-DTA) was introduced 5’ to the genomic fragment using restriction sites EcoRV and XhoI. The loxP-flanked neomycin- and zeomycin-positive selection cassette was introduced 2.4-kb downstream of Bdnf proximal promoter IV into an unconserved region of the large intron between exons VII and VIII by ET homologous recombination cloning in bacteria (Zhang et al., 1998). In addition, a PciI restriction site was introduced immediately 5’ to the neomycin cassette to aid in identification of correct recombinants during the gene targeting procedure in mouse ES cells. This construct was used to generate the loxP control animal.
To generate the \textit{Bdnf} \textit{pIV}^/- targeting construct, a deletion of proximal promoter IV, the exon IV transcriptional start site (TSS), and a portion of exon IV (the deletion spans -160 to +281 relative to the TSS) was created by digesting the loxP control construct with SphI and AgeI, blunting the ends, and ligating them back together. To generate the \textit{Bdnf} TMKI and CREmKI targeting constructs, a 1.8-kb NheI-NheI fragment spanning proximal promoter IV was shuttled from the loxP control construct into pBluescript II to create a smaller vector containing the sequence of interest, and the CREm and TM mutations were introduced into this fragment by site-directed mutagenesis. The NheI-NheI fragment containing either the CREm mutation or the TM mutation was then shuttled back into the loxP control construct using NheI to replace the wildtype promoter sequence with the CREm- or TM-mutated sequence and creating the CREmKI and TMKI targeting vectors. All primer sequences used for cloning are available upon request.

All targeting constructs were confirmed by direct sequencing of their entirety prior to use in gene targeting. The KpnI-linearized targeting vectors were electroporated into sv129 mouse ES cells. Genomic DNA isolated from G418-resistant ES cell clones was digested with PciI and screened for homologous recombination at the \textit{Bdnf} promoter IV locus by Southern blot using a 5’ probe external to the genomic fragment contained within the targeting vector (see Figure 1B). Recombinant clones containing the predicted 5.7-kb (TMKI, CREmKI, loxP control) or 5.3-kb (\textit{pIV} deletion) rearranged fragment were obtained at frequencies of around 30-35%, and appropriate recombination was confirmed with an independent 3’ probe which produced an 8.4 kb fragment. For targeting of TMKI and CREmKI mutations, since recombination can occur between the
mutation(s) at Bdnf promoter IV and the loxP-neomycin selection cassette, we checked for the presence of the appropriate mutation by PCR genotyping as described below and in Figure 1E, and confirmed this by direct sequencing.

For each mutant, two positive ES cell clones were independently transfected with a Cre recombinase-expressing plasmid and appropriate excision of the loxP-flanked neomycin cassette was confirmed by Southern blot. Two confirmed ES cell clones per mutant were injected into C57BL/6 blastocysts to generate two independent lines per mutant, which were subsequently implanted into pseudopregnant females. The resulting chimeric offspring were mated with C57BL/6 mice, and the resulting agouti offspring were screened for the mutation by PCR genotyping (see below). The presence of the mutations was confirmed in the animals by Southern analysis. All experiments described here were performed using animals in a sv129/C57BL6 hybrid genetic background, with the mutation backcrossed into the C57BL/6 background between four and six generations. Results from pIV−/− and CREmKI mice were combined from experiments conducted in two independent lines of each mutation; results from TMKI mice were collected from a single line of the mutation.

**Animal husbandry and colony management**

For all four mutant lines, pIV, TMKI, CREmKI, and loxP control, all crosses generated the expected Mendelian numbers of each genotype. All four mutants were viable and fertile and showed no obvious alterations in cerebral, cerebellar, or hippocampal anatomy (Supplemental Figure 3 and data not shown). In addition, no obvious changes in gait were observed in CREmKI animals at P14 or P21 (data not
shown), as assessed by paw printing. Non-toxic ink was applied to the bottom of the mouse’s paws, the mouse was allowed to walk across a sheet of blank paper, and the mean distance between prints was taken as a measure of gait.

In order to generate TM or CREmKI mutants with loxP control littermates (rather than plain wildtype littermates), the loxP control mutation was bred into the TM or CREmKI line. In the typical cross that was used to generate matched homozygous mutant and loxP control littermates for experiments, both parents were heterozygous at the promoter IV locus for the CREm or TM mutation and homozygous at the loxP locus, such that all offspring were homozygous at the loxP locus and 25% were wildtype at the promoter IV locus and 25% were mutant at the promoter IV locus.

