

Transcriptional Regulation of the Sodium-activated Potassium Channel *SLICK* (*KCNT2*) Promoter by Nuclear Factor- κ B*

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Background: The active role of *SLICK* channels and their regulation in neurons are largely unknown.

Results: *SLICK* transcription is highly dependent upon NF κ B activation in peripheral and central neurons.

Conclusion: *SLICK* is an evolutionarily conserved NF κ B-regulated gene.

Significance: *SLICK* channels may confer neuroprotection during ischemic conditions.

Although recent studies have shown the sodium-activated potassium channel *SLACK* (*KCNT1*) can contribute to neuronal excitability, there remains little information on the physiological role of the closely related *SLICK* (*KCNT2*) channel. Activation of *SLICK* channels may be important during pathological states such as ischemia, in which an increase in intracellular sodium and chloride can perturb membrane potential and ion homeostasis. We have identified two NF κ B-binding sites within the promoter region of the human *SLICK* (*KCNT2*) and orthologous rat *Slick* (*Kcnt2*) genes, suggesting that conditions in which NF κ B transcriptional activity is elevated promote expression of this channel. NF κ B binding to the rat *Slick* promoter was confirmed *in vivo* by ChIP analyses, and NF κ B was found differentially bound to the two sites. We verified NF κ B transcriptional regulation of *SLICK/Slick* by mutational analyses and studying gene expression by luciferase assay in P19 cells, where NF κ B is constitutively active. For the rat gene, activation of the *Slick* promoter was found to be additive in single NF κ B mutations and synergistic in double mutations. Unexpectedly, for the human gene, NF κ B exhibited cooperativity in activating the *SLICK* promoter. The human *SLICK* promoter constructs were then tested under hypoxic conditions in PC-12 cells, where NF κ B is not active. Only under hypoxic conditions could luciferase activity be detected; the double NF κ B mutant construct failed to exhibit activity. Transcriptional regulation of *Slick* by NF κ B was verified in primary neurons. The *Slick* transcript decreased 24 h after NF κ B inhibition. Our data show *SLICK* expression is predominantly under the control of NF κ B. Because neuronal NF κ B activation occurs during stressful stimuli such as hypoxia and injury, our findings suggest that *SLICK* is a neuroprotective gene.

Neuronal potassium channels play an essential role in resting membrane potential, action potential firing, synaptic vesicle

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release, and cell volume regulation (1). *SLACK* (*KCNT1*, *SLO2.2*) is a slowly activating, outwardly rectifying, sodium-activated and chloride-sensitive potassium channel (K_{Na}) found to contribute to neuronal excitability (2). The other member of the K_{Na} channel family, known as *SLICK* (*KCNT2*, *SLO2.1*), has been less studied. The human *SLICK* gene is ~400 kb and shares a striking 98% amino sequence identity with the rat protein sequence, exhibiting strong evolutionary conservation. Interestingly, when recorded in heterologous expression systems, *Slick* channels are more sensitive to intracellular chloride compared with *Slack*, and *Slick* activation is very rapid (3). *SLICK/Slick* channels contain a putative ATP-binding site. However, although rat *Slick* channel gating was found to be sensitive to intracellular ATP, current studies on human *SLICK* expressed in *Xenopus* oocytes did not find similar ATP inhibition (3, 4). Recent findings have shown that in contrast to *Slack* channels, *Slick* channels are the only large-conductance potassium channels sensitive to small changes in cell volume (5). Moreover, computer simulations predict that the instantaneous activation properties of *Slick* could in fact prevent action potential firing (3). These properties suggest that *Slick* channel expression might be tightly regulated, yet little is known about the channel's pathophysiological role in the nervous system.

A number of transcription factors are responsible for neuronal plasticity in response to stress, such as chronic inflammation and ischemia. NF κ B is an inducible transcription factor implicated as a pro-inflammatory and protective mediator in almost all mammalian cell types, with a dominant role for pro-survival in neurons (6). NF κ B in mammals is composed of five family members: RelA (p65), RelB, c-Rel, p50 (NF κ B1), and p52 (NF κ B2) (7). These members can form homo- or heterodimers that initiate or repress transcription. The p65, RelB, and c-Rel proteins contain a C-terminal transactivation domain that is essential for recruitment of the RNA polymerase transcriptional machinery. The p50 and p52 proteins lack this domain and therefore repress transcription if bound, unless bound as a heterodimer to a transactivation domain-containing member. The complexes remain inactive in the cytoplasm, bound by inhibitory family I κ B. Once I κ B is phosphorylated, NF κ B can freely translocate into the nucleus and become active. In *in vivo*

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studies using transgenic mice, loss of p50/p65 DNA binding specifically in neurons resulted in prominent cell death after neurotoxic insults in hippocampal slices (8).

NF κ B is also found to be a critical component of preconditioning, a biphasic endogenous mechanism for neuroprotection against future insults from ischemia and epilepsy (9). The early phase of preconditioning develops within a few minutes and lasts between 1 and 2 h, whereas the late phase develops between 12 and 24 h and lasts 3–4 days (10). *In vivo* models of brain tolerance, including ischemic, epileptic, and polyunsaturated fatty acid-induced preconditioning, determined NF κ B-dependent neuronal protection in the adult rat hippocampus, where activation of NF κ B was an essential step during late preconditioning (9). NF κ B p50/p65 heterodimers were identified bound to DNA as early as 1 h after sublethal ischemia.

