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Activation of NF-κB in Axons and Schwann cells at Site of Sciatic Nerve Crush and Role in Modulating Axon Regeneration in Adult Rats: Studies with Etanercept

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Abstract

An increasing weight of evidence implicates early inflammatory events as inhibitors of functional recovery in both peripheral and central neuropathologies. In this study, we investigated the role of the inflammatory TNF-α/NF-κB axis on events subsequent to sciatic nerve crush injury in rats. Electrophoretic mobility shift assays (EMSA) revealed that within 6 hours post-crush NF-κB DNA binding activity increased significantly in a 1 cm section of sciatic nerve, centered on the crush site. Immunofluorescent staining for NF-κB subunits verified increased nuclear localization of p50, but not p65 or c-Rel, in Schwann cells, with no evidence of immune cell infiltration. In rats injected s.c. with etanercept, a TNF-α receptor chimera which binds free cytokine, the injury-induced rise in NF-κB DNA-binding activity was inhibited. Immunofluorescent staining confirmed that nuclear localization of NF-κB subunit p50 in Schwann cells was significantly lower in etanercept treated vs. control nerves following nerve injury. Axonal growth determined 3 days after nerve crush with immunofluorescent staining for GAP43 demonstrated that regeneration distance of leading axons from the site of nerve crush was significantly greater in etanercept treated animals than saline-treated controls. These data indicate that TNF-α mediates rapid activation of injury-induced NF-κB DNA binding in Schwann cells, and inhibits post-injury axonal sprouting.

INTRODUCTION

Sensory neurons of the adult peripheral nervous system (PNS) are able to survive and regenerate after injury to a much greater extent than neurons from the central nervous system (CNS). Injury to mature peripheral neurons results in a sequence of molecular and cellular responses that are associated with, and may play an important role in, successful axonal regeneration and recovery of function (1). Peripheral nerve transection or crush leads to an acute myelinoaxonal degeneration in the distal area of the damaged nerve, called Wallerian degeneration. This process is associated with macrophage infiltration and Schwann cell proliferation that proceeds axonal re-growth (2). Pro-inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α) and its receptor tumor necrosis factor receptor 1 (TNFR1) are rapidly up-regulated at the site of the peripheral nerve injury and mediate many of the events associated with Wallerian degeneration (3–5). In addition, following nerve injury sensory

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neuron expression of TNF-α rises and is associated with increased anterograde axonal transport of this inflammatory mediator to the crush site (6). By engaging TNFR1, TNF-α activates the transcription factor nuclear factor kappa B (NF-κB) leading to induction of proinflammatory and immunomodulatory genes (4).

NF-κB plays a number of critical roles in both developing and post-injury PNS, including initial myelin formation during development and Schwann cell-mediated re-myelination following nerve injury (7,8). Through its intracellular mediation of $p75^{NTR}$-induced anti-apoptotic signals the NF-κB signaling pathway promotes Schwann cell differentiation and myelination after nerve injury (9). Upon axotomy sensory neurons of activity and this process may be part of the recovery process that may protect neurons against cell death and neurodegeneration during axon regeneration in the adult PNS (10). Furthermore, the activation of the NF-κB signaling pathway in CNS neurons has important consequences for neuronal survival and plasticity (11,12). Sciatic nerve transection leads to an up-regulation of NF-κB in spinal cord neurons through a transactivation process which is consistent with NF-κB acting as a neuronal survival signal (13). However, NF-κB also mediates numerous inflammatory pathways in multiple cells and organ systems, including in the CNS, and inflammation is now recognized to exacerbate most, if not all, neurodegenerative conditions, including Alzheimer's disease, Parkinson's disease and stroke (14,15). In the PNS, nerve injury has been associated with production of pro-inflammatory cytokines in the spinal cord and contributes to nociceptive processing, and blocking NF-κB activity in spinal glia alleviates pain behaviors in rats with chronic nerve constriction injuries (16). Therefore, the actions of TNF-α and NF-κB, while necessary for eventual proper neuronal growth after injury, may also mediate early detrimental inflammatory events

Our previous work had assessed the importance of NF-κB within the sensory neuron perikarya during axon regeneration (10). In the current work we have focused on role of NF-κB at the crush site within the sciatic nerve of the adult rat. The work analyzes the expression of NF-κB subunits within the multicellular nerve crush environment and determines the effect of blocking TNF-α signaling and, therefore, NF-κB activation on short term sciatic nerve regeneration following nerve crush injury.