For routine experimentation, animals were genotyped using a PCR-based strategy. pIV mutants were genotyped directly for the presence of the deletion at proximal promoter IV with primers F: 5’- cagacataactaaggccagccttagaag-3’, R1: 5’-ggaagacccgttttagatgtt-3’, and R2: 5’-ATTGATAGTGGAATTTGCatggcgGAGG-3’. TMKI, CREmKI, and loxP control animals were genotyped for the presence of the loxP site using primers F: 5’-AGGGCGGTGAGCCACAGGCTGTGAGTTTG-3’ and R: 5’ ATCCCCAAAGTCCCCCATCCCCAGTTTCC-3’, and for the presence of a mutation at CaRE3/CRE by amplifying a fragment spanning the promoter IV CaRE3/CRE with primers F: 5’ gttgcctgctagataatgagc-3’ and R: 5’-atatgtactcttgtgacgc-3’ and digesting the product with the PvuII restriction enzyme which cuts only in the mutant CaRE3/CRE. See Supplemental Figure 1 and accompanying figure legend for expected genotyping products and sizes.
**Neuronal cell culture**

For gene expression studies, cortical neurons were prepared from E16.5 mouse embryos as previously described (Xia et al., 1996) and maintained in either Basal Medium Eagle (Sigma) supplemented with 10% calf serum, 15 mM glucose, 1 mM L-glutamine, and 100 U/ml penicillin/streptomycin or Neurobasal with B27 supplement (Invitrogen), 1 mM L-glutamine, and 100 U/ml penicillin/streptomycin for the indicated number of days. Cells were typically seeded at a density of 2x10^5 cells/cm² on dishes coated with polyornithine (30µg/mL). For KCl-mediated depolarization of neurons, neuronal cultures were treated overnight with 1µM tetrodotoxin (Tocris Bioscience, Ellisville, MO) to reduce endogenous neuronal activity prior to stimulation. Neurons were membrane depolarized with 55 mM extracellular KCl as previously described (Tao et al., 1998). For synaptic stimulation, neurons were treated with 10µM glutamate, 20µM NMDA, or 20µM bicuculline.

For analysis of synapse number in cortical neurons, low-density cortical cultures were prepared from E17.5 embryos and seeded at a density of 1.75x10⁴ cells/cm² on a glial feeder layer or 2.5x10⁴ cells/cm² on glass coverslips coated with poly-D-lysine (20µg/mL) and laminin (3.4µg/mL). Similar results in the relative number of synapses seen between CREmKI and loxP control neurons were seen on both substrates and the data from both conditions were combined. Neurons were maintained in Neurobasal with B27 supplement (Invitrogen), 1 mM L-glutamine, and 100 U/ml penicillin/streptomycin for 18 DIV, with replacement of one half of the media in each well every 4-5 days.
Neurons were transfected with a plasmid encoding green fluorescent protein (GFP) at day 5 in vitro by the calcium phosphate method (Xia et al., 1996).

**Visual stimulation and seizure induction**

Eight- to twelve-week old adult mice reared in a 12-hour:12-hour light:dark cycle were transferred to and maintained in constant darkness for 14 days. Animals in the “unstimulated (-)” condition were killed and their eyes enucleated in the dark, prior to bringing the body into light to dissect the brain. Animals in the “stimulated (+)” condition were exposed to light for 90 minutes. Visual cortex and somatosensory cortex were isolated based on anatomical landmarks and immediately flash frozen for protein quantification or directly homogenized into Trizol reagent (Invitrogen) for RNA isolation.

Seizures were induced in 8-12 week old adult mice by intraperitoneal injection of kainic acid (2.5 mg/ml in saline) at a dose of 20 mg of kainic acid per kg body mass. Control animals were injected with an equivalent volume of saline. At the indicated times post-injection, mice were killed, and the entire cortex was isolated for protein or mRNA analysis.

**Gene expression analysis**

For measurements of gene expression in cultured neurons, total RNA was isolated using the RNeasy mini kit (Qiagen, Valencia, CA). For measurements of gene expression in brain tissue, total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA) and subsequently passed over an RNeasy mini column for further clean-up. For each sample, 1 ug of total RNA was digested w/DNAsel (Invitrogen, Carlsbad,
CA) and reverse-transcribed by oligo(dT)-priming using SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA). The amount of each transcript of interest present in the sample was measured by quantitative real-time PCR on 1/20th of the resulting cDNA using SYBR Green detection (Applied Biosystems, Foster City, CA). Standards were performed on each plate in duplicate, and each sample was measured in triplicate. Except for the primers used to detect the Bdnf coding exon, all qPCR primer pairs are exon-spanning. All qPCR primer sequences are available upon request. All mRNA levels of genes of interest were normalized to β-tubulinIII mRNA levels to adjust for small differences in input RNA. Because the absolute value of gene expression measurements for a given transcript varies substantially from plate to plate even among wildtype samples, experiments were normalized as follows.

