Cellular/Molecular

Activation of Nuclear Factor-κB via Endogenous Tumor Necrosis Factor α Regulates Survival of Axotomized Adult Sensory Neurons

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Embryonic dorsal root ganglion (DRG) neurons die after axonal damage *in vivo*, and cultured embryonic DRG neurons require exogenous neurotrophic factors that activate the neuroprotective transcription factor nuclear factor-κB (NF-κB) for survival. In contrast, adult DRG neurons survive permanent axotomy *in vivo* and in defined culture media devoid of exogenous neurotrophic factors *in vitro*. Peripheral axotomy in adult rats induces local accumulation of the cytokine tumor necrosis factor α (TNFα), a potent activator of NF-κB activity. We tested the hypothesis that activation of NF-κB stimulated by endogenous TNFα was required for survival of axotomized adult sensory neurons. Peripheral axotomy of lumbar DRG neurons by sciatic nerve crush induced a very rapid (within 2 h) and significant elevation in NF-κB-binding activity. This phenomenon was mimicked in cultured neurons in which there was substantial NF-κB nuclear translocation and a significant rise in NF-κB DNA-binding activity after plating. Inhibitors of NF-κB (SN50 or NF-κB decoy DNA) resulted in necrotic cell death of medium to large neurons (≥40 μm) within 24 h (60 and 75%, respectively), whereas inhibition of p38 and mitogen-activated protein/extracellular signal-regulated kinase did not effect survival. ELISA revealed that these cultures contained TNFα, and exposure to an anti-TNFα antibody inhibited NF-κB DNA-binding activity by ~35% and killed ~40% of medium to large neurons within 24 h. The results show for the first time that cytokine-mediated activation of NF-κB is a component of the signaling pathway responsible for maintenance of adult sensory neuron survival after axon damage.

Key words: **denervation; dorsal root ganglion (DRG); necrosis; nerve; neuropathy; neurotrophic; sciatic; sensory neurons; transcription; trophic**

Introduction

The vast majority of neurons are bound by the neurotrophic factor theory, which states that neuronal survival during development depends on trophic substances provided by the targets of innervation. In general, this critical dependence continues throughout life, having profound implications in cases of both peripheral and central neuropathy. Adult sensory neurons are notable exceptions to this rule, being able to survive indefinitely after axotomy and loss of target innervation. In addition, these cells uniquely survive in defined media in the absence of exogenous neurotrophic factors (Lindsay, 1988). In contrast, embryonic sensory neurons require target-derived neurotrophins signaling through trk receptors (Davies, 2003), and activation of the anti-apoptotic transcription factor nuclear factor-κB (NF-κB) (Middleton et al., 2000).

Axotomy of adult peripheral neurons stimulates synthesis of cytokines such as interleukin-6 (IL-6) (Murphy et al., 1995) and leukemia inhibitory factor (Banner and Patterson, 1994). The potent proinflammatory cytokine tumor necrosis factor α (TNFα) is also detected in damaged nerve and corresponding lumbar dorsal root ganglia (DRGs) (Shubayev and Myers, 2001, 2002; Schafers et al., 2002, 2003). Although the cytokine source in lumbar DRGs is unknown, Schwann cells comprising the nerve sheaths are thought to be the source of TNFα in the sciatic nerve after damage. Consequently, after nerve damage, sensory neurons within the DRG are thought to be exposed to an inflammatory-like environment (Lu and Richardson, 1991).

Cytokine-mediated inflammatory events are associated with activation of NF-κB. Prototypical NF-κB is retained in the cytoplasm by the inhibitory protein IκBα (Baldwin, 1996), which after appropriate stimulation is phosphorylated by IκB kinase leading to targeted proteolysis of IκB, allowing the active form of NF-κB to cross the nuclear membrane and initiate targeted transcription. Activation of NF-κB represents a highly protective response in cultured brain neurons (Barger et al., 1995; Mattson et al., 1997; Taglialatela et al., 1997). NF-κB protects cells by inducing the expression of genes that promote cell survival such as

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those encoding for anti-oxidant (manganese superoxide dismutase) (Mattson et al., 1997), calcium stabilizing (calbindin D28K) (Cheng et al., 1994), and anti-apoptotic proteins (bcl-2, bcl-XL) (Tamatani et al., 1999). NF-κB is activated in neurons by a number of proinflammatory cytokines, including TNFα (Barger et al., 1995), IL-1α (Nonaka and Huang, 1990), and ciliary neurotrophic factor (CNTF) (Middleton et al., 2000). Harmful stimuli, such as elevated levels of intracellular calcium, oxidation, and DNA damage, also elevate NF-κB-binding activity (Baldwin, 1996; Karin and Lin, 2002).

The ability of NF-κB to protect neurons against harmful stimuli leads to the hypothesis that it may play an important role in adult sensory neuronal survival after loss of trophic support. The recent localization of TNFα in adult DRGs after nerve damage, combined with the potent NF-κB-stimulating activity this cytokine exhibits, makes TNFα an attractive candidate for activation of NF-κB in axotomized DRG. The aim of the current study was to determine the role of the NF-κB/TNFα pathway in maintaining the survival of adult sensory neurons after loss of target-derived neurotrophic support.

Materials and Methods

Sciatic nerve crush. Adult male Wistar rats (250 g) underwent unilateral sciatic nerve crush at mid level while under isoflurane-induced anesthesia. Nerve crush was induced by the use of fine forceps, with firm pressure being applied for 30 s twice. The contralateral flank was also opened, but the nerve was not crushed. Animals were allowed to recover, and then at various times, the animals were killed and the DRGs were removed rapidly (within 5 min) and snap-frozen on dry ice. All experimental procedures on animals were performed according to strict United Kingdom Home Office guidelines and standards set forth by the Canadian Institutes of Health Research and the University of Manitoba.

Sensory neuron cultures and treatments. DRGs from adult male Wistar rats (250 g) were dissociated using a previously described method (Lindsay, 1988). During dissociation of cells, Neurobasal medium was used with 10% heat-inactivated bovine serum or with N2 additives. The cells were plated onto polyornithine–laminin-coated Costar dishes and cultured in Neurobasal media with serum and incubated overnight at 37°C in a 95% air/5% CO_2 humidified incubator. The medium was changed the following day to serum-free Ham's F-12 medium with modified N2 additives [0.1 mg/ml transferrin, 20 nM progesterone, 0.01 mM cytosine arabinoside, 100 μM putrescine, 30 nM sodium selenite, and 1 mg/ml bovine serum albumin (BSA); all additives were from Sigma (Poole, UK), and culture medium was from Life Technologies (Paisley, UK)].

Double-stranded DNA with an NF-κB consensus sequence (κB DNA) was prepared by annealing the complementary single-stranded oligonucleotides with the sequences 5′-GAGGGGACTTTCCCT-3′ and 5′-AGGGAAAGTCCCCTC-3′. Double-stranded DNA with "scrambled" sequences 5′-CTTTGAGGCCCTGGA-3′ and 5′-TCCAGGGCCTCAAAG-3′ was prepared in a similar manner. Decoy DNA, scrambled control DNA, and IκB antisense oligonucleotide (Sigma Genosys, Oakville, Ontario, Canada) with the sequence 5′-GCGCTCGGCCGCCTGGAACATGGC-3′ were prepared in culture media as 100× stock immediately before use. Anti-TNFα antibody (200 μg/ml; Santa Cruz Biotechnology, Santa Cruz, CA) was added to cultures at 1:300 concentration.