Slick is highly expressed in cortical and hippocampal areas, regions that constitutively express NF κ B (high basal levels of active NF κ B) (11). Slick is also moderately expressed peripherally in dorsal root ganglion (DRG)² neurons (12). In this study, we examined the NF κ B-dependent regulation of the *SLICK* gene.

Experimental Procedures

ChIP—The protocol of Maze *et al.* (13) was modified to include a nuclear extraction method previous to chromatin shearing. Briefly, Dynabeads (anti-rabbit M-280, catalog no. 112-04, Novex) were blocked in PBS solution containing 0.5% BSA. Beads were then resuspended in blocking solution containing 10 μ g of antibody (anti-acetylated Lys-9 histone H3 (H3K9, catalog no. ab4441), anti-NF κ B p65 (catalog no. ab7970), and anti-NF κ B p105/p50 (catalog no. ab7971), Abcam) and rotated overnight at 4 °C. Beads were washed the next day with blocking solution. Adult Sprague-Dawley rats were decapitated, and fresh punches (3 \times 1 mm, six punches per animal) were taken from the hippocampus, caudate putamen, and cerebellum before flash-freezing. Six punches were processed per sample and resuspended in hypotonic lysis buffer with protease inhibitors. Tissue sonication and chromatin shearing were carried out as described previously (13). Part of the sample was used to verify sonication electrophoresing on agarose gel, and part of the sample was used as the input control in quantitative real-time PCR. Shearing of DNA to 400 bp was confirmed by electrophoresing sample on agarose gel electrophoresis. DNA absorbance was measured using a spectrophotometer, and equal amounts of DNA were added to the antibody-bound Dynabeads with overnight rotation at 4 °C. Samples were washed, eluted, and digested with RNase for 1 h at 37 °C, followed by proteinase K for 2 h at 55 °C. DNA was recovered using a Qiagen PCR purification kit (catalog no. 28106).

ChIP/Quantitative Real-Time PCR—Primers were designed based on the two rat NF κ B-binding sites, the mammalian conserved site, and the divergent site found only in mice and rats. Gene desert primers were designed by identifying a region in rat chromosome 12 without any genes. SYBR Green PCR Master

Mix (catalog no. 4309155, Applied Biosystems) For quantification, a 50-cycle two-step denaturing/annealing was used, with a 15-s absorbance reading on a Bio-Rad iQ5 cycler. Each sample was performed in triplicate. Acetylated H3K9 was used as a positive control for active transcription. The primers used were as follows: NF κ B conserved distal site, GCATAAGGGTGCCCTGCAATTTTCC (forward) and CCAGCACCCAGGCTGTGGTCCGCC (reverse); NF κ B non-conserved proximal site, GGCTGGGCCTTCAACAACACTGC (forward) and CGTGATGGCTAGGCACTTTCC (reverse); and gene desert, AGGTCCTTAGAGAGAGCAGGA (forward) and GGAAAAGCAGGTGAGTAGCA (reverse).

Cell Culture—PC-12 cells (a gift from Dr. Richard A. Rabin, The State University of New York at Buffalo) and P19 cells (a gift from Dr. Jerome A. Roth, The State University of New York at Buffalo) were maintained in a 5% CO₂ incubator at 37 °C. PC-12 cells were cultured in high-glucose DMEM containing 10% horse serum, 5% fetal bovine serum, and penicillin/streptomycin. P19 cells were cultured in minimum essential medium containing 7.5% newborn calf serum, 2.5% fetal bovine serum, and penicillin/streptomycin.

Dual-Luciferase Assay and Hypoxic Stress—Two kb of sequence upstream of the start site of human *SLICK* and rat *Slick* were cloned into the pGL3 luciferase vector. Site-directed mutagenesis of the putative NF κ B-binding sites was performed using PfuTurbo DNA polymerase (catalog no. 600250, Agilent). PC-12 and P19 cells lines were plated onto 12-well plates, and constructs were transfected using Lipofectamine 2000 (catalog no. 11668-027, Life Technologies, Inc.) the following day. On day 3, cells were washed and lysed according to the Dual-Luciferase reporter assay system (catalog no. E1960, Promega). For hypoxic stress, transfected PC-12 cells were flushed in a hypoxia chamber (containing a Petri dish with sterile water for adequate humidity) for 10 min with a mixture of 5% CO₂ and 95% N₂. The chamber was then sealed, and cells were incubated at 37 °C for 1, 6, and 24 h. Luminescence measurements were obtained on BioTek microplate reader. Luciferase and *Renilla* readings were subtracted from initial blank, divided, and normalized to the pGL3 vector control.

Primary DRG Neuronal Culture—DRG were dissected from day 15 Sprague-Dawley rat embryos. The ganglia were dissociated in trypsin (2.5 mg/ml) for 60 min. Cells were plated on poly-D-lysine (100 μ g/ml)- and laminin (3 μ g/ml)-coated coverslips, pooled one pup per coverslip, and maintained on serum-free C2 media containing β -NGF (100 ng/ml). Unlike neonatal and adult DRG neurons, embryonic DRG neurons require NGF for survival (14). The day after dissection, cells were treated with 1 μ M cytosine β -D-arabinofuranoside for 2 days. The NF κ B inhibitor caffeic acid phenethyl ester (CAPE; EMD Millipore) was dissolved in dimethyl sulfoxide (DMSO) and diluted to a final concentration of 2.5 μ g/ml in culture growth medium to 0.001% DMSO. On day 5 in culture, CAPE or DMSO was added to neuronal coverslips for 30 min, and cells were washed. For transcriptional analysis, RNA extraction was performed after 24 and 48 h.