For measurements of the time course of gene expression in cultured neurons in response to a given stimulus (Figures 2, 3), for a given transcript, the highest gene expression measurement among all the triplicates of all the time points in either genotype in each experiment was assigned a value of 1, and all other measurements were normalized to it. These values were used for statistical analysis by repeated-measures analysis of variance (ANOVA) with StatView4.5 (Abacus Concepts). For data presentation, these normalized values were averaged across all the experiments and presented mean ± SEM. Thus, within a given mRNA transcript, all values at different time points in different genotypes can be directly compared.

For measurements of gene expression in intact brain tissue in response to visual experience (Figure 4A-B, 4D), for a given transcript in a given tissue, the average level of gene expression in the loxP control brain in the absence of stimulation (-) was assigned a
value of 1, and all measurements for that given transcript in that tissue was normalized to it. These values were used for statistical analysis by two-way ANOVA with StatView4.5 (Abacus Concepts). For data presentation, these normalized values for each condition were averaged and presented mean ± SEM. Thus, within a given mRNA transcript in a given tissue, all values for different genotypes under different conditions can be directly compared.

**Protein expression analysis**

Freshly dissected cortex from 8-12 week old mice was weighed and then either flash frozen for later analysis, or immediately lysed by sonication in 20 volumes of extraction buffer (50 mM sodium acetate pH 4.0; 1 M NaCl; 1% BSA; 1% Triton X-100) supplemented with protease inhibitors. Lysates were incubated on ice for 1 hour, after which another 20 volumes of extraction buffer was added and the samples re-sonicated. Insoluble material was removed by high-speed centrifugation (>100,000g), and the pellet was re-extracted in 10 volumes of buffer and combined with the previous supernatant. BDNF protein concentrations were measured by two-site enzyme-linked immunoabsorbant assay (ELISA) using the BDNF E_max ImmunoAssay System (Promega, Madison, WI). Standards using recombinant BDNF were performed on each plate in duplicate, and samples were measured in triplicate. The BDNF concentration for each genotype was measured from at least four independent animals.

**Immunocytochemistry and quantification of synapse density**

Cultured neurons were fixed with 4% paraformaldehyde and 2% sucrose in PBS for 8 minutes at room temperature. Neurons were blocked in 1X GDB (0.1% gelatin,
0.3% Triton X-100, 4.2% 0.4M phosphate buffer, 9% 5M NaCl), incubated with the primary antibody in 1X GDB overnight at 4°C, and incubated with the secondary antibody in 1X GDB for 1-2 hour at room temperature. Neurons were counterstained with the nuclear dye Hoechst 33342 for one minute at room temperature. Primary antibodies were generated in the following species and used at the following concentrations: anti-GAD65 (mouse, 1:1000, Chemicon), anti-VGAT (guinea pig, 1:500, Chemicon), anti-synapsin I (rabbit, 1:500, Chemicon), anti-GABA<sub>AR</sub>γ2 (rabbit, 1:400, Chemicon), anti-GABA<sub>AR</sub>β2/3 (mouse, 1:500, Chemicon), and anti-PSD95 (mouse, 1:200, Affinity BioReagents). Cy3- and Cy-5 conjugated fluorescent secondary antibodies were used at a dilution of 1:500.