MATERIALS AND METHODS

Sciatic nerve crush

Adult male Sprague-Dawley rats (250–300 g) underwent unilateral sciatic nerve crush at the mid-thigh level under isofluorane-induced anesthesia. Nerve crush was performed using flat mosquito needle drivers, twice from opposite directions for 30 s in each direction, in order to ensure a uniform crush across the nerve. The contralateral side was also opened but no crush procedure was performed. Prior to surgery the animals were randomly assigned to one of three treatment groups: 1) saline injected controls, 2) 100 μg etanercept (Enbrel), or 3) 500 μg etanercept. All injections were subcutaneous and performed at the time of the surgery. The animals were allowed to recover and, at various times, underwent perfusion fixation or were humanely euthanized and the required tissues were quickly removed and snap-frozen on dry ice All procedures on animals were performed strictly to standards set forth by the Canadian Council on Animal Care and the University of Manitoba ethics committee.

Sensory neuron cultures

Primary cultures of DRG sensory neurons were obtained from adult male Sprague-Dawley rats weighing between 250 – 300 g as described previously (17–19). Hams F12 media was used during dissociation of the cells with Bottenstein's N2 additives and 0.01 M cytosine arabinoside (CA) to prevent non-neuronal cell proliferation. The cells were plated onto polyornithine-

laminin coated #1 thickness 22 mm diameter glass coverslips and cultured for 40 hours in F12 with N2 and CA at 37°C and 5% CO_2. Two hours following plating the cells were treated with a combination of 1 ng/ml nerve growth factor (NGF), 10 ng/ml neurotrophin-3 (NT-3), 10 ng/ml glial cell line-derived neurotrophic factor (GDNF) and 1 nmol/l insulin. All additives were from Sigma (Aldrich, MO, USA), and the culture media from Gibco (Invitrogen, ON, Canada).

NF-κB activity measurement using electrophoretic mobility shift assay (EMSA)

Extracted sciatic nerves (taking approximately1 cm, centered on the crush) from adult rats were homogenized on ice with small volumes of ice-cold Totex buffer [20 mmol/l HEPES, pH 7.9, 350 mmol/l NaCl, 20 % glycerol, 1 % igepal, 1 mmol/l MgCl2, 0.5 mmol/l EDTA, 0.1 mmol/l EGTA, 0.1 mmol/l phenylmethylsulfonyl fluoride (PMSF), 5 μg/ml aprotinin, and 50 μmol/l dithiothreitol (DTT)]. 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 mmol/l KCl, 0.5 mmol/l EDTA, 0.25 % Nonidet P-40, 2 mmol/l DTT, 0.1 % PMSF, and 20 mmol/l HEPES, pH 7.9; 4 μl of buffer containing 20 % Ficoll 400, 300 mmol/l KCl, 10 mmol/l DTT, 0.1 % PMSF, and 100 mmol/l 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% non-denaturing 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.

Fixation of tissues and cultured cells

Animals to be perfused were anesthetized with 90 mg/kg ketamine and 10 mg/kg xylazine. The animals were fixed using 1 % heparinized saline and 4 % paraformaldehyde (PFA) in 0.1 mol/l phosphate buffer. The tissues, 10 mm of sciatic nerve either side of the crush site, were removed and placed in PFA in 0.1 mol/l phosphate buffer for 3 hours to post fix. The nerve tissues were then washed 3 times and stored in 20 % sucrose and phosphate buffered saline (PBS) solution at 4°C. For immunofluorescence cultured cells were fixed using 2 % PFA in 0.05 mol/l phosphate buffer. The cells were incubated at room temperature for 15 min and fixative removed. Slides were stored in 0.01 mol/l phosphate buffered saline (PBS) awaiting immunofluorescent staining.

