

# Neuronal activation of NF- $\kappa$ B contributes to cell death in cerebral ischemia

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The transcription factor NF- $\kappa$ B is a key regulator of inflammation and cell survival. NF- $\kappa$ B is activated by cerebral ischemia in neurons and glia, but its function is controversial. To inhibit NF- $\kappa$ B selectively in neurons and glial cells, we have generated transgenic mice that express the I $\kappa$ B $\alpha$  superrepressor (I $\kappa$ B $\alpha$  mutated at serine-32 and serine-36, I $\kappa$ B $\alpha$ -SR) under transcriptional control of the neuron-specific enolase (NSE) and the glial fibrillary acidic protein (GFAP) promoter, respectively. In primary cortical neurons of NSE-I $\kappa$ B $\alpha$ -SR mice, NF- $\kappa$ B activity was partially inhibited. To assess NF- $\kappa$ B activity *in vivo* after permanent middle cerebral artery occlusion (MCAO), we measured the expression of NF- $\kappa$ B target genes by real-time polymerase chain reaction (PCR). The induction of c-myc and transforming growth factor- $\beta$ 2 by cerebral ischemia was inhibited by neuronal expression of I $\kappa$ B $\alpha$ -SR, whereas induction of GFAP by MCAO was reduced by astrocytic expression of I $\kappa$ B $\alpha$ -SR. Neuronal, but not astrocytic, expression of the NF- $\kappa$ B inhibitor reduced both infarct size and cell death 48 hours after permanent MCAO. In summary, the data show that NF- $\kappa$ B is activated in neurons and astrocytes during cerebral ischemia and that NF- $\kappa$ B activation in neurons contributes to the ischemic damage.

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## Introduction

The central nervous system reacts to noxious stimuli with a complex genomic response. Recent advances in expression profiling have identified hundreds of genes that are upregulated on damage to brain tissue. In an attempt to investigate transcriptional programs rather than individual genes, the functional role of transcriptional activators has been studied in various paradigms of brain damage. In the case of cerebral ischemia, there is clear evidence for the activation of transcription factors such as hypoxia inducible factor, interferon regulatory factor 1, and NF- $\kappa$ B (Bergeron *et al*, 1999; Iadecola *et al*, 1999; Schneider *et al*, 1999; Stephenson *et al*, 2000).

Originally, NF- $\kappa$ B was discovered in an immunologic context but it is also present in the brain (O'Neill and Kaltschmidt, 1997). Five subunits, p50, p52, p65 (RelA), RelB, and c-Rel, form homo- and heterodimers. In an inactive state, NF- $\kappa$ B dimers are sequestered in the cytoplasm by the specific inhibitors I $\kappa$ B $\alpha$ , I $\kappa$ B $\beta$ , and I $\kappa$ B $\epsilon$ . On stimulation, I $\kappa$ B is phosphorylated by the I $\kappa$ B kinase (IKK) complex, ubiquitinated, and then degraded by the 26S proteasome. However, there are also pathways of NF- $\kappa$ B activation that are independent of IKK or the proteasome (Bui *et al*, 2001; Schölzke *et al*, 2003). In cerebral ischemia, most investigators found activation of NF- $\kappa$ B in neurons (Huang *et al*, 2001; Schneider *et al*, 1999; Stephenson *et al*, 2000). In addition, there is also evidence for the activation of NF- $\kappa$ B in endothelial and glial cells during cerebral ischemia (Carroll *et al*, 1998; Gabriel *et al*, 1999; Terai *et al*, 1996). NF- $\kappa$ B activity in the brain consists mainly of the subunits p50 and p65 (RelA). To show the functional significance of NF- $\kappa$ B activation, we previously used p50-deficient mice. A reduced infarct size in the absence of p50 suggested that NF- $\kappa$ B is involved in ischemic brain damage (Nurmi *et al*, 2004; Schneider *et al*, 1999). However, further interpretation of this result is

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complicated because p50 both represses and transactivates gene transcription, depending on the dimerization partner, the promoter, and cell type that are studied (Franzoso *et al*, 1992; Kang *et al*, 1992; Kurland *et al*, 2001). Moreover, the absence of p50 in all cells and tissues of the body causes slight defects in immune responses (Sha *et al*, 1995) and potentially also other as yet unknown systemic effects (e.g., changed levels of proinflammatory cytokines released from endothelial cells during ischemia), which might influence ischemic brain damage indirectly. To obtain more definite evidence for the functional significance of NF- $\kappa$ B activation in cerebral ischemia, we have now generated transgenic mice that express a specific dominant inhibitor of NF- $\kappa$ B activation under transcriptional control of neuron- or astrocyte-specific promoters. As a dominant inhibitor of NF- $\kappa$ B, we used I $\kappa$ B $\alpha$  mutated at the phosphorylation sites for IKK (serine-32 and serine-36, I $\kappa$ B $\alpha$  superrepressor, I $\kappa$ B $\alpha$ -SR), which has been successfully used as a transgene before (Hettmann *et al*, 1999; Vallabhapurapu *et al*, 2001). Our results show that selective inhibition of NF- $\kappa$ B in neurons reduces ischemic cerebral damage.

## Materials and methods

### Generation of Transgenic Mice

The I $\kappa$ B $\alpha$ -SR transgene is preceded by the  $\beta$ -globin initiation signal to maximize translation efficiency and by a T7 tag to facilitate detection (Vallabhapurapu *et al*, 2001). A 2.1-kb fragment of the human growth hormone gene downstream of the cDNA provides intron and polyadenylation sequences. A 1.8-kb fragment of the rat neuron-specific enolase (NSE) promoter, which has been shown to direct gene transcription into neurons (Forss-Petter *et al*, 1990), was inserted 5' to the I $\kappa$ B $\alpha$ -SR cDNA (pNSE-I $\kappa$ B $\alpha$ -SR). To construct pGFAP-I $\kappa$ B $\alpha$ -SR (GFAP: glial fibrillary acidic protein), the NSE promoter was replaced by a 2.2-kb DNA fragment of the human GFAP promoter that was derived from the plasmid pEGFP-GFAP (Nolte *et al*, 2001). This promoter directs transgene expression into astrocytes (Brenner *et al*, 1994; Nolte *et al*, 2001). Transgenic mice were obtained by pronuclear injection into oocytes that were derived from female F1 hybrids (C57Bl/6  $\times$  DBA2/Crl) and male C57Bl/6 mice. Founders were backcrossed for four to six generations on a C57Bl/6 background. Genomic DNA obtained from tail biopsies was tested by polymerase chain reaction (PCR) using the following primers resulting in a PCR product of 233 bp: nm-map1, 5'-cct gtg ttc act agc aac ctc aaa cag aca cc; nm-map2, 5'-gta atc ctc gtc ctt cat ggc gtc caa ggc-3'.