All imaging and image analysis for synapse density were performed blind to the genotype of the samples. Images were acquired on a Zeiss LSM5 Pascal confocal microscope with a 63X objective at 1024X1024 pixel resolution. Within each set of antibody markers in each experiment, images were acquired with identical settings for laser power, detector gain, amplifier offset, and pinhole diameter in each channel. Settings were selected such that all pixel intensities fall within the dynamic range of the detector. For each neuron, multiple images were collected as a z-stack of 4-5 sections at 0.5μm step size, and maximum intensity projections generated from the z-stack were used for analysis. Synapse density was quantified with Metamorph image analysis software using custom macros. For each channel in each experiment, the average of all the mean pixel intensities for each image was determined, the threshold for each channel was set at three standard deviations above this average mean pixel intensity, and a binary mask that contained all pixels above the threshold was made for each cell. Using the
GFP mask as a guide, the cell body was defined as a region of continuous pixels larger than 60 pixels in diameter and was excluded from analysis by removing the region from the masks of all three channels. Using the binary masks created for each channel, regions of co-localization in all three channels (GFP, pre-synaptic marker channel, post-synaptic marker channel) larger than 2 pixels in size were defined as synaptic puncta. Synapse density was measured as the number of these synaptic puncta divided by the pixel area of the GFP mask. For each experiment, approximately 20 neurons from at least four different coverslips were analyzed for each genotype, and between 3-4 experiments were conducted per pair of synaptic markers. Because there is variability in the absolute value of synapse densities even among control conditions from experiment to experiment, data were normalized as follows. Within a given set of markers in a given experiment, the average wildtype synapse density was set to 1, and the synapse density of each cell in the experiment was normalized to this value. These values were used for statistical analysis by two-way analysis of variance (StatView 4.5, Abacus Concepts) to determine statistical significance.

**Immunohistochemistry and quantification of immunostaining**

P20-P24 mice were anesthetized with Avertin and perfused intracardially at room temperature with 20 mL of PBS followed by 20 mL of 4% paraformaldehyde at a rate of 1mL/minute. Whole brains were isolated and postfixed in 2% paraformaldehyde overnight at 4°C. Brains were embedded in agar and sectioned coronally at 50μm. Sections containing primary visual cortex were immunostained as floating sections. Sections were blocked in 10% goat serum and 0.25% Trixon X-100 in PBS, incubated in primary antibody in blocking solution overnight at 4°C, and incubated in secondary
antibody in blocking solution for 2 hours at room temperature. Sections were
counterstained with the nuclear dye Hoechst 33342 for 5 minutes at room temperature.
Primary antibodies were generated in the following species and used at the following
concentrations: anti-GAD65 (mouse, 1:1000, Chemicon), anti-VGAT (guinea pig, 1:500,
Chemicon), anti-VGLUT1 (guinea pig, 1:250, Chemicon), anti-synapsin I (rabbit, 1:500,
Chemicon), anti-parvalbumin (mouse, 1:2500, Sigma), and anti-NPY (rabbit, 1:2000,
Incstar, Stillwater, MN). Cy2- and Cy3-conjugated fluorescent secondary antibodies
were used at a dilution of 1:500.

Quantification of synaptic marker staining. Image acquisition and quantification
of immunostaining intensity were performed blind to the genotype of the samples. Using
the Hoechst staining as a guide, layer 2/3 of primary visual cortex was located under
epifluorescence. Images were acquired on a Zeiss LSM5 Pascal confocal microscope
using a 63X objective at 1024X1024 pixel resolution. Using the fast-scanning function
of the microscope, the upper and lower limits of the histological section in the z-axis were
identified, and then a single-plane image was acquired exactly through the middle of the
section using identical settings for laser power, detector gain, amplifier offset, and
pinhole for a given synaptic marker within a given experiment. Settings were selected
such that all pixel intensities fell within the dynamic range of the detector. Average
intensity of synaptic staining was quantified in Matlab using custom scripts written with
Imaging toolbox functions. For each channel in each experiment, the average of all the
mean pixel intensities for each image was determined, the threshold for each channel was
set at the average mean pixel intensity, and pixels below this threshold were discarded.
To exclude cell bodies, which are devoid of neuropil containing synaptic staining, areas
of contiguous empty pixels greater than 30 pixels in diameter were removed from the area to be analyzed. The mean pixel intensity of the remaining area was then calculated for each image. For each experiment, at least 16 imaging fields of 142μm² were quantified per genotype from at least 4 separately immunostained hemispheres, and 3-4 experiments were performed per synaptic marker. Because there is variability in the absolute value of immunostaining intensities even among control conditions from experiment to experiment, data were normalized as follows. Within a given marker in a given experiment, the average wildtype staining intensity was set to 1, and the immunostaining intensity of each field was normalized to this value. These values were used for statistical analysis by two-way analysis of variance (StatView 4.5, Abacus Concepts) to determine statistical significance.