Electrophoretic mobility shift assay. Extracted lumbar DRGs from adult rats were homogenized on ice with small volumes of ice-cold Totex buffer [20 mM HEPES, pH 7.9, 350 mM NaCl, 20% glycerol, 1% igepal, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.1 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 5 μg/ml aprotinin, and 50 μM dithiothreitol (DTT)]. Cell extracts from cultures were obtained by scraping with Totex buffer, followed by cell lysis on ice for 30 min, centrifugation at 14,000 rpm for 15 min at 4°C, and retention of the supernatant. Protein levels were determined by the Bradford method (Bio-Rad, Hercules, CA), and samples were stored at −80°C. Equal amounts of protein were incubated in a 20 μl reaction mixture containing 20 μg of BSA; 1 μg of poly(dI-dC); 2 μl of buffer containing 20% glycerol, 100 mM KCl, 0.5 mM EDTA, 0.25% Nonidet P-40, 2 mM DTT, 0.1% PMSF, and 20 mM HEPES, pH 7.9; 4 μl of buffer containing 20% Ficoll 400, 300 mM KCl, 10 mM DTT, 0.1% PMSF, and 100 mM HEPES, pH 7.9; and 20,000–50,000 cpm of ^{32}P-labeled oligonucleotide (S) corresponding to an NF-κB-binding site (5′-AGTTGAGGGGACTTTCCCAGGC-3′). After 20 min at room temperature, reaction products were separated on a 12% nondenaturing polyacrylamide gel. Radioactivity of dried gels was detected by exposure to Kodak X-Omat film, and images on the developed film were scanned into a computer using a UMAX 1200s scanner. Densitometry was performed using Image software (Scion Corp., Frederick, MD). Paint Shop Pro software (Jasc, Minneapolis, MN) was used for preparation of the final figures.

Quantification of neuronal survival. Neuronal survival was quantified by established methods (Mattson et al., 1995). In brief, viable neurons in premarked fields (20× objective) were counted before experimental treatment and at time points after treatment. Neurons that died in the intervals between examination points were usually absent, and the viability of the remaining neurons was assessed by morphological criteria. Neurons with membranes and soma with a smooth round appearance were considered viable, whereas neurons with fragmented or distended membranes and vacuolated soma were considered nonviable. Neurons were counted in four random 20× fields per culture, and the percentage of surviving neurons per culture was calculated. Survival was confirmed by trypan blue exclusion at the last time point.

Neuronal cell diameter measurement. Neurons were cultured for 2 d, then photographed with a microscale and treated with trypan blue, and the neuronal diameter of viable neurons (~90% survival after 2 d) was determined.

Oxidative stress measurement. After experimental treatment, culture media were changed to Locke's buffer. A concentration of 5 μM dihydrorhodamine 123 (DHR123) (Sigma), a dye that enters the mitochondria and fluoresces when bound to hydrogen peroxide or peroxynitrite, was added, followed by a 30 min incubation at 37°C and 95% air/5% CO_2. Coverslips were washed three times with Locke's buffer and attached with dental adhesive to 35 mm Nunc plates containing a 10 mm hole. Locke's buffer was added to the dish, and images were obtained with a Zeiss (Oberkochen, Germany) LSM510 confocal laser-scanning microscope (excitation, 488 nm; emission, 510 nm). The average pixel density was determined using Image software (Scion Corp.).

Caspase-3 activation assay. After experimental treatment, media in each well were changed to Ham's F-12 media containing 0.001% digitonin (Sigma). After a 5 min incubation at 37°C, the media were removed, and 10 μg/ml DEVD-Biotin (Sigma) in F-12 was added to each well for another 20 min at 37°C. Coverslips were washed three times with 2 ml of PBS. Cells were then fixed in cold 4% paraformaldehyde for 10 min. Streptavidin–FITC (5 μg/ml) was then added and incubated for 30 min at room temperature. After two more washes, coverslips were mounted on glass slides with Fluorsafe mounting media, left to dry overnight in darkness, and imaged the next day. Samples were stored at −20°C. The average pixel density was determined using Image software (Scion Corp.).

TNFα ELISA. After 24 h in cell culture, the dishes were placed on ice, and supernatant and cells were collected. Supernatant (3 ml) was collected from each plate and centrifuged at 400 rpm for 10 min to pellet debris and floating cells. The resulting supernatant was frozen at −80°C. Samples were freeze-dried and resuspended in 250 μl of ELISA homogenization buffer (0.5 M NaCl, 2.5 mM $NaH_2PO_4.H_2O$, 7.5 mM Na_2HPO_4, 0.1% Tween 20, 0.75 mM PMSF, 0.75 mM EDTA, and 3.75% protease inhibitor mixture, pH 7.2). For attached neurons, the cells were lysed and sonicated on ice for 10 s. Sciatic nerve, spinal cord, brain, liver, and lumbar DRG from adult rats were dissected, snap-frozen, and each homogenized in 700 μl of ELISA homogenization buffer using a Polytron. Samples were then assayed for TNFα using an in-house-developed sandwich ELISA. Detection was performed using a biotinylated anti-TNFα antibody coupled with avidin HRP (1:5000 dilution). This assay was sensitive to 10 pg/ml TNFα.

Western blotting for NF-κB subunits. Lumbar DRG, lumbar dorsal root, lumbar ventral root, sciatic nerve, and brain from adult rats were homogenized using a Polytron in a buffer containing 50 mM Tris-HCl,