### Middle Cerebral Artery Occlusion

At an age of 4 to 8 months, male mice were anesthetized by intraperitoneal injection of 150  $\mu$ L 2.5% avertin per 10 g

body weight. A skin incision was made between the ear and the orbit on the left side. The parotid gland and the temporal muscle were removed by electrical coagulation. The stem of the middle cerebral artery (MCA) was exposed through a burr hole and was occluded by microbipolar coagulation (Erbe, Tübingen, Germany). Surgery was performed under a microscope (Hund, Wetzlar, Germany). Mice were kept at a body temperature of 37°C on a heating pad. After 48 hours they were deeply reanesthetized with avertin and perfused intracardially with Ringer's solution. Brains were removed and immediately frozen on dry ice. Coronal cryosections (20  $\mu$ m) were cut every 400  $\mu$ m, starting rostrally. Sections were stained with a silver technique to determine the infarct size (Herrmann *et al*, 2003). Stained sections were scanned at 600 dpi and the infarct area was measured (ScnImage, Scion, Frederick, MD, USA). The total infarct volume was obtained from integrating infarcted areas corrected for brain edema (Swanson *et al*, 1990). Surgery and infarct measurement were obtained without a knowledge of the genotype. For the measurement of physiologic parameters in a subgroup of mice, the right femoral artery was cannulated. Samples (100  $\mu$ L per mouse) were collected for analysis of arterial blood gas, hemoglobin, and glucose. For laser Doppler measurements, the probe (P415-205; Perimed, Järfälla, Sweden) was placed 3 mm lateral and 6 mm posterior to the bregma. Relative perfusion units were determined (Periflux 4001; Perimed, Järfälla, Sweden).

### Immunohistochemistry

Coronal cryosections (10  $\mu$ m) were prepared and air-dried for 1 hour. They were fixed in acetone (-20°C) for 2 mins, air-dried again, and blocked with biotin-blocking solution for 20 mins and with peroxidase-blocking reagent for 5 mins (DAKO, Hamburg, Germany). For staining of the T7 epitope, a monoclonal mouse anti-T7 antibody (Novagen, Madison, WI, USA) was pre-labeled with biotin using DAKO ARK (DAKO, Hamburg, Germany). Sections were incubated overnight at 4°C with biotin-labeled anti-T7 (2.5  $\mu$ g/mL) in phosphate-buffered saline (PBS). For detection of the anti-T7 antibody, a streptavidin-peroxidase complex (ARK, DAKO, Hamburg, Germany) was used. Peroxidase was visualized with 3,3'-diaminobenzidine (DAB). For immunohistochemistry of neurofilament-200 kDa, a rabbit anti-neurofilament-200 kDa antibody (1:500, Sigma, Munich, Germany) was used. After washing with PBS, sections were incubated with an alkaline phosphatase-conjugated goat anti-rabbit antibody (1:80, DAKO, Hamburg, Germany) for 30 mins at room temperature. Alkaline phosphatase was visualized with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT).

### Real-time RT-PCR

Mice were reanesthetized and perfused with Ringer's solution 24 hours after middle cerebral artery occlusion (MCAO); the ischemic and the corresponding contralateral cortices were quickly dissected and frozen on dry ice.

**Table 1** Primers for real-time PCR

Name	Forward primer	Reverse primer	Length of amplicon
GAPDH	cattgtggaaggctcatga	tcttctgggtggcagtgatg	53
$\beta$ -Actin	gccctgaggctctttcca	tggatgccacagattccat	55
Cyclophilin	aggtcctggcatctgtccat	gaaccgtttgtttgggtcca	51
GFAP	ctggacaccaaatccgtgtca	tcctgcttcgagtccttaatga	101
c-myc	accagcagcgactctgaagaa	tccacagacaccacatcaatttc	62
TGF- $\beta$ 2	acatcgatagcaaggtgtgaa	cgctctgcacgtcgaagga	66

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFAP, glial fibrillary acidic protein; TGF- $\beta$ 2, transforming growth factor- $\beta$ 2.

Tissues were stored at  $-80^{\circ}\text{C}$ . RNA was extracted with peqGOLD RNAPure (peqLAB, Erlangen, Germany), according to the manufacturer's instructions. In total 10  $\mu\text{g}$  RNA was transcribed with MMLV reverse transcriptase and random hexamers. Primers for the quantitative real-time PCR are listed in Table 1. Polymerase chain reaction was performed according to the following protocol: 10 mins at  $95^{\circ}\text{C}$ , 15 secs at  $95^{\circ}\text{C}$ , and 1 minute at  $60^{\circ}\text{C}$  (40 cycles). Amplification was quantified with the Gene Amp 5700 sequence detector and the SYBR Green kit (PE Diagnostik, Weiterstadt, Germany). A linear concentration-response curve was established by diluting pooled samples. Quantified results for individual cDNAs were normalized to mean levels for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), cyclophilin, and  $\beta$ -actin. The purity of the amplified products was checked by the dissociation curve.

### TUNEL Staining

For terminal deoxynucleotidyl transferase-mediated 2'-deoxyuridine 5'-triphosphate nick end labeling (TUNEL) staining, sections were fixed in 4% paraformaldehyde at room temperature for 30 mins. Then sections were washed twice in PBS for 5 mins and permeabilized for 2 mins with 200  $\mu\text{L}$  permeabilization solution (0.1% Triton X-100 and 0.1% sodium citrate in PBS) at  $4^{\circ}\text{C}$ . After washing, sections were incubated with 50  $\mu\text{L}$  TUNEL Reaction Mix (enzyme solution diluted 1:6 in labeling solution; In Situ Cell Detection Kit, Fluorescein, Roche, Mannheim, Germany) for 1 hour at  $37^{\circ}\text{C}$  in the dark. Then sections were mounted with medium containing 4',6-diamidino-2-phenylindole dihydrochloride (DAPI, Vectashield). TUNEL- and DAPI-positive cells were detected with a fluorescence microscope. In three fields of 0.04  $\text{mm}^2$  at the dorsal, ventral, and middle border of the infarct at the level of the anterior commissure, TUNEL-positive cells and DAPI-positive nuclei were counted. TUNEL-positive cells were expressed as percent of total cell count. Cells were quantified without a knowledge of the genotype.