Immunofluorescent staining for NF-κB subunits

The sciatic nerve tissues were embedded in optimum cutting temperature (OCT) media and stored at −80°C. Embedded tissue samples were sectioned using a HM500 OM cryostat (Microm, Walldorf, Germany). All nerve sections were cut to a thickness of 10 μm. Sections were air dried overnight at 4°C, then used the following day or stored at −20°C in cryoprotectant (30 % ethylene glycol (Sigma, MO, USA), 30 % glycerol (VWR, PA, USA) in 0.01 mol/l PBS (Sigma, MO, USA)). Slides underwent washes in 0.01 mol/l PBS to remove cryoprotectant, PFA and OCT. For cultured cells, following PBS wash, slides were placed in ice cold methanol for 3 min to permeate the membrane and allow intracellular antibody penetration. 0.2 % Triton X100 (Sigma, MO, USA) in 0.01 mol/l PBS was used to permeate tissue sections and further permeate cultured cells. Samples, tissue sections and fixed cultured cells, were washed in 0.01 mol/l PBS. Tissue sections to be treated with the same antibody were dried around and then were drawn around with a hydrophobic immunopen (Chemicon, CA, USA) to separate antibody types and keep solutions on the slide. Slides were blocked using 10 % donkey serum (Jackson, PA, USA) in 0.2 % Triton X100 in 0.01 mol/l PBS for 1 to 2 hours at room

temperature in a humidified chamber. Slides were incubated with the primary antibody (or mixture of primary antibodies for double staining) in 0.2 % Triton X100 (Sigma, MO, USA) in 0.01 mol/l PBS, overnight at 4°C. The primary antibodies used were anti-GAP43 (1:1000), anti-c-Rel (1:200), anti-p50 (1:200), anti-p65 (1:200), and anti-S100 (1:4000). Primary antibodies were all polyclonal from rabbit and were purchased from Santa Cruz Biotechnologies, except the GAP43 antibody which was purchased from Chemicon, and the S100 antibody which was monoclonal from mouse and was purchased from Sigma. The secondary antibody was FITC-conjugated donkey anti-rabbit (1:200 dilutions; Jackson ImmunoResearch, PA, USA), except for detection of S-100 primary antibody which was Cy3-conjugated donkey anti-mouse (1:400 dilution, also Jackson). For a control slide tissues were incubated with 0.2 % Triton X100 in 0.01 mol/l PBS and no antibody. Following incubation slides underwent washes with 0.01 mol/l PBS. The light sensitive fluorescing secondary antibody (or mixture of secondary antibodies for double fluorescence), diluted in 0.01 mol/l PBS only, was then applied and slides incubated for 1 hr at room temperature in a humidified chamber. The chamber was wrapped in black plastic to prevent light reaching the antibody and quenching the fluorescence. Slides underwent washes with 0.01 M PBS. Sections were viewed by light microscopy (Axioskop 2 mot, Zeiss, NY, USA) using the correct wavelength filter and exposures taken by a camera (Axiocam, Zeiss, NY, USA) using Axiovision Ver.4.3 software (Zeiss, NY, USA).

Data analysis

Figures 1 and 2 use the standard Students t-Test to compare the ipsilateral and contralateral sides. Figure 7 uses the standard Student t-Test to compare the % increase in etanercept treated vs. saline treated controls. Figure 11B uses the standard Student t-Test to compare each etanercept treatment with the saline treated control.

RESULTS

Adult rats underwent a unilateral nerve crush of the sciatic nerve at mid thigh. Fig. 1 shows that by 2 hrs after nerve crush there was a non-significant increase in the NF-κB DNA binding levels in the nerve on the side of the nerve crush. At 14 hrs after injury NF-κB binding activity was increased significantly when compared to the contralateral side and both were significantly increased compared to the control (pre-crush) level. NF-κB DNA binding activity was further increased on the ipsilateral side at 24 hrs after nerve crush and remained significantly increased compared to the contralateral side. Both sides remained significantly elevated compared to control nerve.

Expression levels of the NF-κB subunits were measured in the sciatic nerve by semi-quantitative Western blotting. Fig. 2A shows representative Western blots demonstrating p50, p65, and c-Rel expression in various nervous tissue samples. Fig. 2B is a graphical representation of the NF-κB subunit expression in the sciatic nerve proximal (5 mm nerve segment) to the crush site at 12 hrs post-crush compared to nerve sample from the contralateral side. All 3 of the subunits showed a significant increase in expression in the tissues proximal to the crush site compared to tissue from the contralateral side, at 12 hrs post-crush.