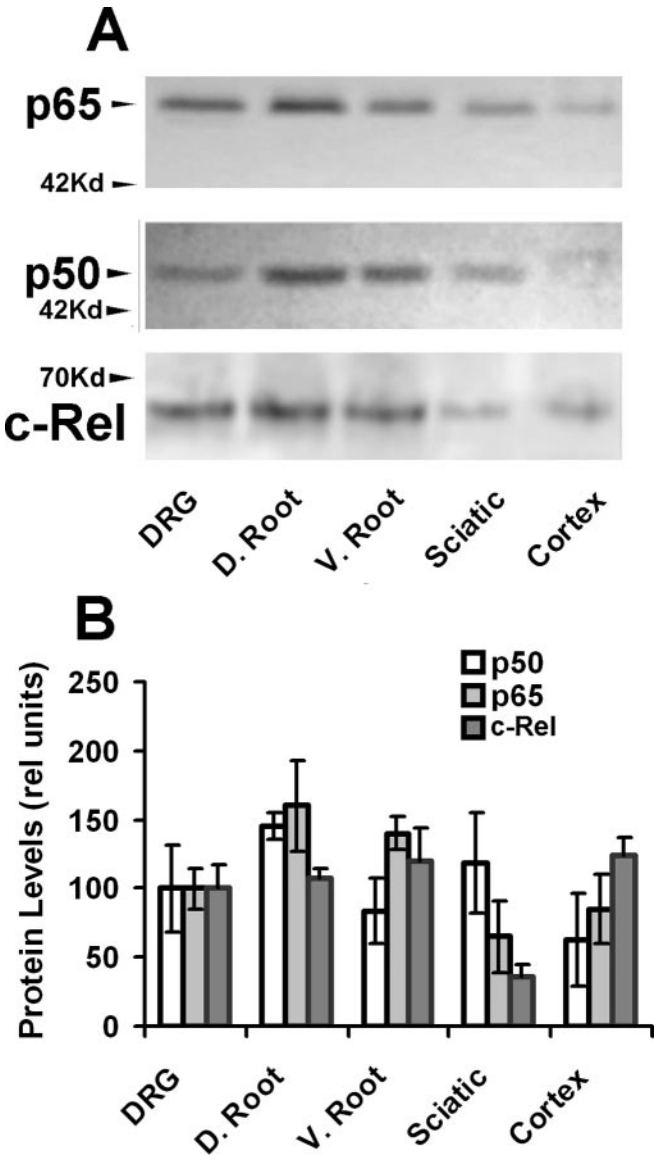


Figure 1. NF-κB subunit protein levels in neuronal tissues. *A*, Immunoblot of p65, p50, and c-Rel subunits of NF-κB in adult rat tissue. *B*, Densitometry of p50, p65, and c-Rel. rel units, Relative units. D. Root, Dorsal root; V. Root, ventral root; Sciatic, sciatic nerve segment (1 cm) at mid-thigh; Cortex, cerebral cortical tissue. All data are mean ± SE; $n = 4$ animals.

pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM PMSF, 1 mM aprotinin, 1 mM leupeptin, 1 mM pepstatin, 1 mM Na_3VO_4, and 1 mM NaF. Samples of 5 μg of total protein were subjected to SDS-PAGE and then transferred to nitrocellulose, which was then incubated with primary polyclonal antibodies to p65, p50, or c-Rel (1:1000, 1:100, and 1;100, respectively; Santa Cruz Biotechnology) in 3% milk–PBS overnight at 4°C, followed by incubation of a secondary antibody (HRP-conjugated anti-rabbit secondary antibody, 1:2500; Cell Signaling) at room temperature for 1 h. Enhanced chemiluminescence (LumiGlu; Cell Signaling) was used to detect the signal from the blot.

Immunofluorescence for NF-κB subunits. Neurons were fixed with 2% paraformaldehyde for 15 min at room temperature. Coverslips were washed three times for 5 min each with PBS and then immersed in ice-cold methanol for 3 min to perforate the membranes. Coverslips were then immersed in PBS plus 0.2% Triton X-100 for 10 min, washed, and then blocked with 10% donkey serum in PBS plus 0.2% Triton X-100 and incubated with primary antibody overnight. The primary antibodies used were anti-p50 (1:500), anti-p65 (1:200), and anti-c-Rel (1:200). All primary antibodies were anti-rabbit and purchased from Santa Cruz

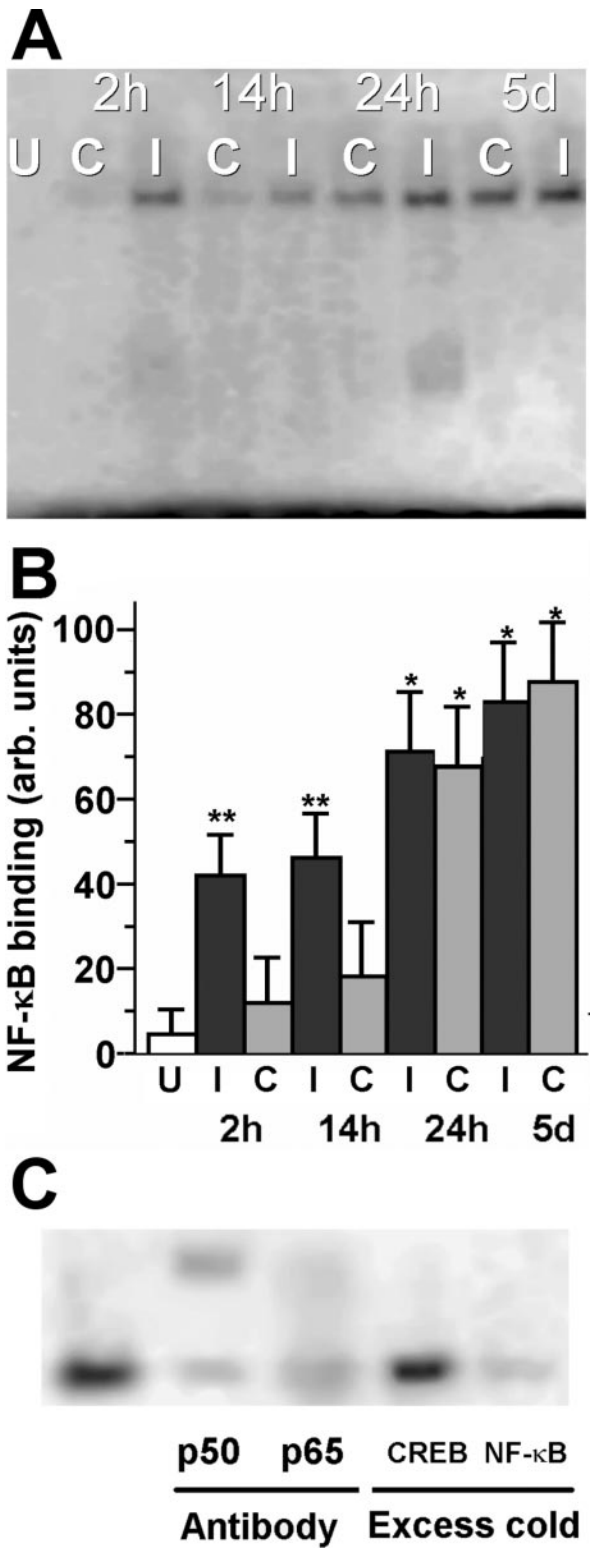
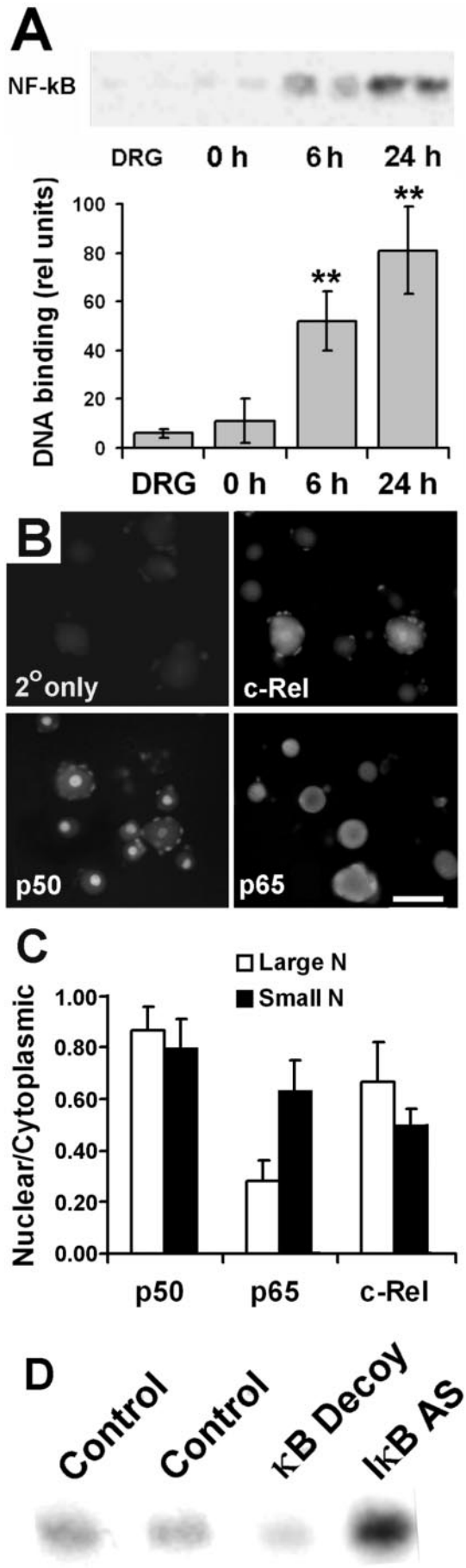