**Quantification of inhibitory neuron numbers.** Inhibitory neurons were counted with the investigator blind to the genotype of the sample. The number of parvalbumin- and NPY-positive inhibitory neurons per unit volume was quantified by stereological methods on an epifluorescence microscope using Stereo Investigator (MBF Bioscience). Within a given experiment, identical camera settings were used for all sections stained with a given marker. Hoechst counterstaining was used as a guide to identify and trace the region corresponding to primary visual cortex. For counting parvalbumin-positive interneurons, the region was tiled with counting frames of size 150μmX150μm at a grid interval of 200μmX200μm; for counting NPY-positive interneurons, the region was completely counted with counting frames of size 200μmX200μm; and for counting total cell nuclei, the region was tiled with counting frames of 100μmX100μm at a grid interval of 150μmX150μm. A sampling run was accepted if the estimated Scheaffer coefficient
of error was less than 0.05. The density of parvalbumin- and NPY-positive cells was normalized to the density of total cell nuclei; no significant differences in the total number of cell nuclei were observed between CREmKI and loxP control visual cortices. Furthermore, in independent experiments which used the monoclonal antibody NeuN (Chemicon) to quantify the number of neuronal nuclei, no significant differences in the total number of neuronal nuclei were observed between CREmKI and loxP control visual cortices (data not shown). For each experiment, at least 8 hemispheres were quantified per genotype from at least 4 separately immunostained brain sections, and 3 experiments were performed per marker. For a given cell type in a given experiment, the average wildtype cell density was set to 1, and the cell density of each hemisphere was normalized to this value. These values were used for statistical analysis by two-way analysis of variance (StatView 4.5, Abacus Concepts) to determine statistical significance.

**Electrophysiology**

Coronal sections (300μm) containing primary visual cortex were cut from P16-P18 mice using a Leica VT1000S vibratome in ice-cold choline dissection media (25mM NaHCO₃, 1.25mM NaH₂PO₄, 2.5mM KCl, 7mM MgCl₂, 25mM glucose, 0.5mM CaCl₂, 110mM choline chloride, 11.6mM ascorbic acid, 3.1mM pyruvic acid). Slices were incubated in artificial cerebral spinal fluid (ACSF, contains 127mM NaCl, 25mM NaHCO₃, 1.25mM NaH₂PO₄, 2.5 KCl, 2 CaCl₂, 1mM MgCl₂, 25 glucose) at 32°C for 30 minutes immediately after cutting, and subsequently at room temperature. All solutions were saturated with 95% O₂, 5% CO₂, and slices were used within 6 hours of preparation.
Whole-cell voltage-clamp recordings were performed in ACSF at room temperature from layer II/III pyramidal neurons in primary visual cortex. Neurons were identified by cell body position and visual morphology under infrared DIC optics. Recording pipettes were pulled from borosilicate glass capillary tubing with filaments using a Sutter P92 Flame/Brown Puller (Sutter Instruments) to yield tips of 3-5 M$\Omega$ resistance. Spontaneous miniature inhibitory postsynaptic currents (mIPSCs) were recorded with pipettes filled with 147mM CsCl, 5mM Na$_2$-phosphocreatine, 10mM HEPES, 2mM MgATP, 0.3mM Na$_2$GTP, and 1mM EGTA. Osmolarity was adjusted to 300 mOsm with water, and pH was adjusted to 7.3 with CsOH. Inhibitory miniature events were pharmacological isolated by bath application of 0.5$\mu$M tetrodotoxin (Tocris Bioscience, Ellisville, Missouri), 10$\mu$M (R)-CPP (Tocris Bioscience), and 10$\mu$M NBQX disodium salt (Tocris Bioscience) to antagonize voltage-gated sodium channels, NMDA receptors, and AMPA receptors, respectively. To record mIPSCs, the membrane potential was held at -70mV, events were filtered at 1kHz, and series resistance was left uncompensated. Data was collected in epochs of 10 seconds for a duration of 10-15 minutes per cell. Input and series resistances were monitored throughout the duration of the recording, and cells were discarded if these parameters changed by more than 10% or if the series resistance was greater than 25 M$\Omega$. To confirm that we were recording from layer II/III pyramidal neurons, 50$\mu$m Alexa Fluor 488 hydrazide (Invitrogen) was included in the internal pipette solution to visualize cell morphology, and the cells were viewed under epifluorescence illumination after the whole-cell patch configuration was achieved. Cells without a characteristic pyramidal morphology were discarded.
mIPSC events were detected and analyzed off-line in IgorPro (Wavemetrics, Lake Oswego, OR) using custom written macros. For each cell, the root mean square (RMS) of the baseline of all its traces was calculated, and the event threshold was set to be 1.5 times the RMS value. Currents were counted as events if they crossed the event threshold, had a rapid rise time (1.5 pA/ms) and an exponential decay (2 < t < 200 ms). For both amplitude and interevent interval, 50 points were randomly selected from each cell, and these points concatenated from all cells of one genotype to generate a distribution of events for that genotype. The cumulative probability and mean ± S.D. of these sampled distributions are presented in Figure 6B and C, respectively. Statistical significance of the data was determined by two methods. First, the cumulative distributions for each genotype generated by equal random sampling of each cell were compared by the Kolmogorov-Smirnov test; \( P < 0.01 \) was considered significant. Second, a Monte Carlo simulation was performed in which points were randomly sampled from each condition and the mean of these samplings compared at least 1000 times. The sum of these comparisons generated a \( P \) value which was considered significant if less than 0.01.