RNA Extraction and cDNA Synthesis—An RNeasy micro kit (Qiagen) was used for RNA extraction from neuronal culture.

² The abbreviations used are: DRG, dorsal root ganglion/ganglia; CAPE, caffeic acid phenethyl ester; DMSO, dimethyl sulfoxide.

RNA was reverse-transcribed with SuperScript III reverse transcriptase (Life Technologies, Inc.).

cDNA Quantitative Real-time PCR—Transcriptional abundance was measured using a thermocycler and SYBR Green PCR Master Mix. For quantification, a 50-cycle two-step denaturing/annealing was used, with a 15-s absorbance reading on a Bio-Rad iQ5 cycler. Each sample was performed in triplicate. The primers used for DRG neuronal culture were as follows: *GAPDH*, AACGACCCCTTCATTGAC (forward) and TCCACGACATACTCAGCAC (reverse); and *Slick*, GGTTTACAGGTCTCGGTGGC (forward) and GAGATGATGAAGGGAACTGC (reverse).

Results

NF κ B Transcriptional Consensus Sites in *SLICK* Are Conserved in Mammals—Promoter analyses for transcription factor binding within the *SLICK* and rat *Slick* genes were performed using the web-based program TFSEARCH utilizing 2 kb of the gene sequence upstream of the start codon (Fig. 1). Consensus NF κ B-binding sites were identified within the *SLICK* promoter region. The *SLICK* promoter region was aligned and compared with other mammalian species, and we determined that NF κ B consensus sites are evolutionarily conserved. One putative site was completely conserved across all mammalian species examined, 828 bp upstream of the human *SLICK* start codon. Another consensus site was identified in human, chimpanzee, rhesus monkey, and dog, 13 bp upstream of the -828 site. In the rat and mouse *Slick* genes, there was an NF κ B consensus site 457 bp upstream of the start codon. Although NF κ B-binding sites are conserved across mammalian species, there are variations in the placement of the sites.

Putative NF κ B-binding Sites Verified in Vivo Are Found Differentially Bound to the *Slick* Promoter—To verify the identified putative NF κ B-binding sites of the *Slick* promoter, ChIP was performed on tissue from the rat hippocampus, caudate putamen, and cerebellum. High-level expression of *Slick* mRNA and Slick protein was found in the rat hippocampus, with moderate expression in the caudate putamen and undetectable expression in the cerebellum as determined by *in situ* hybridization and immunohistochemistry (11). We utilized antibodies against the p50 and p65 subunits of NF κ B, and acetylated H3K9 was used as a positive control for transcriptional activation. Quantitative PCR C_t values were normalized, dividing IP C_t values by input C_t values to provide ΔC_t . These values are relative to 1; therefore, values above 1 represent a higher IP C_t value correlating to a lower IP DNA abundance and therefore less transcription factor binding. Gene desert primers were used as a negative control for nonspecific binding. Gene desert C_t values were undetectable, being above the cutoff threshold (data not shown).³ We compared IP C_t values amplifying the evolutionarily conserved NF κ B-binding site, GGGGATTCC (distal site to the start methionine), and the non-conserved NF κ B-binding site (proximal site to the start methionine) present only in the rat and mouse genes, GGGGATGCC. At the distal NF κ B-binding site, both the p50 and p65 subunits exhibited a

lower mean ΔC_t in the hippocampus compared with the caudate putamen and cerebellum, indicating a higher level of NF κ B binding at this site within the hippocampus (Fig. 2). However, the data did not achieve statistical significance. At the proximal NF κ B-binding site, transcription factor binding was significantly different in the hippocampus *versus* the caudate putamen and cerebellum (Table 1). Specifically, the p65 subunit was significantly less bound in the proximal NF κ B-binding site within the cerebellum compared with the hippocampus. Furthermore, the p50 subunit was significantly less bound in the caudate putamen at the proximal NF κ B-binding site compared with every other p50 distal and proximal NF κ B-binding site. In other words, both the p50 and p65 subunits exhibited significantly higher binding levels at the proximal NF κ B-binding site in the hippocampus compared with the caudate putamen and cerebellum.

It should also be noted that in the hippocampus, p50 subunit binding to the proximal NF κ B-binding site was significantly less compared with the distal NF κ B-binding site. This suggests that the proximal NF κ B-binding site has lower NF κ B-binding affinity. Notwithstanding, these results confirm that NF κ B binds to the *Slick* promoter *in vivo* and indicates differential NF κ B-binding affinity between the two sites. Corroborating the high levels of constitutive NF κ B activity and *Slick* mRNA expression in the adult hippocampus, we found the highest levels of p50 and p65 binding to the *Slick* promoter within the hippocampus.

NF κ B Is Essential for Mammalian *SLICK* Transcription—To identify whether NF κ B can actively recruit the RNA polymerase transcriptional machinery and promote transcription of the *SLICK* gene, 2 kb of sequence upstream of the start site of the rat and human genes were inserted into the pGL3 promoter-less luciferase vector and designated as the wild-type construct. To observe activation of the luciferase gene, the inserted promoter region must contain regulatory elements that will initiate transcription. One or both NF κ B-binding sites were mutated to prevent NF κ B binding (Fig. 3A). The constructs were transfected into two separate neuronal model cell lines, PC-12 and P19 cells, for luciferase assays (Fig. 3B). PC-12 cells contain inactive NF κ B complexes, whereas P19 cells contain constitutively active p50 and p65 subunits (15, 16). In the PC-12 cell line, activation of luciferase was not observed in the rat or human constructs. In the P19 cell line, luciferase activation increased by ~ 17 -fold in both the rat and human wild-type constructs compared with the vector control. Nullifying either NF κ B-binding site in the rat promoter resulted in an additive decrease in luciferase activity, whereas nullifying both NF κ B-binding sites by mutation resulted in a synergistic decrease that did not significantly differ from the empty vector base line. In contrast, nullifying either NF κ B-binding site in the human promoter resulted in a complete loss of luciferase activity, not differing from the empty vector (Fig. 3C). However, we found that nullifying both NF κ B-binding sites resulted in a small but significant increase in luciferase activity over the base line. This suggests that occupancy of either site by NF κ B subunits represses other transcription factors; nonetheless, our data demonstrate that there is strong NF κ B-dependent transcription of *SLICK* across species.