Figure 2. NF-κB binding in DRG after peripheral nerve crush. *A*, EMSA analysis of NF-κB binding in DRGs at the indicated times after sciatic nerve crush (2 h to 5 d). *B*, Densitometric analysis of EMSA. All data are mean ± SE; $n = 3$; $^{*}p < 0.01$ versus untreated; $^{**}p < 0.01$ versus both untreated and right-side sham-operated DRG; $n = 4$ separate animals. U, Untreated; I, ipsilateral to the sciatic nerve crush; C, contralateral control in which a sham operation was performed consisting of exposing, but not damaging, sciatic nerves; arb. units, arbitrary units. *C*, Supershift analysis of NF-κB in DRG after nerve crush. Lumbar DRGs were removed 4 h after sciatic nerve crush. Band mobility was visualized in samples treated with antibodies to p50 or p65. An excess cold probe for cAMP response element-binding protein (CREB) and NF-κB was added to control for probe specificity.



Biotechnology. The secondary antibody was FITC-conjugated donkey anti-rabbit (1:400 dilution; Stratech, Luton, UK). Coverslips were washed and incubated with a secondary antibody for 1 h in the dark. Coverslips were mounted onto slides using Vectorshield antifade mounting medium with 4′,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA). Cells were viewed by light microscopy (Leica DMR microscope), and exposures were produced (Hamamatsu digital camera).

Results

NF-κB subunits are abundant in DRG

Activated neuronal NF-κB is a dimer formed generally from the subunit p50, p65, or c-Rel. Western blot analysis of adult rat tissues determined that protein levels of p50, p65, and c-Rel were present in peripheral nervous tissues in amounts comparable with or greater than that in the cerebral cortex (Fig. 1*A*,*B*).

NF-κB binding increases in DRG after nerve crush

Although of primary importance to cultured embryonic sensory neuron survival (Hamanoue et al., 1999; Middleton et al., 2000), the pattern of NF-κB-binding activity in adult lumbar DRG immediately (within hours) after axotomy has not been reported. Analysis by electrophoretic mobility shift assay (EMSA) of NF-κB DNA binding in lumbar DRG revealed a single band with little nonspecific binding (Fig. 2*A*). Levels of NF-κB binding in lumbar DRGs from unoperated control animals were very low (Fig. 2*A*, top), a notable observation given the abundance of NF-κB subunit protein found in this tissue using Western blotting (Fig. 1). After sciatic nerve crush and resulting axotomy of lumbar DRG neurons, levels of NF-κB binding significantly increased within 2 h in the ipsilateral DRG relative to either untreated or contralateral DRGs (Fig. 2*B*). Levels of NF-κB binding peaked 24 h after surgery, at which point binding levels were ~20-fold greater than untreated. NF-κB binding in the contralateral DRG was significantly lower than the ipsilateral side at 2 and 14 h but increased over time, and by 24 h, contralateral and ipsilateral levels of NF-κB binding were equivalent, and both remained elevated 5 d after surgery. Supershift assays on DRG (Fig. 2*C*), performed using antibodies to p50 and p65, and c-Rel subunits of NF-κB showed a decrease in band intensity with p65 antibody and a shift of almost the entire band with p50, confirming the identity of the band as NF-κB.

NF-κB binding increases in dissociated DRG neurons

Because dissection and dissociation mimic to some degree axotomy, we determined NF-κB-binding levels in the DRG neuron sample both before plating and at various times after culture.

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Figure 3. NF-κB binding in cultured DRG neurons. *A*, EMSA analysis of NF-κB-binding activity at various points after DRG dissection and cell culturing. DRG, whole DRGs processed immediately after dissection from animal; 0 h, DRG neurons after dissociation but before plating; 6 and 24 h, DRG neurons 6 and 24 h after culture. The top panel shows an EMSA blot, whereas the graph describes the data statistically. Data are mean ± SE; $n = 3$; $^{**}p < 0.01$ versus DRG control. rel units, Relative units. *B*, Immunofluorescence staining of DRG cultures for NF-κB subunits. Cultures were grown for 5 h and then fixed and immunostained for the NF-κB subunits p50, p65, or c-Rel and visualized with FITC. Scale bar, 50 μm. *C*, Quantitation of immunocytochemistry. FITC intensity was measured both in the nucleus and in a portion of the cytoplasm. Data are presented as the average nuclear intensity divided by the average cytoplasmic intensity of signal (ratio, 0.0–1.0). Medium to large N, Neurons ≥40 μm; Small N, neurons <40 μm. All data are mean ± SE; $n = 18$–24. *D*, Decoy DNA and IκB antisense exposure regulates NF-κB DNA-binding activity. Sensory neurons were cultured and immediately treated with either 10 μM NF-κB double-stranded decoy DNA or 20 μM IκB antisense DNA. After 12 h, gel-shift assay was performed to determine NF-κB DNA-binding activity.