**Chromatin immunoprecipitation**

Typically one hemisphere (half of a forebrain) was used for a single immunoprecipitation experiment. Chromatin immunoprecipitation was performed essentially as in (Wells and Farnham, 2002). To crosslink protein-DNA complexes, forebrains from P24-P32 mice were finely chopped into small pieces and crosslinked in 1% formaldehyde for 10 minutes at room temperature. Cross-linking was quenched by adding 125mM glycine and incubating for 5 minutes at room temperature. Cross-linked
tissue was washed three times with ice-cold PBS and homogenized with 12 strokes in 50 mM Hepes-KOH, pH 7.5, 140 mM NaCl, 1 mM EDTA, pH 8.0, 10 % Glycerol, 0.5 % NP-40, 0.25 % Triton X-100 supplemente with protease inhibitors (Complete tab, Roche Diagnostics, Mannheim, Germany). Nuclei were pelleted and washed with 200 mM NaCl, 1 mM EDTA, pH 8.0, 0.5 mM EGTA, pH 8.0, 10 mM Tris-HCl, pH 8.0 with protease inhibitors. The nuclei were resuspended in 2-3 pellet volumes of 1 mM EDTA, pH 8.0, 0.5 mM EGTA, pH 8.0, 10 mM Tris-HCl, pH 8.0 with protease inhibitors and sonicated using a Misonix 3000 Sonicator (Misonix, Farmingdale, NY). 12 pulses of 15 seconds each were delivered at a power setting of 7.5 (equivalent to 30-33 watts), which resulted in genomic DNA fragments of sizes ranging from 400 bp to 2 kb. Nuclear lysates were centrifuged at 20,000g for 20 minutes to remove insoluble material.

Salt and detergent were added to the lysates to adjust the buffer to 0.1% SDS, 0.5% Triton X-100, 20mM Tris-Cl, pH 8.0, 150 mM NaCl, 1mM EDTA, pH8.0, and 0.5mM EDTA, pH 8.0 (for anti-CREB ChIP) or 1% Triton X-100, 0.1% DOC, 10mM Tris-Cl, pH8.0, 0.3M NaCl, 1mM EDTA pH 8.0, and 0.5mM EGTA, pH 8.0 (for anti-MEF2D, anti-CBP, anti-pol II, and anti-H3). Pre-cleared lysates were incubated with the following antibodies at 4°C overnight. For anti-CREB ChIP, 4ug of rabbit polyclonal anti-CREB antibody (Millipore, Cat. #06-863) was pre-incubated with 16 μg of either the HCREB antigen peptide against which the antibody was raised (negative control IP) or an irrelevant peptide (2D2, the MEF2D antigen peptide) for use in immunoprecipitation. For anti-MEF2D ChIP, 4 ug of rabbit polyclonal anti-MEF2D antibody (kind gift of Dr. T.K. Kim) was pre-incubated with 16 μg of either the 2D2 antigen peptide against which the antibody was raised (negative control IP) or an irrelevant peptide (HCREB) for use in
immunoprecipitation. 4 μg of mouse monoclonal anti-CBP antibody (clone AC238, Millipore, Billerica, MA), 4μg of mouse monoclonal anti-RNA pol II antibody (Thompson et al., 1989) (clone 8WG16, Covance, Princeton, NJ), and 2 ug of rabbit polyclonal anti-histone H3 antibody (ab1791, Abcam, Cambridge, MA) were used per IP. For anti-CBP, anti-pol II, and anti-H3 ChIPs, normal mouse or rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA) was used for the negative control immunoprecipitation. Immunocomplexes were collected with 40ul/IP of Protein A/G PLUS agarose (Santa Cruz Biotechnology) and washed as follows. For anti-CREB ChIP, IPs were washed twice in 0.1% SDS, 0.5% Triton X-100, 20mM Tris-Cl pH 8.0, 150mM NaCl, 1mM EDTA, pH 8.0; twice in 0.1% SDS, 0.5% Triton X-100, 20mM Tris-Cl pH 8.0, 150mM NaCl, 2mM EDTA; once in LiCl buffer (0.25M LiCl, 10mM Tris-Cl pH 8.0, 1% IGEPAL-CA360, 1% deoxycholic acid, 1mM EDTA pH 8.0); and twice in TE (10mM Tris-Cl, 1mM EDTA pH8.0). For anti-MEF2D, CBP, pol II, and H3 ChIP, IPs were washed twice in low salt buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl, pH 8.1, 150mM NaCl); twice in high salt buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl, pH 8.1, 500mM NaCl); twice in LiCl buffer; and once in TE. For all IPs, all washes were for 5 minutes at 4°C, except for the last wash, which was for 5 minutes at room temperature.

