

Supplemental Material

Table S1. Oligonucleotide primers used for site-directed mutagenesis.¹

Mut Δ CRE (sense) 38-mer:

5'-GGAGGTATCATATGACAAGGCAGCGTGGAGCCCTCTCG-3'

Mut Δ NF- κ B (sense) 33-mer:

5'-GCGTGGAGCCCTCTCGTGACTTTCCCATTCACC-3'

Mut Δ -117 to -79 (sense) 41-mer:

5'-GGAGGTATCATATGACAGCCACTTTCCCATTCACCGAGGAG-3'

Mut IS (sense) 33-mer:

5'-CAGCGTGGAGCCCTCTACTGGACTCCCACCCAC-3'

Mut CRE + Mut IS (sense) 38-mer:

5'AGGTATCATATGACAGCTCCACTCAAGGCAGCGTGGAG3'

Mut NF- κ B + Mut IS (sense) 38-mer:

5'CGTGGAGCCCTCTACTATACTCCCACCCACTTTCCCAT 3'

¹Only transcribed (sense) strand sequences are shown.

Figure S1. Generation of knockout and conditional *Bhlhb2* alleles. The targeting vector, wild type *Bhlhb2* genomic region encoding five exons (indicated as boxes), and three different mutant alleles are shown. The sizes of homology arms in the targeting vector are indicated. Restriction fragment sizes are also indicated as well as the positions of the two external probes and one internal probe used for genotyping analysis. The *neo* allele is the result of the gene-targeting event in ES cells. The *cko* allele is the conditional knockout allele derived from the *neo* allele after Flpe-mediated excision of the *neo* cassette between 2 *frt* sites. The *ko* allele is the null allele derived from the *cko* allele through Cre-mediated recombination between the 2 *lox2272* sites. neo, PGK-em7-neomycin dual selection cassette for bacteria and ES cells; TK, thymidine kinase cassette.

Figure S2. Genotyping *Bhlhb2* knockout mice. Ear DNA from mice from heterozygous intercross was amplified by PCR as described in Materials and Methods. The first primer pair (amplified a 727 bp product only from the intact *Bhlhb2* gene (labeled WT with an arrow), while the second primer set produced a 555 bp band representing the null allele (labeled KO with an arrow).

Gel shift assays. Results from the NF- κ B gel shift experiments are shown in Figure S3. NMDA (50 μ M) treatment for 40 min increased DNA binding activity to an NF- κ B target sequence based on the rat promoter 4 sequence that was blocked by a 50-fold molar excess of unlabeled (cold) NF- κ B probe. To characterize the components in the protein–DNA complex, we performed a “supershift” gel shift experiment using antibodies specific for the p50 and p65 subunits of NF- κ B. When nuclear extracts were incubated with the anti-p65 antibody, a slow migrating or “supershifted” band was seen, consistent with a protein–antibody–DNA complex, whereas incubation with an anti-p50 antibody markedly attenuated the specific band. Thus, the protein complex was composed of p50:p65 subunits. The increase in NF- κ B-specific DNA binding activity was preceded by an increase in I- κ B- α phosphorylation (phospho-I- κ B- α), a necessary step to activate NF- κ B through the canonical pathway (Anest et al., 2003; Yamamoto et al., 2003), which occurred within 20 min following NMDA treatment (Figure S4). CREB was also activated under these conditions, as determined by protein blot (phospho-serine¹³³) (Figure S4) and by an ELISA plate assay (Figure S5).

Figure S3. NF- κ B DNA-binding activity is increased in NMDA-treated cultured hippocampal neurons. NMDA (50 μ M) was added to cultured rat hippocampal neurons as described in Materials and Methods. Nuclear extracts were prepared and incubated (2 μ g protein) with a labeled double-stranded DNA oligonucleotide the specifically binds NF- κ B in the

presence or absence of 50-fold excess cold oligonucleotide or anti-p65 or anti-p50. Data are representative of results from three experiments.

Figure S4. Time course for induction of I- κ B- α and CREB phosphorylation in NMDA-

treated hippocampal neurons. Cultured hippocampal neurons were incubated with a subtoxic concentration of NMDA (50 μ M) for the indicated times on day in vitro eight. Phosphorylated (activated) I- κ B- α (Ser³²), total I- κ B- α , phosphorylated (activated) CREB (Ser¹³³) and total CREB levels were determined as described in Materials and Methods.

Figure S5. ELISA assay for CREB-dependent DNA binding activity in hippocampal

neuron cultures pretreated with NMDA. Neurons pretreated with NMDA showed a two-fold increase in CREB-mediated DNA binding activity that was blocked by the NMDA receptor inhibitor MK801. Competition with an excess of CREB “wild type oligo,” an unlabeled ds CREB probe was seen, but not with an excess of a mutated CREB ds DNA (labeled “mutated oligo”).