### Immunoblot

Tissue lysates (in 50 m mol/L Tris-HCl, pH 7.0, 1% sodium dodecyl sulfate (SDS), 2% 2-mercaptoethanol) were resolved by 10% SDS-polyacrylamide gel electrophoresis

and transferred to Hybond nitrocellulose (Amersham, Freiburg, Germany). Membranes were rinsed in TBST (20 m mol/L Tris-HCl, pH 7.6, 150 m mol/L NaCl, 0.1% Tween 20), incubated in 5% nonfat dry milk dissolved in TBST for 2 hours, and then incubated with rabbit anti-I $\kappa$ B $\alpha$  antibodies (0.4  $\mu\text{g}/\text{mL}$ , Santa Cruz, Heidelberg, Germany) or mouse anti-T7 (0.3 ng/mL, Novagen, Madison, WI, USA) in TBST. Antibody-antigen complexes were detected with ECL reagents (Amersham, Freiburg, Germany).

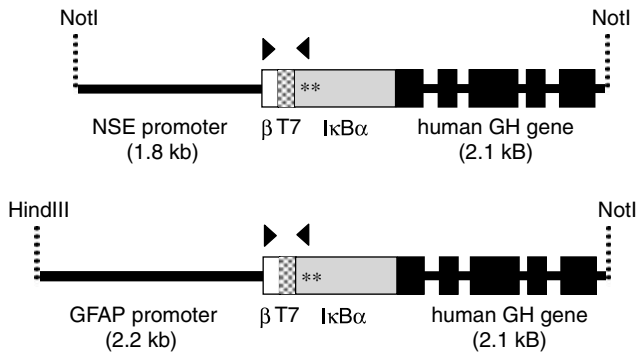
### Cell Culture and Transfection

Cortical neurons were derived from embryonic day 16 (E16) mice. The cells from individual brains were dissociated and cultured in 24-well plates precoated with poly-D-lysine (50  $\mu\text{g}/\text{mL}$ ) at a density of 200,000 cells/well. These cells were then incubated in neurobasal medium (Invitrogen, Karlsruhe, Germany) supplemented with B27 (Invitrogen), 0.5  $\mu\text{mol}/\text{L}$  L-glutamine, penicillin (100 IU/mL), and streptomycin (100  $\mu\text{g}/\text{mL}$ ). After 10 days *in vitro*, cells were transfected using Lipofectamine 2000 (Invitrogen) and 1  $\mu\text{g}/\text{well}$  of the NF- $\kappa$ B reporter plasmid pNF- $\kappa$ B-Luc comprising five tandem repeats of NF- $\kappa$ B binding sites (Stratagene, Amsterdam, Netherlands), according to the manufacturer's protocol. After 24 hours, cells were stimulated as indicated and harvested. To control for cell viability after stimulation with 10  $\mu\text{M}$  camptothecin, cells were cotransfected with 0.1  $\mu\text{g}/\text{well}$  of the reference plasmid pRL-TK (Promega, Mannheim, Germany). Renilla luciferase was measured with the Dual-Luciferase Reporter Assay, according to the manufacturer's protocol (Promega). Firefly luciferase activity was measured as described (Sallmann *et al*, 2000). Cell death was quantified after 16 hours of exposure to 10  $\mu\text{mol}/\text{L}$  camptothecin by staining neurons with Vectashield and counting the cells with condensed and normal nuclei.

The preparation and culture of primary mouse astrocytes has been described before (Schwaninger *et al*, 2000). Cells were transfected with pNF- $\kappa$ B-Luc (2  $\mu\text{g}/6\text{-cm}$  well) and GFAP-I $\kappa$ B $\alpha$ -SR using Transfast (Promega, Mannheim, Germany), according to the manufacturer's instructions. Cotransfections were performed with a constant DNA concentration, which was maintained by adding pBlue-script (Stratagene, La Jolla, CA, USA). At 42 hours after transfection, cells were stimulated for 6 hours and harvested.

### Statistical Analysis

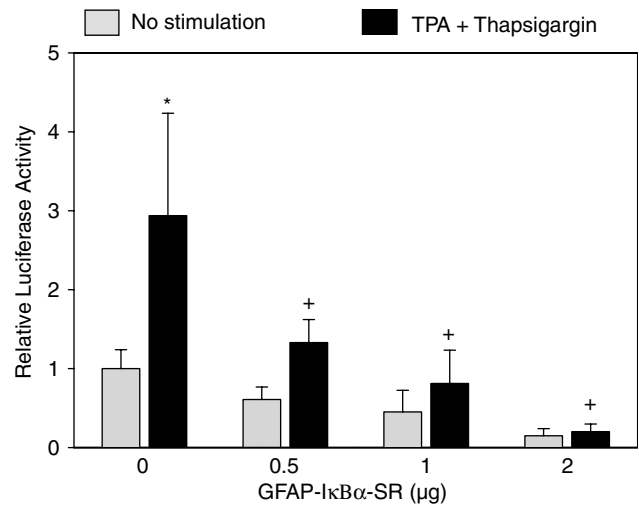
Data are illustrated as mean  $\pm$  s.d. Statistical comparisons of three or more groups were made by analysis of variance followed *post hoc* by Tukey's honestly significant difference (Tukey-HSD) or Fisher's protected least-squares difference (LSD) as indicated. Two groups were compared by a two-sided *t*-test. Values were considered to be significant at  $P < 0.05$ .



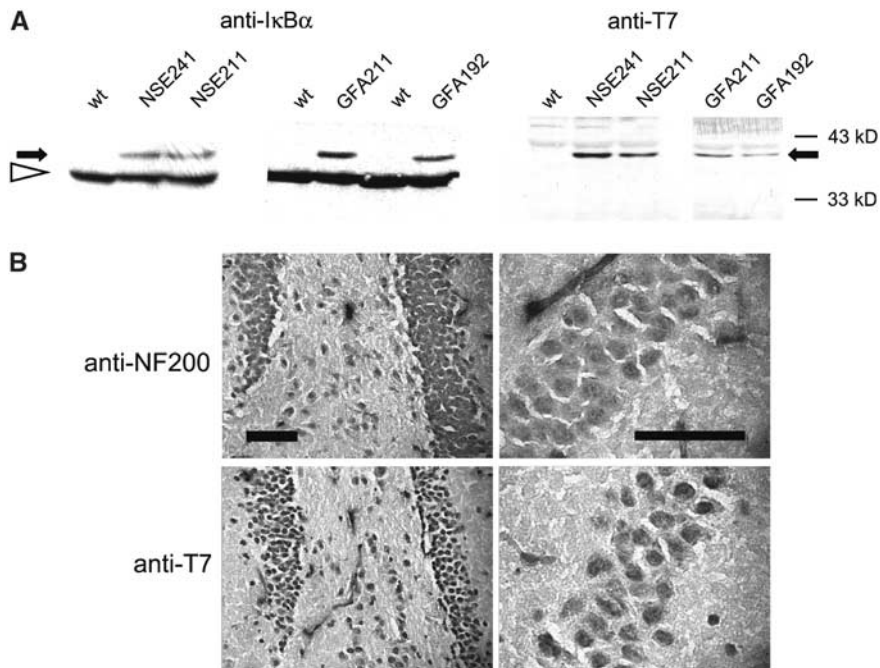
**Figure 1** Schematic drawing of the DNA fragments for the generation of NSE-I $\kappa$ B $\alpha$ -SR (upper panel) and GFAP-I $\kappa$ B $\alpha$ -SR transgenic mice (lower panel).  $\beta$ ,  $\beta$ -globin translational initiation site; T7, T7-antibody tag; arrowheads, primers for genotyping; \*, mutation of serine to alanine; GH, growth hormone sequences.