For chromatin immunoprecipitates used to check for the ability of the antibodies to pull down their target factors under crosslinking conditions, washed immunocomplexes were mixed with 20μl of 2X Laemmli sample buffer and boiled for 10 minutes to elute and reverse crosslinks. Samples were separated by SDS-PAGE and probed with an independent antibody (mouse monoclonal anti-CREB, MAB5432,
Millipore; mouse monoclonal anti-MEF2D, BD Biosciences, San Jose, CA). For chromatin immunoprecipitates used for the detection of co-immunoprecipitating DNA, the immunoprecipitates were eluted twice from the beads by incubation at 65°C with 100 μl of 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0, 1 % SDS. Crosslinks in the eluates and corresponding input samples were reversed by incubation at 65°C overnight. The samples were digested with proteinase K and extracted with phenol/chloroform. Then, the DNA fragments were pelleted by ethanol precipitation and treated with RNaseA, before a final purification over a Qiaquick PCR purification column. The amounts of DNA fragments of interest in chromatin immunoprecipitates was measured by quantitative real-time PCR using SYBR green detection. All qPCR primer sequences are available upon request. For each IP, the corresponding input DNA was serially diluted (1:20, 1:100, 1:500) to create a standard series against which the ChIP sample could be normalized to control for differences in starting material. To be able to compare and combine ChIP data for a given antibody across different experimental repeats, the fold enrichment of DNA fragments in each experimental IP sample was calculated relative to the negative control IP. Statistical significance was determined with two-way analysis of variance (ANOVA) using StatView4.5 (Abacus Concepts). For data presentation, normalized values were averaged across all experiments and presented mean ± SEM.
Supplemental References


SUPPLEMENTAL FIGURES

Supplemental Figure 1

PCR genotyping of genomic DNA isolated from tails of indicated mutant mice.  i) PCR genotyping to detect the pIV deletion: WT, 853 bp; mut., 535 bp; ii) PCR genotyping to detect the presence of the loxP site:  WT, 113 bp; loxP, 176 bp; iii) PCR genotyping to detect the mutant CaRE3/CRE.  A short fragment spanning the CaRE3/CRE is PCR amplified and digested with the restriction enzyme PvuII, which will cleave only fragments which contain the mutant CaRE3/CRE:  WT, 442 bp; mut., 207bp + 235 bp (two bands not resolved in this gel).
Supplemental Figure 2

The activity-dependent expression of the remaining major Bdnf transcripts I, II, and VI in pIV\(^{-/-}\), TMKI, and CREmKI cortical neurons in response to membrane depolarization. Levels of Bdnf exon I (A-C), exon II (D-F), and exon VI (G-I) mRNA in 5DIV cortical neurons prepared from pIV\(^{-/-}\) and wildtype littermates (A,D,G), homozygous TMKI and loxP control littermates (B,E,H), or homozygous CREmKI and loxP control littermates (C,F,I) that were either left untreated or membrane depolarized with high extracellular potassium for the indicated amounts of time. Activity-dependent expression of Bdnf transcripts I or II is not statistically different between pIV\(^{-/-}\), TMKI, or CREmKI and their...
respective controls at all timepoints (P>0.05, repeated-measures ANOVA), except t=6 hrs for TMKI vs. loxP control. Interestingly, membrane depolarization causes a statistically significant 2-3 fold increase in the induction of exon VI-containing transcripts in all three promoter IV mutants, pIV−/−, TMKI, or CREmKI, compared to the inductions observed in their respective controls (G-I, P<0.01, repeated-measures ANOVA with pairwise comparisons at each timepoint 3 hours or longer, Bonferroni-Dunn post-hoc test), which may account for a significant portion of the residual induction in total Bdnf levels in the mutants (Figure 2D-F). This finding suggests that, under strong membrane-depolarizing conditions, CREB-dependent promoter IV activity may serve to negatively regulate exon VI-containing transcripts. Data are from n=3 (pIV−/− and CREmKI) or n=2 (TMKI) independent experiments in which each pair was measured in triplicate and are presented as mean ± SEM.