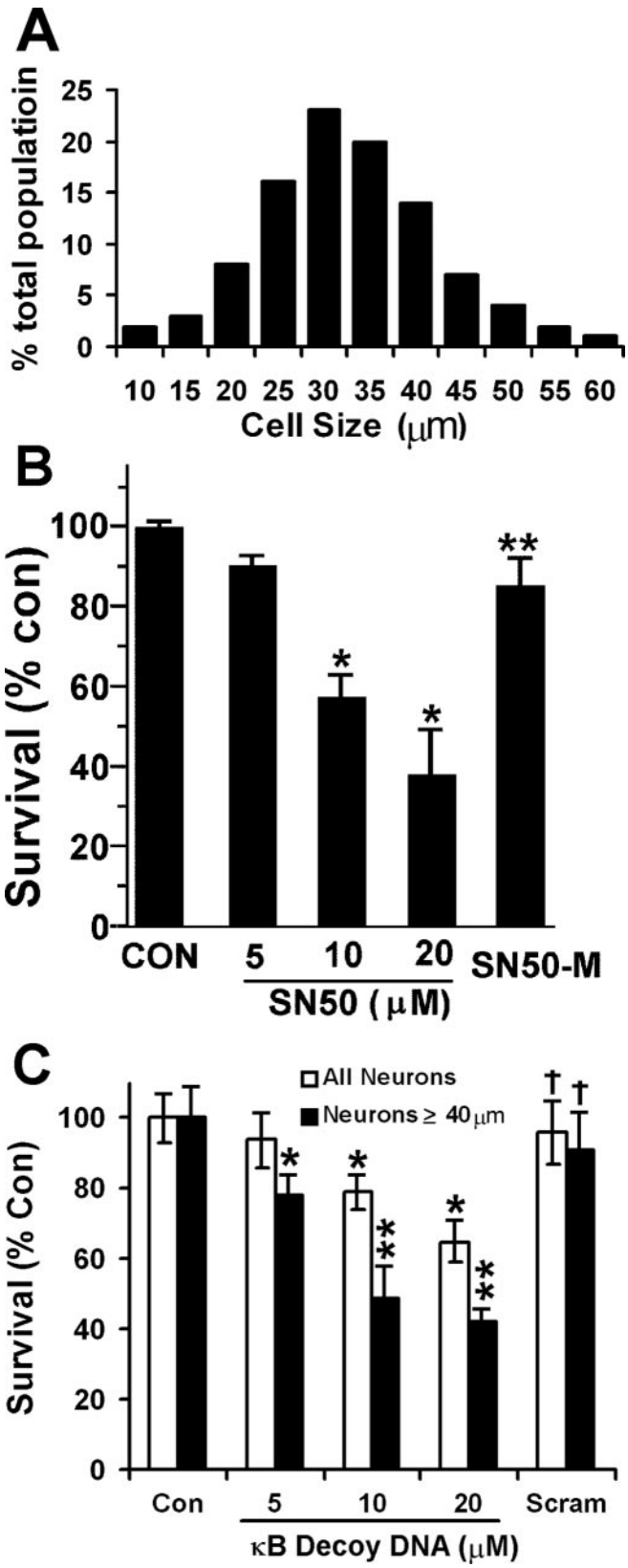

Figure 4. Inhibition of NF-κB causes neuronal death, increasing with cell size. *A*, Cells were cultured for 2 d, treated with trypan blue, and then photographed in the presence of a microscale bar. Approximately 90% of neurons were viable, and distribution of living neurons by cell diameter was determined. *B*, *C*, DRG cells were cultured for 2 d, photographed, and then exposed to various inhibitors of NF-κB. The original cell diameter was determined from the size of the cell before treatment. Neuronal survival was determined morphologically and confirmed at the latest time point by trypan blue staining. *B*, Cells were exposed to the peptide inhibitor SN50 (5, 10, and 20 μM) or the control (CON) peptide SN50-M (20 μM) for 12 h, and survival was determined in medium to large neurons (≥40 μm in diameter). Data are mean ± SE; $n = 4$; $^{*}p < 0.01$ versus control; $^{**}p < 0.01$ versus 20 μM SN50. *C*, DRG cultures were treated with NF-κB decoy (5, 10, or 20 μM), and survival was determined in all neurons (□) and in neurons ≥40 μm (■) 12 h after treatment. As a control (Con), cells were treated with a double-stranded DNA composed of a scrambled sequence of the NF-κB decoy DNA (Scram). Data are mean ± SE; $n = 4$; $^{*}p < 0.05$ versus control; $^{**}p < 0.01$ versus control; $^{\dagger}p < 0.05$ versus 20 μM NF-κB decoy DNA.

EMSA analysis of NF-κB DNA-binding activity revealed a single band (Fig. 3*A*, top), similar to that observed in lumbar DRGs *in vivo*. NF-κB-binding levels were increased significantly within 6 h of plating and by 24 h rose to a peak of ~20-fold greater than freshly dissected DRG (Fig. 3*A*, bottom), similar to the increase seen *in vivo* after axotomy.

To further characterize this phenomenon, neurons were stained with anti-p50, anti-p65, or anti-c-Rel antibody for immunofluorescent localization 5 h after plating. Strong p50 and c-Rel staining was seen in the cytoplasm and nuclei of all neurons (Fig. 3*B*), whereas faint nuclear staining for p65 was detected in all neurons (Fig. 3*B*). Quantitation of the intensity of signal in nucleus relative to cytoplasm reveals a differential pattern of NF-κB nuclear localization between cell types. Immunofluorescent p50 was primarily nuclear in all cell types, whereas immunofluorescent p65 was localized to nuclei in small (<40 μm) neurons but was almost absent in the nuclei of large (≥40 μm) neurons. Immunofluorescent c-Rel was localized to nuclei of satellite cells, present in cytoplasm but more abundant in nuclei of large neurons, but very faint in both cytoplasm and nuclei of small neurons. This demonstrates that large and small neurons mobilize different NF-κB subunits to the nucleus in response to culture conditions.

The gel-shift assay shown in Figure 3*D* demonstrates the effectiveness of both NF-κB decoy in reducing and IκB antisense in increasing NF-κB-binding activity in cultured sensory neurons. Cells were cultured and treated immediately with either 20 μM NF-κB decoy DNA or 20 μM IκB antisense. Twelve hours later, the gel-shift assay demonstrates an approximate twofold decrease in NF-κB-binding activity after decoy DNA treatment compared with control, or a twofold increase relative to control after IκB antisense treatment. Note, however, that any decoy DNA that may still be extant in the cells after 12 h might interfere directly with the gel-shift assay.

Inhibition of NF-κB, but not mitogen-activated protein kinases, causes neuronal death

To determine whether this rapid rise of NF-κB-binding activity in cultured cells played a role in survival, neurons were treated with an NF-κB inhibitor, SN50, a peptide that contains the nuclear translocation signal of NF-κB and thus may block movement of the active complex into the nucleus. To partially characterize the cell culture system, a detailed size analysis of cultured cells was performed. This revealed a highly heterogeneous neuronal population varying in size from 10 μm to >60 μm (Fig. 4*A*). Approximately 30% of neurons were >40 μm, and preliminary observations indicated that these were particularly vulnerable to NF-κB inhibition. Treatment with SN50 killed neurons in a dose–response manner (Fig. 4*B*), with 20 μM causing ~60% death in medium to large neurons, whereas a scrambled form of the peptide had no effect on survival. Confirming these results, inhibition of NF-κB DNA-binding activity by double-stranded

Table 1. Effect of inhibition of MEK and p38 on sensory neuron survival

Treatment	Percentage of survival (mean ± SE)
Control	100 ± 7.81
10 μM U0126	93.1 ± 5.18
10 μM decoy DNA	61.6 ± 7.37*
10 μM U0126 plus 10 μM decoy DNA	58.1 ± 3.68*
2 μM SB203580	89.3 ± 7.04
2 μM SB203580 plus 10 μM decoy DNA	69.9 ± 6.4**

Cultured adult sensory neurons were treated with inhibitors of NF-κB, MEK, and p38 for 24 h, and neuronal survival was assessed by trypan blue exclusion. Cells were treated with 10 μM decoy, 10 μM U0126 (MEK inhibitor), or 2 μM SB203580 (p38 inhibitor). Survival was determined 24 h later. Data are means ± SE, $n = 3$; *$p < 0.01$, **$p < 0.05$ versus control.