### Results

The DNA constructs that were used for the generation of transgenic mice are schematically depicted



**Figure 2** Expression of I $\kappa$ B $\alpha$ -SR in astrocytes inhibits NF- $\kappa$ B activation. Primary astrocytes were transiently transfected with the construct pGFAP-I $\kappa$ B $\alpha$ -SR and stimulated with 300 n mol/L TPA plus 1  $\mu$  mol/L thapsigargin as indicated. Luciferase activity is expressed relative to the unstimulated control group of the experiment. Values are means  $\pm$  s.d. of two independent experiments each performed in triplicate.  $P < 0.0001$  (one-way ANOVA). \* $P < 0.0001$  compared with the unstimulated control group. + $P < 0.0001$  compared with the stimulated group without pGFAP-I $\kappa$ B $\alpha$ -SR (Tukey-HSD *post hoc* test).



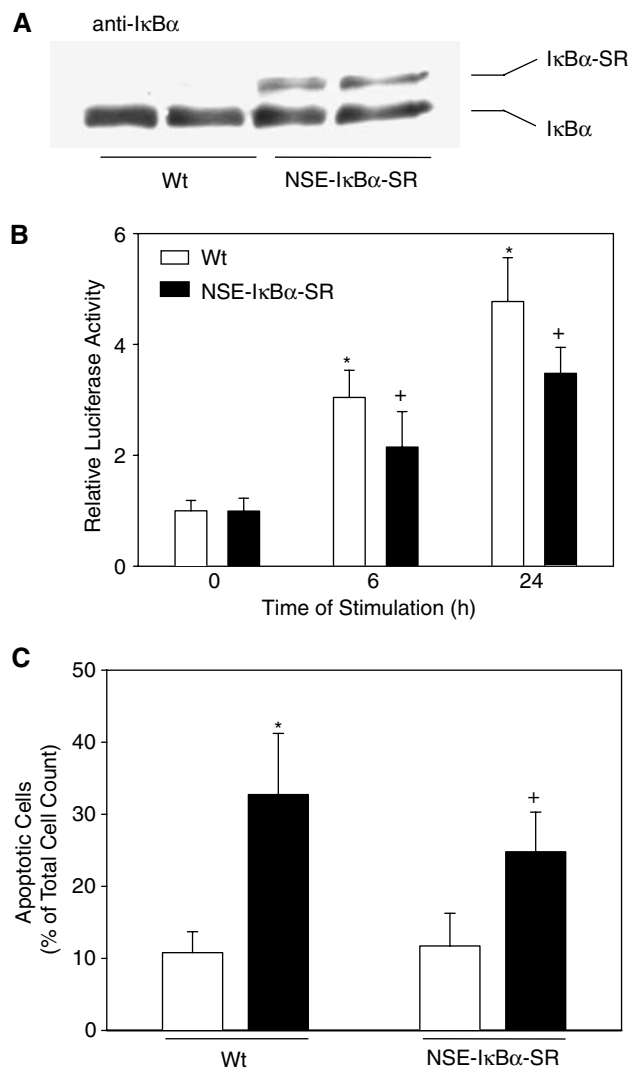
**Figure 3** Characterization of the expression of the I $\kappa$ B $\alpha$ -SR transgene. (A) In brain extracts of transgenic mice, I $\kappa$ B $\alpha$ -SR (arrow) could be detected as a band with slightly higher molecular weight than endogenous I $\kappa$ B $\alpha$  (arrowhead, left panel) due to the T7 tag. The same band was also stained with anti-T7 antibody (right panel). (B) I $\kappa$ B $\alpha$ -SR is expressed in neurons in NSE-I $\kappa$ B $\alpha$ -SR211 mice. Sections of the dentate gyrus of the hippocampus were stained for the neuronal marker neurofilament-200 kDa (NF200, BCIP/NBT, blue) and the T7 tag (3,3'-diaminobenzidine, brown). Bar, 100  $\mu$ m.

in Figure 1. For selective inhibition of NF- $\kappa$ B in neurons or astrocytes, the superrepressor of NF- $\kappa$ B I $\kappa$ B $\alpha$ -SR was put under transcriptional control of the rat 1.8-kb, neuron-specific NSE or the human 2.2-kb, astrocyte-specific GFAP promoter, respectively. To verify that the T7-tagged, mutated form of I $\kappa$ B $\alpha$  (I $\kappa$ B $\alpha$ -SR) acts as an inhibitor of NF- $\kappa$ B, we transfected the construct pGFAP-I $\kappa$ B $\alpha$ -SR together with an NF- $\kappa$ B reporter gene into primary mouse astrocytes. NF- $\kappa$ B activity stimulated by phorbol 12-myristate 13-acetate (TPA) plus thapsigargin was inhibited by expression of I $\kappa$ B $\alpha$ -SR in a dose-dependent manner (Figure 2).

We obtained three founders for the NSE-I $\kappa$ B $\alpha$ -SR construct and four founders for the GFAP-I $\kappa$ B $\alpha$ -SR construct. Two founder lines for each construct were further investigated (NSE-I $\kappa$ B $\alpha$ -SR211, NSE-I $\kappa$ B $\alpha$ -SR241, GFAP-I $\kappa$ B $\alpha$ -SR211, GFAP-I $\kappa$ B $\alpha$ -SR192). The transgenes did not have any obvious effect on behavior or breeding nor was gross brain morphology altered by the transgene (data not shown).

The transgenic I $\kappa$ B $\alpha$ -SR has a higher molecular weight than the endogenous form because of the T7 tag. This allowed a direct comparison of the expression of transgenic and endogenous I $\kappa$ B $\alpha$  by immunoblotting. Transgenic I $\kappa$ B $\alpha$ -SR levels in brain extracts were lower than endogenous I $\kappa$ B $\alpha$  in all transgenic lines (Figure 3A). The specificity of the band in immunoblots was confirmed by detection of the transgene with an antibody against T7 (Figure 3A). Transgene expression was restricted to the brain and could not be detected in heart, kidney, liver, or spleen (data not shown).