The functional significance of the observation that mutations in promoter IV can cause a significant increase in the activity-dependent activation of promoter VI in response to strong membrane depolarization is unclear. The substantial increase in promoter VI activity in promoter IV mutants is not observed in response to synaptic stimulation in cultured neurons (Figure 3) or in response to sensory experience-dependent neuronal activity in the intact brain (Figure 4A). These data suggest that the upregulation of activity-dependent Bdnf promoter VI activity in promoter IV mutants occurs only in response to very strong, non-physiological levels of stimulation that are not relevant in the intact nervous system of CREmKI mice.

However, the observation that even a subtle mutation in promoter IV that impairs its activity-dependent regulation is sufficient to cause a significant upregulation in the
induction of exon VI-containing transcripts under extreme stimulation conditions may be
providing clues into the mechanisms by which stimulus-specific gene expression from
the many Bdnf promoters is coordinated. For instance, CREB-binding at promoter IV
may serve to directly repress promoter VI activity; alternatively, exon IV-containing
transcripts or promoter IV-derived BDNF protein may act to negatively regulate
promoter VI. Another possibility is that the activity-dependent expression of the tight
cluster of promoters IV through VII is regulated through looping of a long-range, cis-
acting locus control region such that, in the absence of a CREB-dependent activating
complex at promoter IV, transcription of downstream promoters is enhanced. Further
investigation into the functions of promoter IV-derived Bdnf and potential changes in
local chromosomal conformations in CREmKI mutants may yield additional insights into
the mechanisms of neuronal activity-dependent Bdnf transcriptional regulation.
Supplemental Figure 3

BDNF protein levels in pIV+/− (A) and CREmKI (B) cortex is approximately 50% reduced compared to control littermates. BDNF protein levels were quantified by ELISA in whole cortical samples isolated from adult Bdnf+/− or CREmKI animals, and their respective littermates controls, reared under normal housing conditions. Asterisk indicates P<0.01 by Student’s t-test. Data are mean±SEM and from n=4 animals/condition.
CREmKI brains appear grossly normal at an anatomical level and are comparable in size to loxP control brains. A) Brains from CREmKI and loxP control littermates were sectioned at 35μm on a cryostat and stained with hematoxylin and eosin. B) Higher magnification view of CREmKI and loxP control cortices demonstrating grossly normal cortical layering in CREmKI brains.
**Supplemental Figure 5**

Visual experience-dependent gene expression in the somatosensory cortex of CREmKI and loxP control animals. Adult CREmKI and loxP control mice were reared in complete darkness for 14 days, after which they were either maintained in darkness (-) or exposed to light for 90 minutes (+). RNA was isolated from the somatosensory cortex of each animal and the levels of the indicated transcripts were measured by quantitative real-time PCR. For each mRNA transcript, gene expression across all conditions was normalized to the average value of the loxP control unstimulated (-) condition. In contrast to gene expression in the primary visual cortex (Figure 4A-B), none of the transcripts are well-induced in the somatosensory cortex in response to visual stimulation.
Supplemental Figure 6

Neuronal activity-dependent gene expression in response to kainic acid-induced seizures in CREmKI brains. Adult CREmKI or loxP control animals were injected with saline (timepoint 0) or with the chemoconvulsant kainic acid, and total RNA was collected from whole cortex at the indicated times post-injection. Levels of Bdnf exons I (A), II (B), VI (C), and NP2 (D) mRNA were measured by qPCR. Asterisk indicates P<0.01, repeated-measures ANOVA, pairwise comparisons at indicated timepoints by Bonferroni-Dunn post-hoc test. As in the case with visual stimulation, neuronal activity-dependent induction of most immediate-early genes including NP2 (D) and c-fos (data not shown) appears enhanced in CREmKI brains in response to seizures as compared to loxP control brains. Data are mean ± SEM from n=6-7 animals per condition, with each animal measured in triplicate.
Preliminary data suggest that CREmKI and loxP control animals have similar body masses from ten days through approximately six months of age. Males (A) and females (B) were separately housed from weaning age to the day of weighing in groups of 2 to 4 and fed *ad libitum*. Each point represents a unique animal, n=41 to 61 animals/condition.