NF-κB decoy DNA caused the death of ~55% of medium- to large-sized neurons within 12 h of treatment (Fig. 4*C*), whereas a scrambled form of double-stranded DNA had no effect on survival. These NF-κB inhibitors, which work through different mechanisms, both killed DRG neurons in a dose-dependent manner, whereas control substances had no effect on survival.

Activation of the mitogen-activated protein (MAP) kinase, extracellular-regulated kinase (ERK), and phosphoinositide 3-kinase (PI 3-K) is an important survival response to growth factors and cytokines. Inhibition of these kinases induces death in both central and peripheral embryonic neurons, whereas the MAP kinase p38 may be induced after loss of trophic support (Xia et al., 1995; Miller and Kaplan, 2001; Davies, 2003). However, in contrast with the dramatic effects of NF-κB inhibition, MAP/ERK kinase (MEK) and p38 inhibition had no effect on survival of cultured adult DRG neurons of all sizes (Table 1). Treatment of 24 h cultured neurons with U0126, a MEK inhibitor, or SB203580, an inhibitor of p38, did not kill cultured adult sensory neurons, nor did either treatment significantly change the killing efficacy of NF-κB decoy DNA. In addition, the PI 3-K inhibitor LY294002 (up to 10 μM) also failed to kill these neurons and confirmed a previous study (Dodge et al., 2002).

Inhibition of NF-κB leads to oxidative stress and necrotic cell death

An observation of cell death over time indicated that significant death of medium to large neurons begins at ~4 h after treatment (data not shown); thus, early markers of neuronal morbidity were examined. Within 2 h of NF-κB inhibition by SN50, there was a significant increase in mitochondrial oxidative stress, as measured by DHR fluorescence (Fig. 5*A*, confocal image and statistical analysis), indicating rapidly increased oxidative stress in mitochondria after NF-κB inhibition. The rapidity of death and evident cell swelling after NF-κB inhibition suggested that death may not have followed a typical apoptotic process. When DRG neurons treated with SN50 were tested for caspase 3-like activation, there was no evidence of any increase in protease activation [Fig. 5*B* (fluorescent image), *C* (statistical analysis)] when compared with amyloid-β-treated hippocampal neurons, which acted as positive controls. In addition, neither treatment with ZVAD-fmk nor DEVD-fmk, two broad-spectrum caspase inhibitors, decreased neuronal death caused by inhibition of NF-κB by SN50 (Fig. 5*D*). These observations indicate that death caused by NF-κB inhibition was more characteristic of necrosis than caspase-mediated apoptosis.

Cultured DRG cells contain and release TNFα

Recently, TNFα has been reported to accumulate in DRG neurons after sciatic nerve crush (Schafers et al., 2002, 2003). A TNFα ELISA was used to measure levels of TNFα protein released by the DRG cultures into the culture media and to determine TNFα levels in the neuronal protein fraction (Table 2). Both the culture media and DRG protein fraction had detectable levels of TNFα protein, confirming endogenous production of this cytokine.

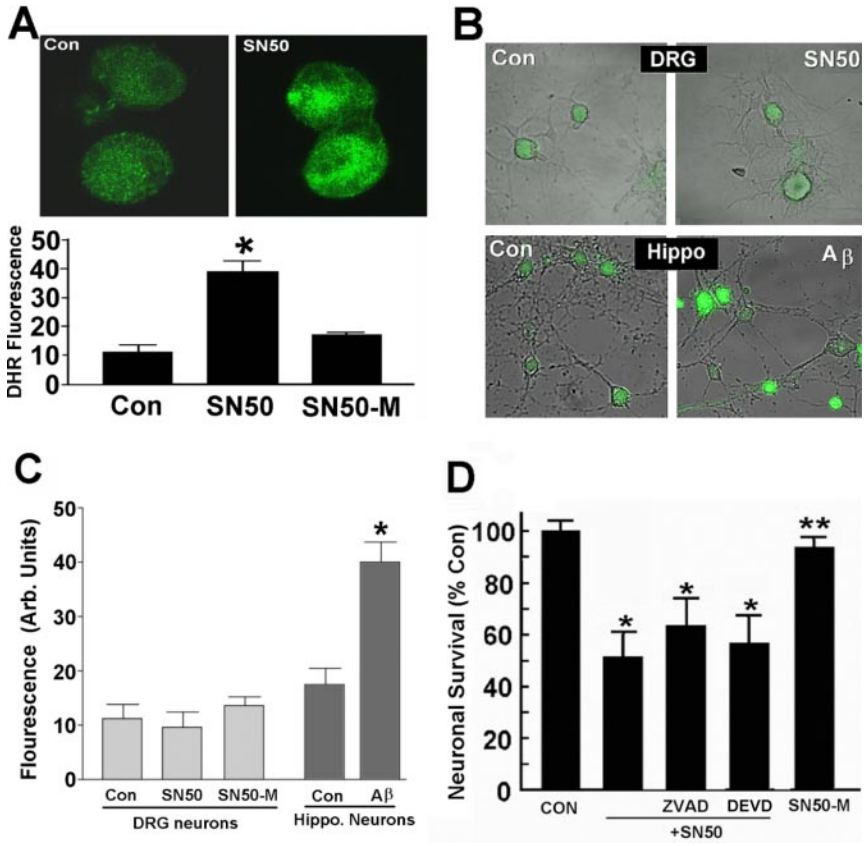

Figure 5. SN50-mediated inhibition of NF-κB induces oxidative stress. *A*, DRG cells were cultured for 2 d and then exposed to vehicle (Con), 10 μM SN50, or the inactive control peptide SN50-M for 2 h. Cells were then treated with 5 μM DHR 123 (DHR), a cell-permeable dye that fluoresces in the presence of hydrogen peroxide or peroxynitrite. Cells were analyzed for fluorescence using a LSM510 confocal microscope. All images were acquired using the same settings so that fluorescent intensity could be quantified. Top, Sample image showing DHR fluorescence is punctuate, indicating localization to mitochondria. Bottom, Statistical representation of fluorescent intensity. Data are mean ± SE; $n = 20$ sampled neurons; *$p < 0.01$ versus both control and SN50-M treated cells. *B*, DRG neurons were plated for 2 d and then treated with vehicle or 10 μM SN50 for 6 h. Cells were then exposed to an avidin–biotin-linked peptide substrate that fluoresces at 510 nm when bound to active caspase 3-like proteases. As a positive control, embryonic rat hippocampal neurons (Hippo), cultured for 7 d, were exposed to 100 nM Ab1-42, which causes significant caspase 3 activation. Fluorescent images of vehicle (Con) and SN50-treated DRG neurons and Aβ42-treated hippocampal neurons are shown. *C*, Images were quantified and graphed. Data are mean ± SE; $n = 7$; *$p < 0.01$ versus control. *D*, In a separate test, DRG neurons were exposed to either 10 μM SN50 or 10 μM SN50-M. Some cultures exposed to SN50 were treated immediately before with the broad-spectrum caspase inhibitors ZVAD or DEVD. Neuronal survival was determined in all neurons after 12 h by morphological criteria and verified by trypan blue dye exclusion. Data are mean ± SE; $n = 3$; *$p < 0.01$ versus control; **$p < 0.01$ versus SN50-treated cells.