The cell specificity of the transgene expression in NSE-I $\kappa$ B $\alpha$ -SR mice was further verified by immunohistochemistry. The neuronal cell layer of the dentate gyrus of the hippocampus that expresses the neuronal marker neurofilament-200 was also stained by an antibody against the transgene epitope T7 (Figure 3B). In addition, neurons in the granular layer of the cerebellum and in the cortex were positive for the T7 marker (not shown). Primary cortical neurons *in vitro* expressed the transgene as shown by immunoblots of cell extracts (Figure 4A). To test whether transgene expression interferes with NF- $\kappa$ B activation, we used a reporter gene assay in primary cortical neurons from mice with the NSE-I $\kappa$ B $\alpha$ -SR transgene and from wild-type littermates. NF- $\kappa$ B-driven luciferase expression was stimulated by 10 ng/mL TNF- $\alpha$ , a classical NF- $\kappa$ B inducer. In cortical neurons from transgenic mice, NF- $\kappa$ B activation was preserved, but was significantly lower (Figure 4B). To investigate the effect of NF- $\kappa$ B inhibition on neuronal cell survival, we used camptothecin, a DNA-damaging agent (Park *et al*, 1997). DNA damage is an important component of the pathophysiology of cerebral ischemia, well in advance of DNA fragmentation caused by the apoptotic process (Chen *et al*, 1997; Cui *et al*, 2000; Tobita *et al*, 1995). Camptothecin (10  $\mu$ mol/L for 16 h) stimulated NF- $\kappa$ B-driven luciferase expression



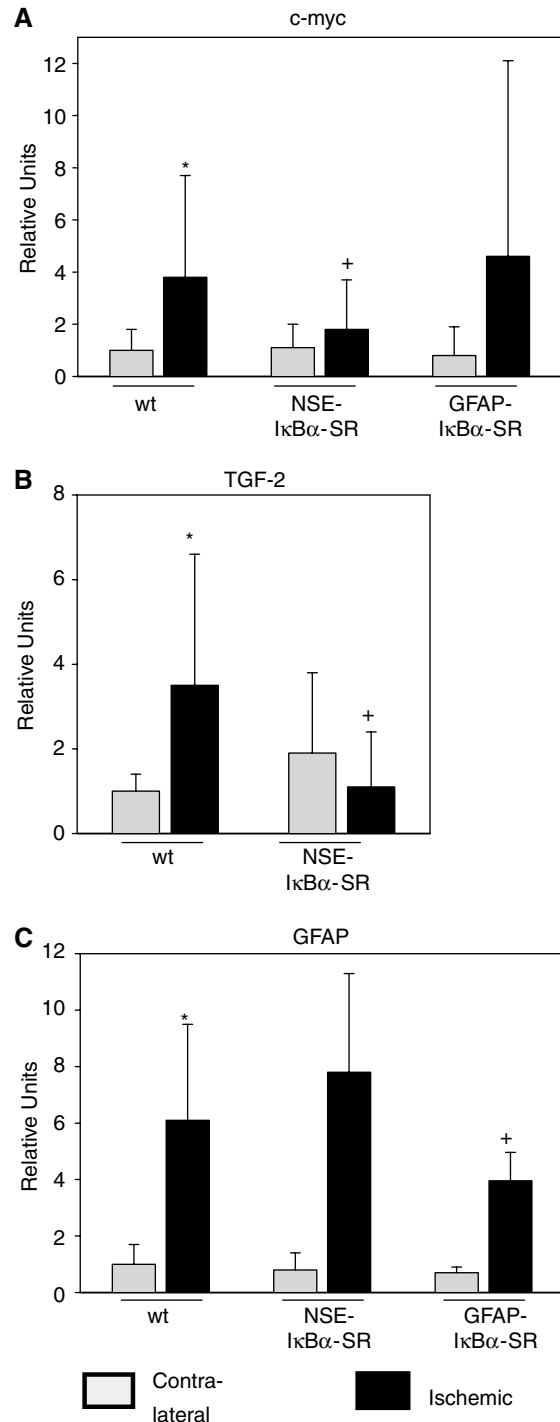
**Figure 4** Expression and functions of I $\kappa$ B $\alpha$ -SR in primary cortical neurons of NSE-I $\kappa$ B $\alpha$ -SR211 mice. **(A)** I $\kappa$ B $\alpha$ -SR is expressed in cortical neurons of NSE-I $\kappa$ B $\alpha$ -SR211 mice but not in the cells of wild-type (Wt) littermates as shown by immunoblotting with an anti-I $\kappa$ B $\alpha$  antibody. **(B)** Stimulation of NF- $\kappa$ B by 10 ng/mL TNF- $\alpha$  for 6 or 24 hours was partially inhibited in primary cortical neurons of NSE-I $\kappa$ B $\alpha$ -SR211 mice compared with cells of wt littermates. Cells were transfected with pNF- $\kappa$ B-Luc, in which five binding sites of NF- $\kappa$ B control the transcription of the luciferase gene. Values are means  $\pm$  s.d. of the luciferase activity expressed relative to the unstimulated control ( $n=5$  to 25).  $P<0.0001$  (one-way ANOVA). \* $P<0.0001$  compared with the unstimulated control. + $P<0.0001$  compared with the stimulation in wt cells (least-squares difference (LSD) *post hoc* test). **(C)** Induction of apoptosis by 10  $\mu$ mol/L camptothecin for 16 hours was partially inhibited in primary cortical neurons of NSE-I $\kappa$ B $\alpha$ -SR211 mice compared with wt littermates. Control subjects received the solvent dimethyl sulfoxide (DMSO). Cells were stained by DAPI and condensed apoptotic nuclei were counted.  $P<0.0001$  (one-way ANOVA). \*,  $P<0.0001$  compared with DMSO-treated wt cells. +,  $P=0.001$  compared with camptothecin-treated wt cells (LSD *post hoc* test). Values are means  $\pm$  s.d. ( $n=12$ ).