³ D. L. Tomasello, A. M. Gancarz-Kausch, D. M. Dietz, and A. Bhattacharjee, unpublished data.

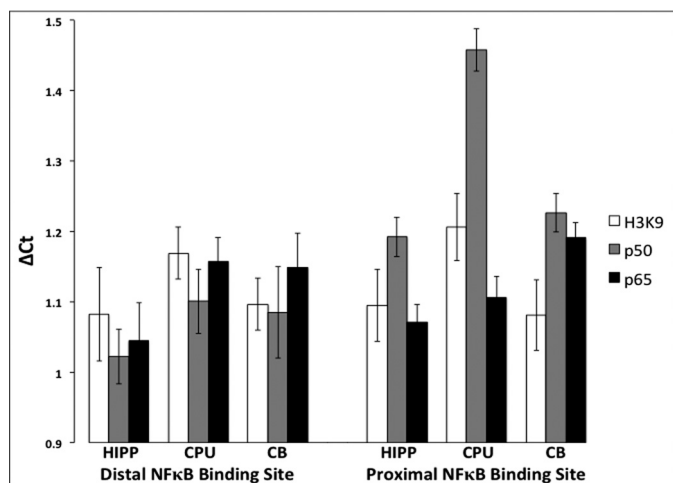


FIGURE 2. Differential NFκB binding to the *Slick* gene verified *in vivo*. Shown are the results from ChIP of NFκB subunits p65 and p50 and acetylated H3K9 bound to the *Slick* promoter region of rat hippocampal (HIPP), caudate putamen (CPU), and cerebellar (CB) tissue. Primers were designed based on the identified putative mammalian NFκB-binding sites, the evolutionarily conserved distal NFκB-binding site, and the proximal NFκB-binding site identified in only rats and mice. Acetylated H3K9 was used as a positive control for active transcription. Relative ΔC_t was calculated by normalizing IP to input. Quantitative real-time PCR was performed in triplicate. Error bars represent S.E. ($n = 4-6$).

Hypoxic Induction of *SLICK* Transcription Occurs through NFκB—To further demonstrate the dependence of *SLICK* transcription on NFκB, we induced NFκB activation in PC-12 cells by hypoxia. Hypoxia is known to cause IκBα degradation, increase NFκB DNA-binding activity, and cause the transactivation of a reporter gene construct containing multiple NFκB DNA-binding sites (17). The human wild-type and double NFκB-binding site mutant *SLICK* constructs were transfected into PC-12 cells and exposed to hypoxic stress for 1, 6, and 24 h. Luciferase activation was observed in the wild-type construct compared with the vector control at each time point (Fig. 4). The degree of activation was comparable with that seen in P19 cells (Fig. 3), where NFκB is constitutively active. The rat *Slick* wild-type construct showed a similar increase in luciferase activity when transfected in PC-12 cells after 45 min of hypoxic stress (data not shown).³ The double NFκB-binding site mutant construct exhibited a small but significant increase in activity at 1 h, similar to what we saw in P19 cells, but this disappeared at 6 and 24 h. We assume that another transcription factor that is repressed by NFκB binding during normal conditions loses activity during prolonged hypoxia. Collectively, our results reveal that the *SLICK* gene can be induced by hypoxia via NFκB activation.

Decreased *Slick* Expression after NFκB Inhibition in Primary Neurons—Although our data support *Slick* regulation by NFκB, we further confirmed this by studying the effects of NFκB inhibition on endogenous *Slick* transcription in native neurons. Using CAPE, a p65 translocation blocker, we assessed *Slick*

expression by quantitative real-time PCR in primary DRG neurons as described previously (18). We elected to use DRG neuronal cultures to study the transcriptional regulation of *Slick*, as both adult and embryonic DRG neurons express *Slick* channels (12). This contrasted with embryonic hippocampal neuronal cultures, where we observed little to no *Slick* expression at this early developmental stage (data not shown).³ Additionally, embryonic DRG neurons were cultured in the presence of NGF, a neurotrophic factor essential for survival of these neurons. NGF-dependent survival was found to be contingent upon NFκB activation (19). Furthermore, cytosine β-D-arabino-furanoside was added to cultures to remove mitotic glial supporting cells, ensuring that the effects seen could be attributed to neuron-specific inhibition of NFκB. We found that when DRG neurons were treated with CAPE for 30 min and RNA was isolated after 24 h, *Slick* expression was significantly decreased by ~50% compared with the DMSO control (Fig. 5). *Slick* transcriptional expression was normalized to the housekeeping gene *GAPDH*. We also examined *Slick* transcript levels 48 h after CAPE exposure, and although there was still slightly diminished expression, the data were not statistically significant. This is likely due to the short incubation time and eventual washing out of the inhibitor. Still, our data confirm that endogenous *Slick* expression, assessed in primary neurons, is dependent upon NFκB activation.