Table 2. DRG cultures exhibit endogenous production of TNFα

Sample	TNFα (pg/mg total protein)
DRG culture, protein sample	38.81 ± 7.72
DRG, tissue sample	7.14 ± 0.49
Sciatic nerve, tissue sample	2.40 ± 0.52
Spinal cord, tissue sample	13.47 ± 0.47
Adult brain, tissue sample	3.97 ± 0.05
Liver, tissue sample	11.77 ± 0.4
DRG cell culture supernatant	924.9 ± 240.8 pg/ml

Levels of TNFα were measured by ELISA in cultured rat DRG ($n = 4$) and various rat tissues ($n = 3$). Cultured cells were harvested by scraping 24 h after plating. At the time of harvesting, 3 ml of tissue culture supernatant was collected separately from each plate and centrifuged at 400 rpm for 10 min to pellet floating cells and debris. The resulting supernatant was freeze-dried and resuspended in 250 μl of homogenization buffer used to homogenize protein samples. Three-milliliter media controls were also freeze-dried, resuspended in the same manner, and assayed for TNFα by ELISA. Tissue measurements represent three replicates of the same tissue homogenate on the same ELISA plate.

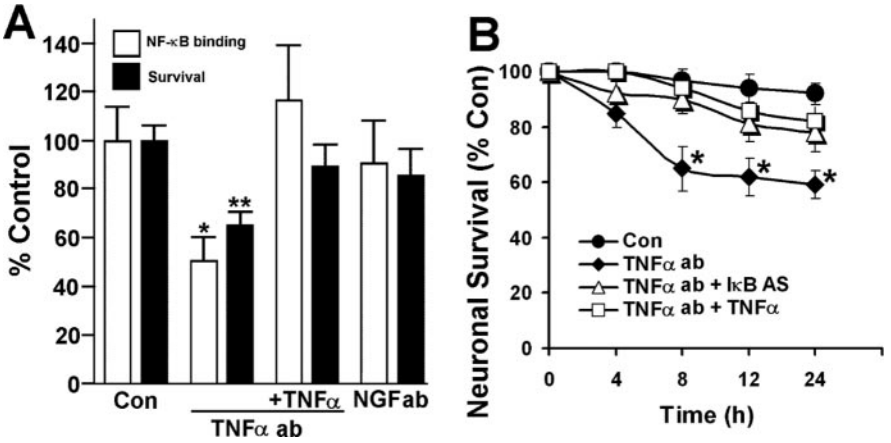

Figure 6. Endogenous TNFα regulates NF-κB binding and survival. *A*, DRG neurons were treated with vehicle (Con), anti-TNFα antibody (TNFα ab), or anti-NGF antibody (NGF ab), followed in some cases by 250 ng/ml TNFα. In some cultures, NF-κB binding was determined after 12 h, whereas in others, survival was determined after 24 h. *B*, DRG neurons were treated with anti-TNFα antibody, then treated with vehicle, IκB antisense (IκB AS), 250 ng/ml TNFα, or both. Survival was determined at the times shown. Data are mean ± SE; $n = 4$; $^*p < 0.05$ versus untreated.

TNFα acts in an autocrine or local paracrine manner to support neuronal survival

In 500 mm^2 plates in 2 ml of media, adult DRG cells were diluted until there were <10 cells per plate, of which ~35% were medium to large neurons. At least 80% of these cells survived 2 d in culture. To determine the role of TNFα in the NF-κB-mediated survival of cultured DRG neurons, cells were cultured immediately after dissection in media containing a function-blocking anti-TNFα antibody. Inhibition of TNFα action resulted in the death of ~40% of medium to large neurons (Fig. 6*A*) by 2 d in culture. In denser cultures, anti-TNFα antibody also muted the rise in NF-κB DNA-binding levels, which were ~60% of untreated levels after 12 h in culture (Fig. 6*A*). Both of these effects were abolished by the addition of 250 ng/ml TNFα to the antibody solution before the addition to cells. Although embryonic neurons have previously been reported to depend on nerve growth factor (NGF)-induced NF-κB activation, adult cultured neurons treated with function-blocking anti-NGF antibody demonstrated neither decreased survival after 2 d in culture nor decreased NF-κB-binding activity after 12 h in culture. To determine the cause and effect relationship between TNFα and NF-κB, cells were pretreated for 12 h with 20 μM IκB antisense DNA, to drive NF-κB DNA-binding activity in the absence of TNFα action. This pretreatment abolished the killing effect of anti-TNFα antibody on medium to large DRG neurons (Fig. 6*B*), indicating that the survival properties of the cytokine were attributable to stimulation of NF-κB-binding activity. In the presence of 1:300 dilution of anti-TNFα antibody, the addition of 10 ng/ml brain-derived neurotrophic factor (BDNF) rescued a small number of neurons, but the addition of NGF had no effect (Table 3). Furthermore, BDNF also rescued ~20% of cells treated with NF-κB decoy DNA, whereas NGF again had no effect (Table 3). In contrast, TNFα was not able to rescue neurons treated with decoy DNA.

Discussion

The survival response of adult DRG neurons after peripheral nerve crush is surprisingly effective, protecting not only against axonal damage and loss of trophic support but conferring protection against an array of insults that normally kill cultured embryonic sensory neurons. This exceptional vitality seems to be independent of any external factor (Lindsay, 1988), and thus DRG neurons possess a unique self-contained survival strategy that may have profound implications for neurodegenerative diseases. Our current study demonstrates that this neuroprotective pathway is mobilized after axon damage in adult sensory neurons and includes NF-κB activated in an autocrine or local paracrine manner via TNFα.

Nerve crush-induced activation of NF-κB in DRG

Adult sensory neurons in the intact lumbar DRG *in vivo* have very low NF-κB DNA-binding activity (Fig. 2*A*,*B*), indicating that the normal survival mechanism(s) sustaining these cells may not involve NF-κB. In contrast, cortical and hippocampal neurons have been reported to require NF-κB for survival (Barger et al., 1995; Mattson et al., 1997; Bhakar et al., 2002), and we have observed a greater level of NF-κB DNA binding in intact cortical tissue than in the intact DRG (G. W. Glazner and J. Schapansky, unpublished observation). However, there is a notable abundance of NF-κB subunit protein expression in lumbar DRG, far above that found in cortical tissue, indicating the ability to mobilize substantial NF-κB (Fig. 1*A*,*B*). Indeed, within 2 h of sciatic nerve crush, NF-κB DNA-binding activity was increased >10-fold in ipsilateral lumbar DRG but not in contralateral DRG (Fig. 2*B*). NF-κB DNA-binding levels rose in the DRGs after nerve crush too quickly to be explained by a stimulatory factor delivered through even the most rapid retrograde transport from the crush site to the DRG. The initial signal is unlikely to be blood-borne because binding levels rise more quickly in the ipsilateral DRG than in the contralateral DRG, although the equalization of NF-κB binding between right and left sides at later time points may be attributable to a paracrine effect. It may be that a change in electrical signaling triggers the cascade that results in NF-κB activation, because this would explain the rapid and unilateral nature of the initial (2 h) increase in NF-κB.