Immunofluorescent staining was performed to examine the localization of the NF-κB subunits within the sciatic nerve. Within the 5 mm proximal nerve segment at 12 hrs post sciatic nerve crush, p50 (Fig. 3) and c-Rel (Fig. 6) levels appeared greater only in the Schwann cells at the crush site compared to naïve tissue, and the levels in the blood vessels appeared the same between crushed and naïve nerve for both subunits. In tissues stained for p65 (Fig. 4), staining appeared greater in the Schwann cells of the 12 hr crushed nerve, with no change in the amount of staining seen in the blood vessels. Staining for p65 was also present in the axons, but only in the crushed nerve. Fig. 5B shows high power images of the p65 expression and axonal

localization in the sciatic nerve at the crush site following 12 hr sciatic nerve crush. The transverse sciatic nerve sections show positive staining for the p65 subunit in Schwann cells, blood vessels and within the axons.

In addition to observing the localization of p50 and p65 in the sciatic nerve after a nerve crush, DRG neurons were axotomized and cultured for 40hrs and stained for p50, p65 and β-tubulin (III). The β-tubulin (III) is a neuron specific axonal cytoskeletal component, and thus an effective marker of neurites that extend from the cell body of the dissociated DRG neurons (Figs. 5E and 5F). When NF-κB subunit staining of the neurites was compared with the secondary antibody background controls, only the p65 subunit appeared to be positively expressed in the neurites, co-expressing with the β-tubulin (III) (Figs. 5D and 5H). The p50 subunit remained within the cell body (Fig. 5C) and without any apparent neurite staining (Fig. 5G). The p65 subunit expression in the neurite appeared to be uniform, with no accumulation at the growth cones.

The previous figures show that NF-κB expression and DNA binding activity was up-regulated in the sciatic nerve following a sciatic nerve crush, similar to the enhancement in TNF-α expression seen in previous studies (4,6). Since TNF-α increases the NF-κB binding activity in DRG neurons *in vivo* (20) and *in vitro* (10) (and unpublished observations), we examined the relationship of TNF-α and NF-κB *in vivo* following nerve injury. Adult rats were administered vehicle, 100 or 500 μg etanercept by s.c. injection at the time of nerve crush. After 3 days, animals were euthanized and tissues harvested (Figs. 7 – 11). Etanercept injection blocked injury-induced enhancement of NF-κB DNA binding activity in sciatic nerve and reduced to a lesser degree injury-induced enhancement in the DRG compared to the saline injected controls (Fig. 7).

Immunostaining of longitudinal sections of naive (no sciatic nerve crush) sciatic nerve demonstrated faint cytoplasmic and nuclear expression of NF-κB subunits p50, p65, and c-Rel in presumably mostly Schwann cells (Figs. 8A, 9A and 10A respectively). In vehicle (saline) treated animals, 3 days after nerve crush, the cytoplasmic content of all three NF-κB subunits was increased (Figs. 8C, 9C, and 10C). However, inspection of levels of nuclear staining showed p50 was increased in nearly all nuclei (Fig. 8D), while c-Rel was only increased in some nuclei (Fig. 10D), and the p65 levels were not increased in nuclei (Fig. 9D). Animals treated with 500 μg of etanercept showed a similar increase in cytoplasmic expression of the NF-κB subunits (Figs. 8E, 9E, and 10E) as that seen in the vehicle treated animals. The nuclear staining, however, showed decreased levels of p50 and c-Rel expression compared to the saline injected samples while p65 expression remained relatively unchanged.

Three days after nerve crush, axon regeneration was measured using immunofluorescent staining for GAP-43 from the center of the crush site to the furthest point that the longest regenerating axon had extended into the nerve trunk (Fig. 11 top panel). The mean regeneration distance was increased significantly in animals treated with 500 μg etanercept compared to saline-treated controls (Fig. 11).