Discussion

In this study, we identified NFκB as a positive regulator of *SLICK* gene expression. Analysis of the promoter region of *SLICK* identified two NFκB-binding sites, with one site completely conserved across all mammalian species examined. The putative sites were verified *in vivo* using ChIP assay, and we determined differential binding of p50 and p65 at the proximal NFκB-binding site compared with the conserved distal NFκB-binding site. Mutating the consensus NFκB-binding sites caused a substantial reduction in *SLICK* transcriptional activity. *SLICK* transcriptional activation was seen after hypoxic stress and was found to be dependent upon NFκB activation, as mutating the two NFκB-binding sites largely inhibited activation of *SLICK*. We identified decreased *Slick* expression after using an NFκB inhibitor in primary neurons. We conclude that the *SLICK* gene is stringently regulated by NFκB activation.

NFκB subunits p50 and p65 are constitutively active in the glutamatergic neurons of the hippocampus and cortex, and extensive supporting evidence has attributed this high basal expression to be important for synaptic plasticity and morphology (see Ref. 20 for review of supporting evidence). Our ChIP data defined the proximal NFκB-binding site of the *Slick* gene as a determining factor of differential transcriptional expression because of variable binding of the NFκB p50 and p65 subunits at this site when analyzed in three different brain regions. Using DNA binding preferences by EMSA-Seq, Wong *et al.*

FIGURE 1. Identification of conserved NFκB-binding sites across mammalian species. Shown is the alignment of nucleotide sequences of the *SLICK* gene in higher and lower order mammalian systems, including human (*Homo sapiens*), chimpanzee (*Pan troglodytes*), rhesus monkey (*Macaca mulatta*), dog (*Canis lupus*), cow (*Bos taurus*), guinea pig (*Cavia porcellus*), rat (*Rattus norvegicus*), and mouse (*Mus musculus*). Sequences were aligned using the web-based program ClustalW from the European Bioinformatics Institute. NFκB consensus sites were predicted using the web-based program TFSEARCH from the Computational Biology Research Center. Putative NFκB-binding sites are highlighted in gray, and the start methionine codon is indicated in boldface. Asterisks indicate conservation.

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TABLE 1

Statistical analysis of ChIP ΔC_i values by individual *t* tests

Significant comparison	<i>p</i>
Hippocampal p50 distal NF κ B-binding site versus hippocampal p50 proximal NF κ B-binding site	$p \leq 0.05$
Hippocampal p65 proximal NF κ B-binding site versus cerebellar p65 proximal NF κ B-binding site	$p \leq 0.05$
Hippocampal p50 distal NF κ B-binding site versus caudate putamen p50 proximal NF κ B-binding site	$p \leq 0.0001$
Caudate putamen p50 distal NF κ B-binding site versus caudate putamen p50 proximal NF κ B-binding site	$p \leq 0.001$
Cerebellar p50 distal NF κ B-binding site versus caudate putamen p50 proximal NF κ B-binding site	$p \leq 0.01$
Hippocampal p50 proximal NF κ B-binding site versus caudate putamen p50 proximal NF κ B-binding site	$p \leq 0.001$
Cerebellar p50 proximal NF κ B-binding site versus caudate putamen p50 proximal NF κ B-binding site	$p \leq 0.01$