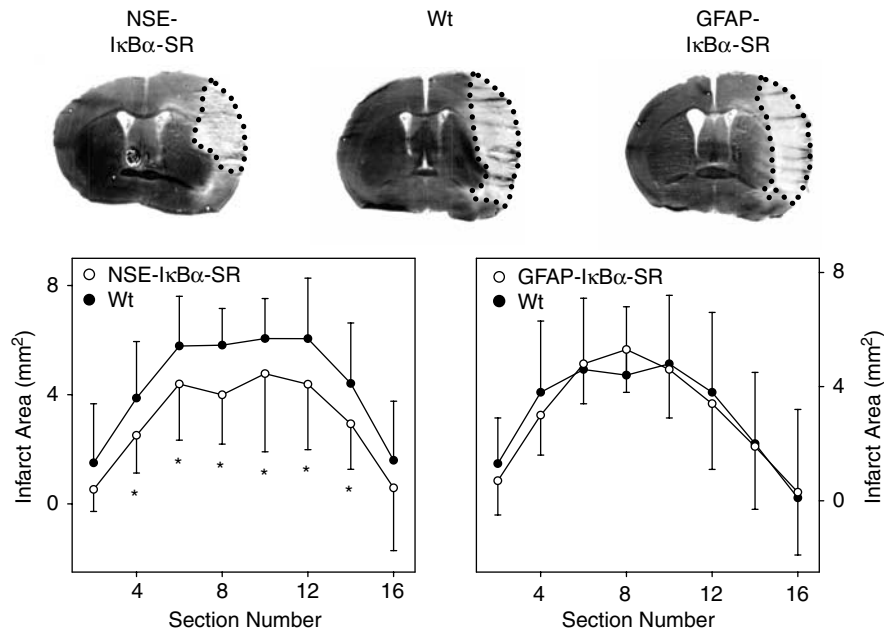
4.0  $\pm$  1.0-fold over controls ( $n=6$ ,  $P<0.0001$ ). Camptothecin exposure for 16 h also induced neuronal apoptosis that was quantified by counting cells with condensed nuclei. In prior experiments nuclear condensation corresponded closely to TUNEL staining. The percentage of apoptotic neurons was significantly smaller in primary cortical cultures expressing the transgene I $\kappa$ B $\alpha$ -SR (Figure 4C). Thus, in primary neurons of NSE-I $\kappa$ B $\alpha$ -SR mice, the transgene is expressed and inhibits NF- $\kappa$ B activation and apoptosis.

To evaluate the effect of the I $\kappa$ B $\alpha$ -SR transgene on NF- $\kappa$ B activation *in vivo*, we measured mRNA accumulation of specific NF- $\kappa$ B target genes in the cortex by real-time RT-PCR. The promoter of the *c-myc* gene contains two functional NF- $\kappa$ B sites and the transforming growth factor- $\beta$ 2 (TGF- $\beta$ 2) promoter contains a putative NF- $\kappa$ B site (Duyao *et al*, 1990; La Rosa *et al*, 1994; Malipiero *et al*, 1990). Both *c-myc* and TGF- $\beta$ 2 are known to be induced in neurons by cerebral ischemia (Ata *et al*, 1999; Huang *et al*, 2001). Middle cerebral artery occlusion for 24 hours induced a significant increase in the mRNA concentration of *c-myc* and TGF- $\beta$ 2 in wild-type mice (Figures 5A, B). However, in NSE-I $\kappa$ B $\alpha$ -SR mice, MCAO did not stimulate mRNA levels of *c-myc* and TGF- $\beta$ 2, supporting the notion that the NSE-I $\kappa$ B $\alpha$ -SR transgene inhibits the transcriptional activity of NF- $\kappa$ B in neurons after MCAO. Glial fibrillary acidic protein is a cell-specific marker for astrocytes and contains a promoter element that

binds the NF- $\kappa$ B subunit p50 (Chen and Swanson, 2003; Krohn *et al*, 1999). In MCAO, GFAP expression was upregulated in both wild-type and NSE-I $\kappa$ B $\alpha$ -SR mice, indicating astrocytic activation (Figure 5C). In GFAP-I $\kappa$ B $\alpha$ -SR mice, however, the induction of GFAP was significantly reduced (Figure 5C). Immunohistochemical study of GFAP after 48 hours of MCAO supported a reduced induction of GFAP in GFAP-I $\kappa$ B $\alpha$ -SR mice compared with wild-type littermates and NSE-I $\kappa$ B $\alpha$ -SR mice

**Figure 5** Cell-type-specific expression of I $\kappa$ B $\alpha$ -SR reduces the induction of NF- $\kappa$ B target genes by cerebral ischemia. mRNA accumulation was quantified by reverse transcription and real-time polymerase chain reaction (PCR) 24 hours after onset of middle cerebral artery occlusion (MCAO). **(A)** mRNA of *c-myc* was upregulated in the ischemic cortex of wild-type (wt) littermates compared with the contralateral side 24 hours after onset of permanent MCAO. Neuronal expression of I $\kappa$ B $\alpha$ -SR (NSE-I $\kappa$ B $\alpha$ -SR211 line) inhibited *c-myc* upregulation, while astrocytic expression of I $\kappa$ B $\alpha$ -SR (GFAP-I $\kappa$ B $\alpha$ -SR211 line) had no effect. Values are means  $\pm$  s.d. ( $n=8$  to 20) expressed relative to the mRNA level in the contralateral hemisphere of wt mice.  $P<0.05$  (one-way ANOVA).  $*P<0.05$  compared with the contralateral side (Tukey-HSD *post hoc* test).  $+P<0.05$  compared with the ischemic cortex in wt mice (LSD *post hoc* test; Tukey-HSD *post hoc* test not significant). **(B)** mRNA of TGF- $\beta$ 2 was upregulated in the ischemic cortex of wt littermates but not of NSE-I $\kappa$ B $\alpha$ -SR211 mice. Values are means  $\pm$  s.d. ( $n=8$ ).  $P<0.01$  (one-way ANOVA).  $*P<0.01$  compared with the contralateral side.  $+P<0.05$  compared with the ischemic cortex in wt mice (Tukey-HSD *post hoc* test). **(C)** mRNA of the astrocyte-specific marker gene glial fibrillary acidic protein (GFAP) was induced in the ischemic cortex of wt littermates and NSE-I $\kappa$ B $\alpha$ -SR211 mice while astrocytic expression of I $\kappa$ B $\alpha$ -SR (GFAP-I $\kappa$ B $\alpha$ -SR211 line) partially inhibited GFAP induction. Values are means  $\pm$  s.d. ( $n=8$  to 20).  $P<0.001$  (one-way ANOVA).  $*P<0.001$  compared with the contralateral side.  $+P<0.01$  compared with the ischemic cortex in NSE-I $\kappa$ B $\alpha$ -SR211 mice (Tukey-HSD *post hoc* test).





**Figure 6** Neuronal, but not astrocytic, expression of the NF- $\kappa$ B inhibitor I $\kappa$ B $\alpha$ -SR reduces the infarct size. After 48 hours of permanent middle cerebral artery occlusion (MCAO), infarct areas were determined on consecutive coronal brain sections using silver staining. Typical sections at the level of the anterior commissure are shown at the top. Infarct areas of NSE-I $\kappa$ B $\alpha$ -SR211 mice, GFAP-I $\kappa$ B $\alpha$ -SR192 mice, or the respective wild-type littermates (Wt) are plotted from rostral to caudal. Values are means  $\pm$  s.d. ( $n = 12$  to 15) of the average infarct area on two consecutive coronal brain sections. The anterior commissure is seen on section 10. \* $P < 0.05$  compared with wt (ANOVA, LSD *post hoc* test).