DISCUSSION

Following peripheral nerve damage, a series of well-described molecular and anatomical events, including Wallerian degeneration of the distal nerve stump and re-growth of axons, leads to whole or partial recovery of nerve function. Among the early nerve-centered inflammatory events are increased local levels of TNF-α and activated NF-κB (3,4,6,21,22). We previously reported that NF-κB DNA binding activity increases in lumbar DRG within 2 hrs of sciatic nerve crush that lasts at least 5 days (10). However, here we demonstrate that NF-κB DNA binding in the nerve itself is not increased significantly until approximately 14 hrs

post crush and remains elevated for at least 24 hrs as measured by EMSA. Western blot analysis shows that at 12 hrs there is a significant increase in expression of all 3 of the NF-κB subunits in the tissue proximal to the crush site at 12 hrs post crush. Twelve hours following sciatic nerve crush the expression of p50, p65 and c-Rel was greatly increased in Schwann cells as assessed using immunofluorescent staining. This would agree with previous work by Ma and Bisby (1998) who found that NF-κB increased within Schwann cells close to the site of nerve injury (23). This very rapid up-regulation of NF-κB subunits following nerve damage must have been due to factors endogenous to the nerve and not the result of invading macrophages. Much of the up-regulated p65 is likely to be a homodimer, but due to the increases in expression seen in both p50 and c-Rel at 12 hrs after nerve crush there are also likely to be p65/c-Rel and p65/p50 heterodimers. By 12 hrs post crush, Wallerian degeneration is at a stage where Schwann cell morphological changes are beginning with myelin degeneration and ovoid formation. Many of the events involved in Wallerian degeneration may be mediated by TNF-α and its receptor TNFR1, and by activating TNFR1, TNF-α activates NF-κB leading to a major role for NF-κB in the proinflammatory process of Wallerian degeneration (4).

Although the level of p65 in the blood microvessels of the sciatic nerve was not altered after sciatic nerve crush injury, the positive staining for p65 in what is proposed to be microcapillary endothelial cells suggests a role for p65 in blood vessel function. Similarly, previously published data found that p65, but not p50, was positively expressed and found to increase expression in vessel endothelial cells in human brain tissues following contusion (24). TNF-α is also known to be expressed in blood vessel endothelial cells in the sciatic nerve where it was thought to increase permeability of the blood vessels following injury, by activating the gelatinases including the matrix metalloproteinases MMP-2 and MMP-9, thus allowing greater macrophage infiltration (4). In the present study as there was no change seen in the level of p65 in the capillary endothelial cells in the sciatic nerve following crush injury it seems unlikely that there is a link between TNF-α and p65 in this tissue, and the mechanism would be different from that seen in vessel endothelial cells in brain tissue.

The subunit p65 was also found to be present in the axons at the crush site 12 hrs post crush, suggesting that p65 was either axonally transported to the axon or secreted into the axon from the surrounding Schwann cells. Examination of Fig. 5C-H shows that, in dissociated DRG neurons cultured for 40 hrs, p65 was expressed throughout the neurite while p50 was found only in the cell body with no neurite staining. Further work is required to determine the exact cellular source of the NF-κB p65 subunit, however, our *in vitro* studies suggest p65 may be anterogradely transported in the nerve. In fact, we have preliminary data showing anterograde accumulation of p65 at the site of a nerve crush using a 12 hr double ligature paradigm (25, 26). Moreover, recent studies show that important components of the NF-κB signal transduction pathway (IKBα and IKK) were present in axonal compartments at nodes of Ranvier in central and peripheral nerves (27).

The physiological effect of TNF-α on NF-κB activation and nerve regeneration following nerve crush was determined by using the blocker etanercept, which binds to and sequesters soluble TNF-α (28). Animals receiving a single 500 μg s.c. injection of etanercept immediately prior to sciatic nerve crush had significantly reduced crush-induced elevation in NF-κB three days later. Thus TNF-α had a direct and robust control over NF-κB in the sciatic nerve at the site of injury after sciatic nerve crush. Inhibition of TNF-α also increased the initial rate of axon regeneration (Figure 11). However, since TNF-α is blocked systemically it is unknown if this is due to inhibition of TNF-κ locally at the crush site or at the DRG since there is significant anterograde transport of TNF-α in the sciatic nerve (6).