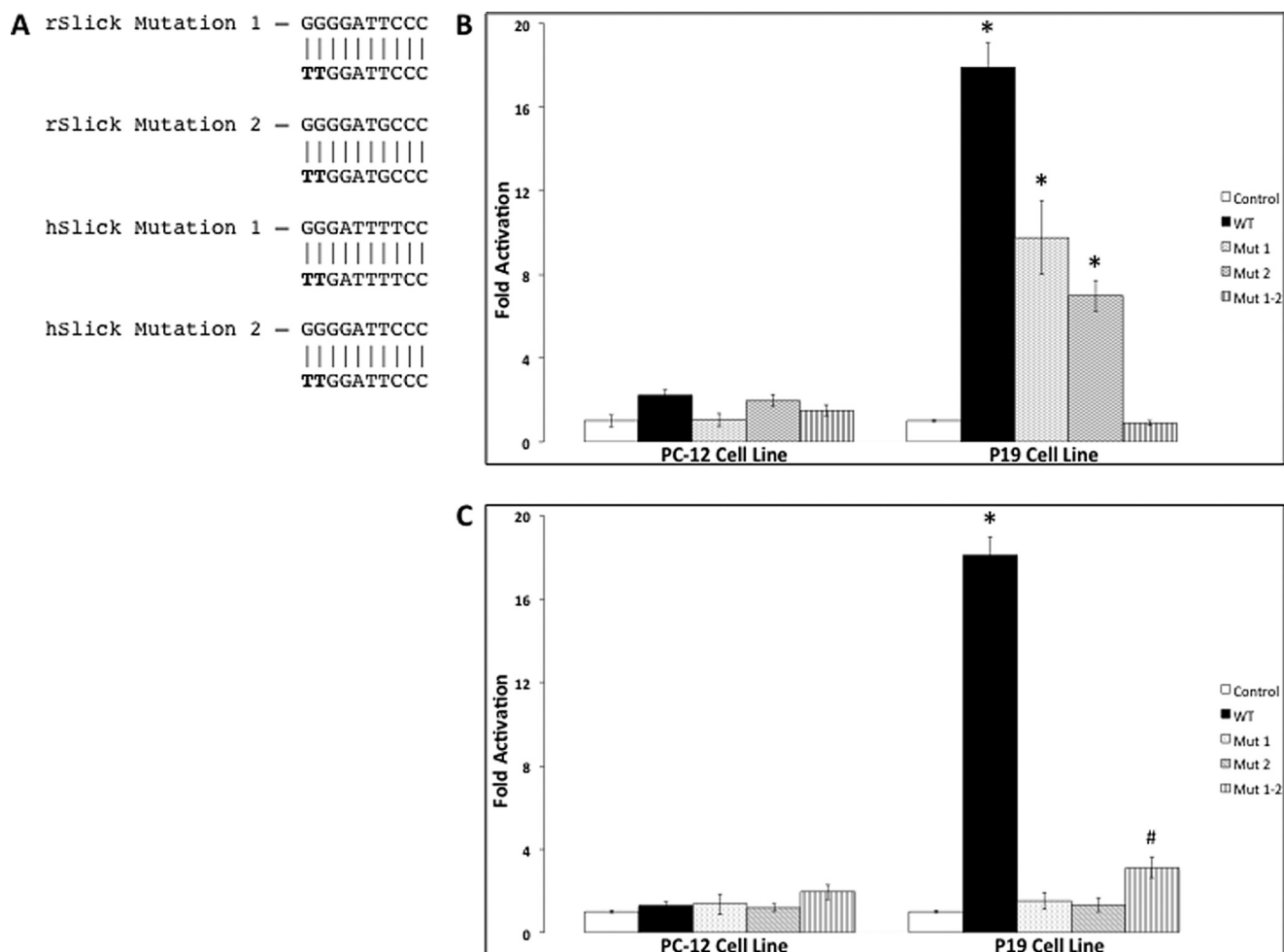


FIGURE 3. NF κ B binding to the *SLICK* gene promotes transcriptional activation. *A*, schematic of the mutations made by site-directed mutagenesis to the putative NF κ B-binding sites of the rat (*r*) *Slick* and human (*h*) *SLICK* genes. The promoter regions of the rat *Slick* (*B*) and human *SLICK* (*C*) genes were cloned into the pGL3 promoter vector. Mutations were made in the rat constructs only in the evolutionarily conserved distal NF κ B-binding site (*Mut 1*), only in the proximal NF κ B-binding site (*Mut 2*), or in both sites (*Mut 1–2*). Mutations were made in the human constructs only in the distal NF κ B-binding site (*Mut 1*), only in the evolutionarily conserved proximal NF κ B-binding site (*Mut 2*), or in both sites (*Mut 1–2*). The constructs were transfected into neuronal model cell lines in which NF κ B can be inducibly expressed (PC-12 cells) or constitutively expressed (P19 cells). Dual-Luciferase assays were performed using a plate reader to measure luciferase activity, with *Renilla* activity as an internal control. The pGL3 vector by itself was used as a control to normalize relative expression of each construct. Experiments were performed in triplicate between multiple experiments ($n = 2–7$). *, $p \leq 0.001$; #, $p \leq 0.01$ compared with the control by one-way analysis of variance. Error bars represent S.E.

(21) identified the relative binding affinities of nine distinct human NF κ B dimers on canonical and non-canonical sequences. In their report, the evolutionarily conserved distal NF κ B-binding site was analyzed and found to bind all NF κ B dimers with relatively high affinity. The exact sequence of the proximal NF κ B-binding site was not assessed in their study; however, a very similar sequence was reported to also have considerable binding affinity for all dimers, but an overall lower binding affinity compared with the distal NF κ B-binding site. Our ChIP data support these findings, as p50 and p65 exhibited

a lower binding affinity for the proximal NF κ B-binding site compared with the distal NF κ B-binding site.

The NF κ B-binding affinity we observed in the hippocampus from our ChIP correlates with the luciferase expression we observed in the rat *Slick* promoter. The high levels of constitutive NF κ B activity in P19 cells resemble those found in adult hippocampal neurons (22). Therefore, when sufficient levels of both p50 and p65 are available, binding is observed at both NF κ B-binding sites, explaining the additive decrease observed after mutation of the distal or proximal NF κ B-binding site.