(data not shown). These data indicate a partial but significant inhibition of NF- $\kappa$ B activity in astrocytes and neurons in the respective transgenic line.

To investigate the consequences of cell type-specific inhibition of NF- $\kappa$ B on ischemic damage, we compared the infarct size in I $\kappa$ B $\alpha$  transgenic lines and wild-type littermates after 48-hour MCAO. In NSE-I $\kappa$ B $\alpha$ -SR211 mice, the infarct size was significantly smaller (26 %) than in controls (Figure 6, left panel;  $18.8 \pm 2.4 \text{ mm}^3$  ( $n = 15$ ) in NSE-I $\kappa$ B $\alpha$ -SR211 versus  $25.4 \pm 2.1 \text{ mm}^3$  ( $n = 12$ ) in controls;  $P < 0.05$ ). To exclude an insertional effect of the transgene, the experiment was repeated with another founder line (NSE-I $\kappa$ B $\alpha$ -SR241) expressing the same transgene. In this line, infarct size was also significantly smaller than in wild-type littermates (reduction by 43%,  $n = 10$ ;  $P < 0.05$ ). Physiologic parameters during the surgery did not differ between NSE-I $\kappa$ B $\alpha$ -SR mice and wild-type littermates. In addition, the reduction in cerebral blood flow between groups was nearly identical (Table 2). In contrast to NSE-I $\kappa$ B $\alpha$ -SR lines, the infarct size in two GFAP-I $\kappa$ B $\alpha$ -SR mouse lines did not differ from controls (GFAP-I $\kappa$ B $\alpha$ -SR192, Figure 6, right panel; GFAP-I $\kappa$ B $\alpha$ -SR211, data not shown). These data demonstrate that inhibition of NF- $\kappa$ B in neurons, but not in astrocytes, reduces the infarct size after permanent MCAO.

One possible explanation for the observed reduction in infarct size is protection of neurons against ischemic cell death. Staining with the fluorescent dye fluoro-jade B, which specifically marks dying neurons but does not distinguish between necrotic

and apoptotic neurons (Schmued and Hopkins, 2000), showed slightly reduced neuronal cell death in the outer zone of the infarct in NSE-I $\kappa$ B $\alpha$ -SR mice, but this did not reach statistical significance (data not shown). TUNEL staining is considered to be more specific for apoptotic cell death than fluoro-jade B. In accordance with this notion, only about 40% of fluoro-jade B-positive cells were TUNEL positive. The number of TUNEL-positive cells was significantly reduced in infarcts of NSE-I $\kappa$ B $\alpha$ -SR mice. In GFAP-I $\kappa$ B $\alpha$ -SR mice, there was a slight reduction in the number of TUNEL-positive cells that did not reach statistical significance (Figure 7,  $P = 0.075$ ). Previous work has shown that, after MCAO, most TUNEL-positive cells in the brain are neurons (Schneider, 1999). These data support the concept that NF- $\kappa$ B in neurons contributes to the induction of apoptotic neuronal cell death.

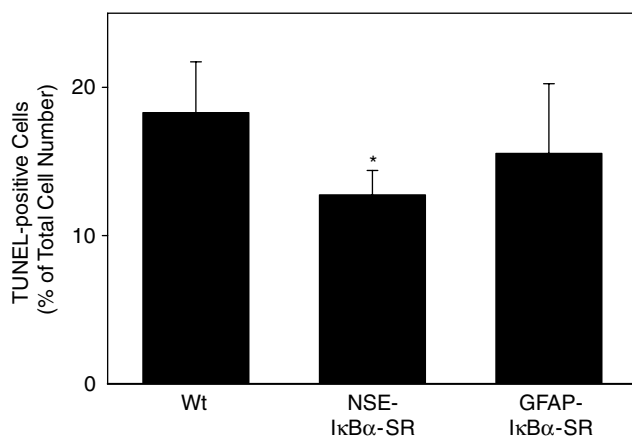
## Discussion

To investigate the contribution of the transcription factor NF- $\kappa$ B to brain pathology, we have generated mice that express the NF- $\kappa$ B superrepressor I $\kappa$ B $\alpha$ -SR under transcriptional control of an NSE and an astrocyte-specific promoter (GFAP). Mice with neuronal and astrocytic expression of the superrepressor have an apparently normal phenotype, although formal behavioral tests have not yet been performed. In addition, body weight was the same as in littermates. Transgene expression effectively

**Table 2** Physiologic parameters 15 mins before and 15 mins after MCAO in control and NSE-I $\kappa$ B $\alpha$ -SR241 mice

Parameter	Wild-type		NSE-I $\kappa$ B $\alpha$ 241-SR	
	Pre-MCAO	Post-MCAO	Pre-MCAO	Post-MCAO
MABP (mm Hg)	63.9 $\pm$ 6.7	44.6 $\pm$ 6.1	62.9 $\pm$ 16.1	43.3 $\pm$ 10.6
Heart rate (per minute)	377.8 $\pm$ 22.3	384.0 $\pm$ 45.8	380.8 $\pm$ 49.5	381.4 $\pm$ 53.2
Glucose (mg/dL)	202.8 $\pm$ 23.8	316.1 $\pm$ 70.4	240.9 $\pm$ 52.4	292.3 $\pm$ 103.3
Arterial pH	7.18 $\pm$ 0.04	7.10 $\pm$ 0.05	7.21 $\pm$ 0.05	7.16 $\pm$ 0.05
Arterial pCO <sub>2</sub> (mm Hg)	61.4 $\pm$ 7.2	61.5 $\pm$ 4.7	53.1 $\pm$ 8.6	58.1 $\pm$ 16.2
Arterial pO <sub>2</sub> (mm Hg)	80.8 $\pm$ 10.5	89.6 $\pm$ 10.5	85.0 $\pm$ 14.6	94.3 $\pm$ 23.0
Base excess	-8.2 $\pm$ 2.1	-11.5 $\pm$ 3.3	-9.8 $\pm$ 3.7	-12.0 $\pm$ 5.5
Hb (g/L)	14.0 $\pm$ 1.4	13.3 $\pm$ 1.6	13.9 $\pm$ 1.6	12.5 $\pm$ 1.6
Laser Doppler (relative units)	82.5 $\pm$ 30.7	14.1 $\pm$ 7.1	80.3 $\pm$ 34.7	14.5 $\pm$ 5.1
Body weight (g)	31.5 $\pm$ 0.8		32.1 $\pm$ 1.3	

None of the parameters differed significantly between wild-type littermates and NSE-I $\kappa$ B $\alpha$ -SR241 mice (*t*-test). Values are means $\pm$ s.d., *n* = 8. MABP, mean arterial blood pressure; Hb, hemoglobin concentration.