NF-κB subunit staining of sciatic nerve sections 3 days after sciatic nerve crush showed an increase in the expression of p50, p65, and c-Rel compared to naïve (non-crush) tissue. The

increase in expression of NF-κB subunits at 3 days post crush may be due to invading macrophages, which deliver TNF-α to the site of injury, in addition to endogenous factors causing an up-regulation of all 3 NF-κB subunits. TNF-α is a key modulator in Wallerian degeneration and is required to prepare the nerve for successful axon regeneration (6,21,29, 30). The inflammatory response at the site of injury appears to be either delaying the onset of axonal sprouting following injury, with the initial fibroblast and Schwann cell production of TNF-α, or is slowing the rate of axon elongation through the second TNF-α peak generated by invading blood-borne macrophages (2 to 3 days after axotomy), or by other methods (3,31, 32). Having shown that NF-κB activation is mediated by TNF-α at the site of injury following a sciatic nerve crush, the likely mechanism by which TNF-α slows regeneration is the downstream activation of NF-κB, in particular transcription via the p65, p50 and/or c-Rel subunits.

Re-establishment of neuronal function following damage involves both tissue repair and neuronal regeneration. Part of the early repair process, inflammation, appears in many neurodegenerative diseases to be detrimental to eventual return of function. We have described the early inflammation-induced expression, localization, and activation of NF-κB in the sciatic nerve following nerve crush, and demonstrated this to be under the regulation of TNF-α. As has been demonstrated with other neuropathological processes, inflammation appears to exert an inhibitory effect on axonal regeneration following nerve damage. Recent clinical trials have shown the utility of anti-inflammatory treatments for neuropathy and neuropathic pain. Our current results provide experimental evidence supporting this therapeutic approach, and elucidates the role of TNF-α in these early inflammatory events.

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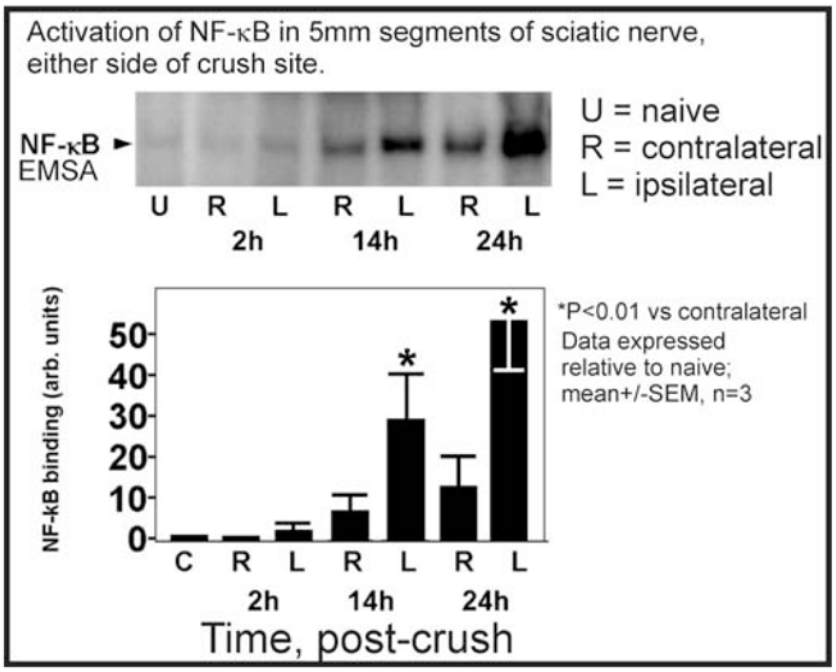

FIGURE 1.

Sciatic nerve crush induces activation of NF-κB at the crush site. Adult Sprague-Dawley rats underwent unilateral sciatic nerve crush at mid-thigh. The crush site (5mm proximal and distal) was removed at various time points and NF-κB binding activity determined. The top panel shows the autoradiogram illustrating the main NF-κB binding complex (supershift analysis revealed p50 and p65 subunit components – not shown). The data are presented below: C = naïve, R = contralateral, L = ipsilateral to nerve crush. The results show that by 14 hr post-crush there is significant NF-κB activation at the crush site. By 24 hr there is some evidence of NF-κB activation on the contralateral side. Values are means +/− SEM (n=3); *P<0.05 vs contralateral side.