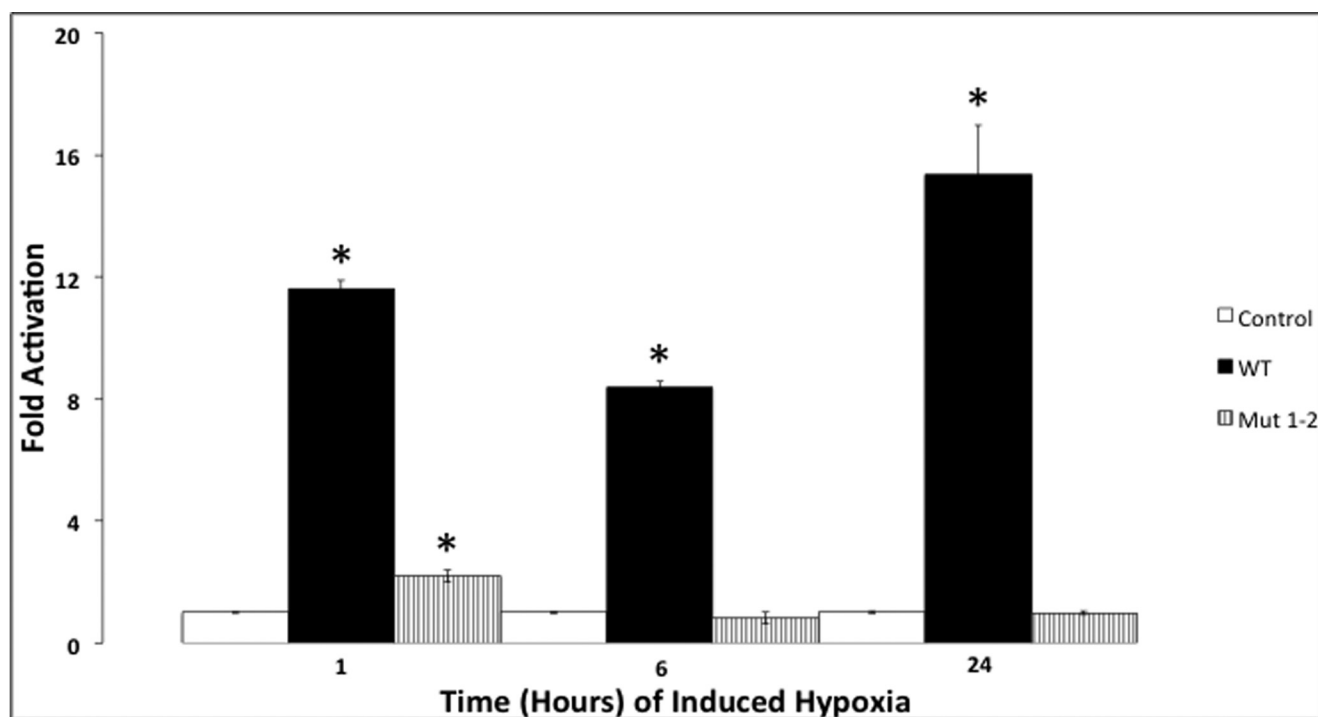


FIGURE 4. **Hypoxic induction of NF κ B increases *SLICK* transcription.** The pGL3 promoter human *SLICK* constructs were transfected into the hypoxic and neuronal model cell line (PC-12) and incubated in a hypoxia chamber for 1, 6, and 24 h at 37 °C. Dual-Luciferase assays were performed using a plate reader to measure luciferase activity, with *Renilla* activity as an internal control. The pGL3 vector by itself was used as a control to normalize relative expression of each construct. Experiments were performed in triplicate and performed twice. *, $p \leq 0.001$ compared with the control by one-way analysis of variance. Error bars represent S.E. Mut 1-2, double NF κ B-binding site mutant.

Interestingly, we found some differences between the human *SLICK* and rat *Slick* promoters. Nullifying either NF κ B-binding site in the rat *Slick* promoter resulted in an additive decrease in luciferase activation and a synergistic decrease in activation when both sites were mutated. This differed from the human *SLICK* promoter, where single mutations were sufficient to obliterate transcriptional activity. This may be due to the distance between the NF κ B-binding sites. Giorgetti *et al.* (23) found NF κ B to additively contribute to polymerase recruitment, which is defined by the strength of the environmental input. The synergistic decrease in luciferase activation of the double NF κ B-binding site mutation in the rat *Slick* promoter supports this idea. However, NF κ B cooperativity was observed for the human *SLICK* promoter, a result that is contrary to the conclusions put forth by Giorgetti *et al.*, who found that NF κ B bound independently and without cooperativity. However, it should be noted that although the evidence supports p65 binding as non-cooperative, other NF κ B subunits were not examined. NF κ B binding predicted by TFSEARCH indicated a high confidence level of c-Rel also potentially binding to the evolutionarily conserved site at -828 bp, along with the other site 13 bp upstream. Immunochemical supershift analyses determined that P19 cells express p50, p65, and c-Rel subunits (22). As c-Rel can activate transcription, it is possible that c-Rel binding may be cooperative at this site. Giorgetti *et al.* (23) also examined intersite NF κ B binding of up to 400 bp for cooperativity. The human NF κ B intersite difference is only 13 bp, much smaller than the average intersite distance examined. Cooperativity may arise because of the limited spacing between the two sites. In specific cases, p50/p52 dimers have been found to exhibit

cooperativity in a viral enhancer (24). Furthermore, cooperativity of the human NF κ B-binding sites would allow for maximal *SLICK* transcriptional activation after a small increase in NF κ B activation, whereas the additive properties in the rat NF κ B-binding sites would not. Our data suggest that cooperative NF κ B binding in the human *SLICK* promoter is an evolutionary adaptation that might be important for more complex brains.

With our ChIP data and the fact that *Slick* channels are highly expressed in adult pyramidal cells of the rat hippocampus and cortex, we can infer that *SLICK* channels are likely involved in synaptic plasticity (11). However, the two NF κ B-binding sites in the human *SLICK* promoter are predicted to bind to c-Rel as well. The c-Rel protein drives neuroprotective effects during glucose oxygen deprivation, and the neuronal survival factor interleukin-1 β can activate c-Rel (25, 26). Therefore, c-Rel activation may be important for inducing *SLICK* expression during ischemia. Of note, we also identified putative HIF1 (hypoxia-inducible factor 1)-binding sites in the *SLICK* promoter (data not shown).³ HIF1-binding sites were not present in the rat promoter. Although we determined that *SLICK* transcriptional initiation occurs primarily through NF κ B by hypoxia experiments, we cannot completely rule out an involvement of HIF1-dependent regulation of *SLICK*.