**Figure 7** Neuronal, but not astrocytic, expression of the NF- $\kappa$ B inhibitor I $\kappa$ B $\alpha$ -SR reduces ischemic cell death. TUNEL-positive cells were counted at the border of the infarct at the level of the anterior commissure and expressed as percent of DAPI-positive cells. Values are means $\pm$ s.d. (*n* = 5 for NSE-I $\kappa$ B $\alpha$ -SR and GFAP-I $\kappa$ B $\alpha$ -SR; *n* = 10 for wild-type, Wt). \**P* = 0.05 (ANOVA, Tukey-HSD *post hoc* test).

reduced the induction of neuron- or astrocyte-specific target genes of NF- $\kappa$ B after MCAO. However, only neuronal expression of I $\kappa$ B $\alpha$ -SR interfered with infarct volume after 48 hours of permanent MCAO. The neuroprotective effect of neuronal I $\kappa$ B $\alpha$ -SR was reproduced in another founder line. Our data provide definitive evidence for the detrimental role of NF- $\kappa$ B during the pathogenesis of stroke. Previous transgenic approaches could not clarify this point because deficiency of p65, the main transactivating subunit of NF- $\kappa$ B in the brain, causes embryonic death (Beg *et al*, 1995) and the use of p50 $^{-/-}$  mice is obscured by reports that p50 can both activate and repress NF- $\kappa$ B activity. Future work will have to investigate whether inhibition of NF- $\kappa$ B also leads to a long-term reduction of the infarct size.

Several investigators have found that NF- $\kappa$ B is activated in neurons after cerebral ischemia (Huang *et al*, 2001; Schneider *et al*, 1999; Stephenson *et al*, 2000). However, previous *in vitro* work of the role of NF- $\kappa$ B in neuronal cell death only provided ambiguous results in contrast to nonneuronal cell types. Several studies have found an antiapoptotic effect of NF- $\kappa$ B in neurons *in vitro* (reviewed in Mattson and Camandola, 2001). However, NF- $\kappa$ B can also promote neuronal cell death *in vitro* (de Erasquin *et al*, 2003; Pizzi *et al*, 2002; Qin *et al*, 1999). Indeed, cortical neurons expressing the NF- $\kappa$ B superrepressor were partially protected against camptothecin-induced apoptosis (Figure 4C). Using the same superrepressor of NF- $\kappa$ B, Fridmacher *et al* (2003) recently observed that inhibition of neuronal NF- $\kappa$ B reduced spontaneous cell death but increased FeSO<sub>4</sub>-induced cell death *in vitro*. Whether the effect is pro- or anti-apoptotic depends on the stimulus, the cell type, the activated subunits, and the duration of NF- $\kappa$ B activation (Kaltschmidt *et al*, 2002; Pizzi *et al*, 2002; Ryan *et al*, 2000). In the ischemic brain, multiple potential stimuli of NF- $\kappa$ B are released, which makes it difficult to predict the net effect on NF- $\kappa$ B activation from *in vitro* data. Our results show *in vivo* that neuronal activation of NF- $\kappa$ B in cerebral ischemia contributes to ischemic brain damage. Possibly, the neuroprotective effect of the super-repressor is due to the moderate expression level in our mouse lines because there is evidence that a critical NF- $\kappa$ B dosage is required for cell survival and either too much or too little activation is detrimental (Goudeau *et al*, 2003).

Numerous target genes of NF- $\kappa$ B are upregulated in cerebral ischemia. Candidates, which may mediate the toxic effect of NF- $\kappa$ B in neurons, are c-myc and TGF- $\beta$ 2 (Ata *et al*, 1999; Huang *et al*, 2001). c-myc functions as a proapoptotic regulator in cells of various types and under a variety of conditions (Packham and Cleveland, 1995). It has been linked to N-methyl-D-aspartate (NMDA) receptor- and

NF- $\kappa$ B-mediated apoptosis in neurons (Qin *et al*, 1999). Administration of TGF- $\beta$ 1 has been reported to protect against focal cerebral ischemia (Prehn *et al*, 1993). However, endogenous TGF- $\beta$  is required *in vivo* for apoptosis of axotomized motoneurons as shown by the application of neutralizing antibodies (Kriegstein *et al*, 2000).

In GFAP-I $\kappa$ B $\alpha$ -SR mice, expression of the transgene I $\kappa$ B $\alpha$ -SR in astrocytes apparently inhibited NF- $\kappa$ B activity because the upregulation of GFAP was reduced (Figure 3). Glial fibrillary acidic protein is a marker of astroglia activation and a target gene of NF- $\kappa$ B (Krohn *et al*, 1999), implying a role of NF- $\kappa$ B in glial activation *in vivo*. However, inhibition of astrocytic NF- $\kappa$ B had no effect on infarct size. A neuroprotective function of GFAP has been reported in cerebral ischemia (Nawashiro *et al*, 2000). Nevertheless, the moderate inhibition of GFAP induction in our mice had no detrimental effect or was overridden by the effect on other target genes. Regulation of the endogenous GFAP gene suggests that I $\kappa$ B $\alpha$ -SR, which is directed by the human GFAP promoter, also exerts negative feedback on its own expression but is still upregulated in ischemic cortex compared to the nonischemic side.

Pharmacological studies support our concept of the neurodegenerative function of NF- $\kappa$ B in cerebral ischemia although the inherent unspecificity of drugs only provides correlative evidence. The antioxidant LY341122 (Stephenson *et al*, 2000), the salicylate triflusal (Acarin *et al*, 2001), the proteasome inhibitor MLN519 (Williams *et al*, 2003), the ubiquitin ligase inhibitor pyrrolidine dithiocarbamate (Hayakawa *et al*, 2003; Nurmi *et al*, 2004), and the cannabinoid dexamabinol (Jüttler *et al*, 2004) all inhibit NF- $\kappa$ B activation and exert neuroprotective effects in cerebral ischemia. The most specific step in the NF- $\kappa$ B signaling is the activation of the IKK complex (Li and Verma, 2002). Because the mutation of IKK phosphorylation sites in I $\kappa$ B $\alpha$  prevents degradation of I $\kappa$ B $\alpha$  and inhibits NF- $\kappa$ B, our results from NSE-I $\kappa$ B $\alpha$ -SR transgenic mice suggest that activation of NF- $\kappa$ B through IKK is a critical step in cerebral ischemia. Pharmacological IKK inhibitors might, therefore, represent a specific and effective treatment of stroke.

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