Binding activity accumulated for 24 h, at which point it reached a peak level ~20-fold that of the initial activity that was maintained for the remainder of the study (out to 5 d). The binding activity in the contralateral side rose to a similar degree, but more slowly, not peaking until 24 h after nerve injury. This is

Table 3. Effect of NGF and BDNF on anti-TNFα and decoy DNA induced sensory neuron death

Treatment	Percentage of survival (mean ± SE)
Untreated	100 ± 12
TNFα ab (1:300)	54 ± 8 *
TNFα ab plus NGF (10 ng/ml)	60 ± 10*
TNFα ab plus BDNF (10 ng/ml)	78 ± 7 **
TNFα ab plus TNFα (250 ng/ml)	96 ± 5[a]
DD (10 μM)	48 ± 6 *
DD plus NGF (10 ng/ml)	53 ± 9 *
DD plus BDNF (10 ng/ml)	75 ± 5[a]
DD plus TNFα (250 ng/ml)	62 ± 13*
DD plus TNFα ab (1:300)	58 ± 8*

Cultured adult sensory neurons were treated with function-blocking anti-TNFα antibody (ab) for 30 min, followed by NGF, BDNF, or TNFα, or treated with NF-κB decoy DNA (DD) for 30 min, followed by NGF, BDNF, or TNFα. Survival was determined 24 h later. Data are mean + SE; *$p < 0.05$ versus untreated; **$p < 0.05$ versus untreated and TNFα treated.

[a]$p < 0.05$ versus untreated and decoy DNA treated.

the first report of both the notable speed and extent of increased NF-κB DNA-binding activity in DRGs immediately after sciatic nerve damage. The observation that nerve injury induced an increase in NF-κB in lumbar DRG within 2 h indicates that a factor local to the DRGs, rapidly induced by a specific signal generated by nerve damage, may be responsible for induction of NF-κB DNA-binding activity.

Role of NF-κB in neuronal survival and death

Activation of NF-κB is well characterized as a very powerful neuroprotective response, both in central and peripheral embryonic neurons. *In vitro*, upregulation of NF-κB protects hippocampal and cortical neurons against multiple insults (Barger et al., 1995; Mattson et al., 1997; Bhakar et al., 2002). In embryonic sympathetic and sensory neurons of the peripheral nervous system, NF-κB activity is induced by exogenous factors and is neuroprotective (Maggirwar et al., 1998; Middleton et al., 2000). Indeed, in our hands, two separate treatments that inhibited NF-κB activation in dissociated adult DRG neurons killed 40–60% of medium to large neurons and 30–50% of total neurons (Fig. 4). The pattern of morbidity and death was consistent with necrosis because there was no stimulation of caspase-like activity, caspase inhibitors did not protect the cells, and morphologically cells underwent rapid swelling and loss of membrane integrity (Fig. 5). The observation of increased oxidative stress in mitochondria after only 2 h of NF-κB inhibition suggests that an anti-oxidant protein with rapid turnover, dependent on NF-κB, is being lost by NF-κB inhibition. NF-κB is known to regulate the mitochondrial anti-oxidant manganese superoxide dismutase (Mattson et al., 1997), a decrease in which may partially explain increased mitochondrial oxidative stress, which is linked to both necrosis and apoptosis. In contrast to the present model, which used adult neurons, cultured embryonic sensory neurons die by apoptosis unless supplied with exogenous factors such as CNTF, NGF, or BDNF (Acheson et al., 1995; Middleton et al., 2000; Miller and Kaplan, 2001; Davies, 2003). In embryonic sensory neurons, CNTF, and to a lesser extent NGF, activates NF-κB binding that aids neuronal survival, whereas the survival properties of BDNF are NF-κB independent (Hamanoue et al., 1999; Middleton et al., 2000). In our study, death caused by NF-κB inhibition was partially reversed by BDNF but unaffected by NGF (Table 3). In addition, embryonic neurons require activation of PI 3-K, whereas adult neurons do not. So, after maturation and in response to nerve damage, lumbar DRGs initiate a novel signaling system whereby they auto-activate NF-κB binding and are thus uniquely independent of target-derived neurotrophic survival factors.

TNFα regulates NF-κB activation and sensory neuron survival

The cytokine TNFα is a potent activator of NF-κB, which is the primary transcriptional enhancer of TNFα, leading to a positive feedback loop seen in immune cells during inflammatory responses (Baldwin, 1996; Baud and Karin, 2001; Karin and Lin, 2002). There is significant endogenous TNFα protein present in intact DRG neuronal cell soma, primarily in small neurons. However, after nerve injury, TNFα protein accumulates in medium and large neurons with the induction of IL-6 mRNA preceding this event (Murphy et al., 1995; Schafers et al., 2002, 2003). Furthermore, TNF receptor 1 (TNFR1) and TNFR2 are present on adult sensory neurons in intact DRG (Shubayev and Myers, 2001) and cultured neonatal sensory neurons, in which application of exogenous TNFα causes increased intracellular calcium (Pollock et al., 2002). In addition, intraplantar injection of TNFα *in vivo* increases NF-κB activity (Wood, 1995). In the present study, the DRG culture exhibited endogenous production of TNFα (Table 2), and treatment with function blocking anti-TNFα antibody decreased NF-κB binding and killed sensory neurons, a phenomenon reversed by either activation of NF-κB by 12 h treatment with 20 μM IκB antisense or exposure to TNFα (Fig. 6). These observations indicate that local TNFα release activates NF-κB-binding activity, which is required for survival in a subset of medium to large neurons.

TNFα and NF-κB in neurodegeneration

TNFα is increased in diseases of the brain, such as Alzheimer's disease, Parkinson's disease, multiple sclerosis, and stroke (Fillit et al., 1991; Gary et al., 1998; Barone and Feuerstein, 1999; Kruger et al., 2000; Nagatsu et al., 2000; Lue et al., 2001), and can be trophic or toxic, depending on the tissue and experimental paradigm. Excess TNFα can kill human cortical neurons and oligodendrocytes (Heller et al., 1992; D'Souza et al., 1995; Rothwell and Luheshi, 1996), perhaps because of inflammatory reactions. However, in isolated cell culture of purified neurons, TNFα is neuroprotective, primarily because of NF-κB activation (Cheng et al., 1994; Barger et al., 1995; Liu et al., 2000). Furthermore, enhanced TNFα signaling is associated with hyperalgesia in otherwise undamaged peripheral nerve (Wagner and Myers, 1996; Sorkin et al., 1997). In diabetic sensory neuropathy, the constitutive level of NF-κB binding in lumbar DRG is significantly reduced and may expose these neurons to enhanced neurodegenerative insult (Purves and Tomlinson, 2002).

Adult DRG neurons can be disconnected from both proximal and distal targets by rhizotomy and sciatic nerve transection and survive indefinitely, and these neurons can be dissociated and cultured in defined media, in the complete absence of exogenous cytokines and growth factors, and survive at a very high rate. This study shows that this unique pathway is exceedingly rapid in induction, is autocrine or locally paracrine, independent of any exogenous factor, and dependent on NF-κB DNA binding, stimulated in part by endogenous TNFα.

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