Slick channels are also expressed in DRG neurons, and the regulation by NF κ B may be important during stressful conditions such as nerve injury and neuropathy. Given the regenerative properties of these injured neurons, cytokine and/or neurotrophin signaling through NF κ B may allow neurons to adapt to neuropathic conditions and maintain normal firing. Although we saw a significant reduction in the *Slick* transcript

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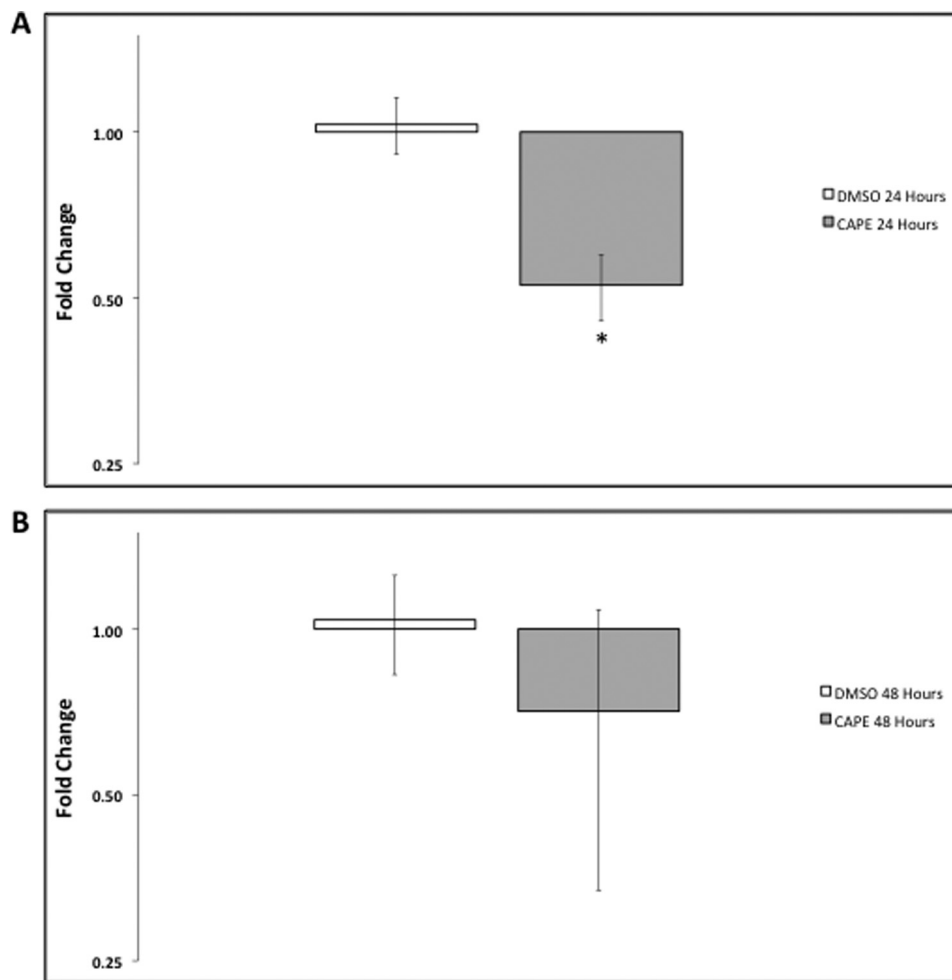


FIGURE 5. **Inhibition of NF κ B significantly decreases *Slick* the transcript in primary neurons.** *A*, a 30-min incubation of neurons with the NF κ B inhibitor CAPE significantly reduced *Slick* transcript levels assayed 24 h later compared with the DMSO control. *B*, transcript levels returned to near control levels at 48 h compared with the DMSO control. The -fold change ($\Delta\Delta C_t$) was normalized to GAPDH ($n = 6$ for 24 h, $n = 3$ for 48 h). *, $p \leq 0.01$ by *t* test. Error bars represent S.E.

in dissociated DRG neurons after NF κ B inhibition, we were not able to detect changes in protein levels after 48–96 h (data not shown).³ This is likely due to the typical longer half-lives of ion channels and also the effect we observed with CAPE inhibition of NF κ B. Using the protocol described by Chiechio *et al.* (18), the significant decrease in the *Slick* transcript observed at 24 h dissipated after 48 h. Therefore, the level of transcript reduction we saw may not be sufficient to significantly impact protein levels. It should be noted that we tried the potent NF κ B-specific peptide inhibitor SN50M (Enzo Life Sciences, Farmingdale, NY), but found substantial neuronal death within 24 h (data not shown),³ as might be expected for a prosurvival transcription factor such as NF κ B (19). Still, we cannot conclude that there are no post-transcriptional mechanisms that also govern *Slick* channel expression.

The small-conductance calcium-activated potassium channels of the SK2 subtype were also shown to have two NF κ B-binding sites and to be regulated by NF κ B (27). SK2 channels are important for synaptic plasticity and are neuroprotective during cerebral ischemia (28, 29). Indeed, SK2 channel openers were shown to reduce neuronal cell death during ischemia (29). Potassium channels are important for countering the ischemia-induced excitotoxicity in neurons (30, 31). During ischemic excitotoxicity, neurons

experience elevations in intracellular calcium, sodium, and chloride, causing changes in neuronal cell volume and eventual death. Potassium channels that are responsive to intracellular ion perturbations would be uniquely suited to confer neuroprotection. This may be one reason why *SK2* and *SLICK* genes are under the control of NF κ B. Elevating NF κ B signaling could limit ischemia-associated neuronal death by induction of the *SK2* and *SLICK* genes (17). Simply targeting *SLICK* channels directly during ischemia or neuropathy, channels that are uniquely sensitive to cell volume perturbations, could have neuroprotective effects (5). This remains to be explored.

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