Figure S6. Basal and NMDA receptor-mediated changes in promoter function by mutant

CRE or NF- κ B sites in a *Bdnf* reporter plasmid lacking a functional E-box site. Luciferase-*Bdnf* promoter 4 reporter plasmids having an intact E-box element, mutated E-box element alone or a mutated E-box site in conjunction with CRE or NF- κ B mutations were constructed by site-directed mutagenesis (Materials and Methods). *Bdnf* promoter 4-firefly luciferase constructs were cotransfected into primary hippocampal neurons with a pRL-SV40 *Renilla* luciferase plasmid to control for transfection efficiency and assayed for promoter activity as described in Materials and Methods. Under basal conditions, neurons carrying the mutated E-box only had significantly increased levels of luciferase activity compared to the intact promoter, similar to the response observed for the same E-box mutation, but quantified by DsRed fluorescence (Figure

4A). However, incorporating a second mutation into either the CRE or the NF- κ B site, on the mutated E-box background, significantly reduced basal luciferase activity. Transfected neurons were also treated with 50 μ M NMDA for six hours and luciferase levels determined. Cells transfected with *pbdnf*-GL4.10 carrying the E-box mutation alone showed a 2-fold increase in luciferase activity relative to its matched untreated control. This response was significantly lower compared to neurons transfected with the intact *pbdnf*-GL4.10 plasmid (2.7-fold increase). When neurons having mutant reporter gene plasmids with double mutations (E-box and CRE site) or (E-box and p65 NF- κ B site) were treated with NMDA and assayed for luciferase activity 6 h later, each of the double mutants had significantly enhanced luciferase activity relative to their untreated control compared to the reporter activity seen in cells transfected with an E-box mutant plasmid alone relative to its untreated control. Results are expressed as relative luciferase activities (firefly/*Renilla* means \pm SE, n = 5 transfections per construct and condition). $**P < 0.01$ mutant E-box vs the intact promoter (*pbdnf*-GL4.10), basal conditions (no treatment); $\#P < 0.05$, $##P < 0.01$ for double mutants (mutated E-box and mutated NF- κ B or mutated E-box and mutated CRE) vs E-box mutant alone under basal conditions, Student's *t* test. $^{\Delta}P < 0.05$ for fold change for each double mutant or the intact promoter vs E-box mutant following 6 h treatment with 50 μ M NMDA.

Figure S7. BHLHB2 occupancy of *Bdnf* promoter 4 in cortex of *Bhlhb2* mutant mice.

Chromatin immunoprecipitation (ChIP) assays showing BHLHB2 occupancy within the *BDNF* promoter 4 in cortical tissue prepared from a *Bhlhb2* $+/+$ mouse but not a *Bhlhb2* $-/-$ mouse. Immunoprecipitated Acetylated histone H3 (AcH3) served as a positive control. The predicted 252 bp PCR product is indicated by an arrow. The PCR assay also shows “inputs” from each

genotype group prior to immunoprecipitation. Samples immunoprecipitated with nonimmune rabbit IgG were used as negative controls. Buffer-only “no antibody” controls produced similar results as “IgG” negative controls.

Figure S8. *Bhlhb2* mRNA levels in hippocampus of *Bhlhb2* mutant mice. Hippocampi from *Bhlhb2* mutant mice were collected for quantitative real-time PCR as described in Materials and Methods. *Bhlhb2* mRNA levels were normalized to *Gapdh* mRNA representing each of the three *Bhlhb2* genotypes. Expression values are expressed relative to *Bhlhb2* +/+ mice \pm SEM (n = 3 mice per genotype group).

Figure S9. KA treatment induces *Bdnf* exon 1, *Bdnf* exon 4, and *c-fos* mRNA levels in *Bhlhb2* +/+ mice. Mice were injected intraperitoneally with KA (10 mg/kg). After 90 min, hippocampi from *Bhlhb2* mutant mice were collected for quantitative real-time PCR. Expression values are expressed relative to no KA control \pm SEM. $P < 0.01$ relative to no treatment.

References

- Anest V, Hanson JL, Cogswell PC, Steinbrecher KA, Strahl BD, Baldwin AS (2003) A nucleosomal function for IkappaB kinase-alpha in NF-kappaB-dependent gene expression. *Nature* 423:659–663.
- Yamamoto Y, Verma UN, Prajapati S, Kwak YT, Gaynor RB (2003) Histone H3 phosphorylation by IKK-alpha is critical for cytokine-induced gene expression. *Nature* 423:655–659.