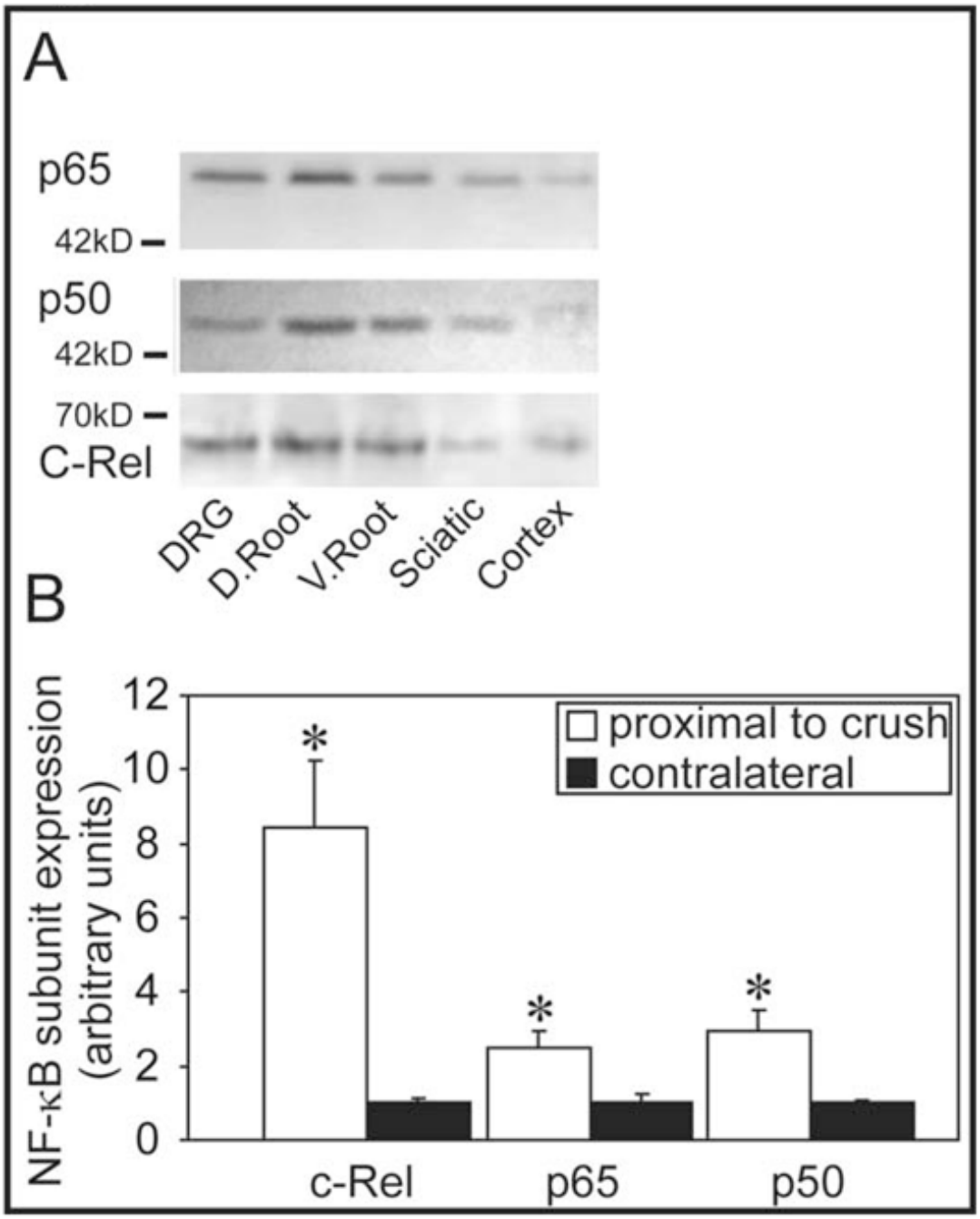

FIGURE 2.

Expression of NF-κB subunits in nervous tissue and effect of nerve crush on levels of expression. The upper panel, **A**, shows representative Western blots with the bands demonstrating 3 major groups of NF-κB subunits, p50, p65, and c-Rel expressed in various nervous tissue samples. Lower panel, **B**, bar graph of NF-κB subunit expression in the sciatic nerve proximal to the crush site at 12 hrs post-crush compared to sciatic nerve from the contralateral side. At 12 hrs post-crush all 3 subunits are significantly increased in the tissues proximal to the crush site compared to the contralateral side. Means +/− SEM (n=4). *P<0.05 vs contralateral.

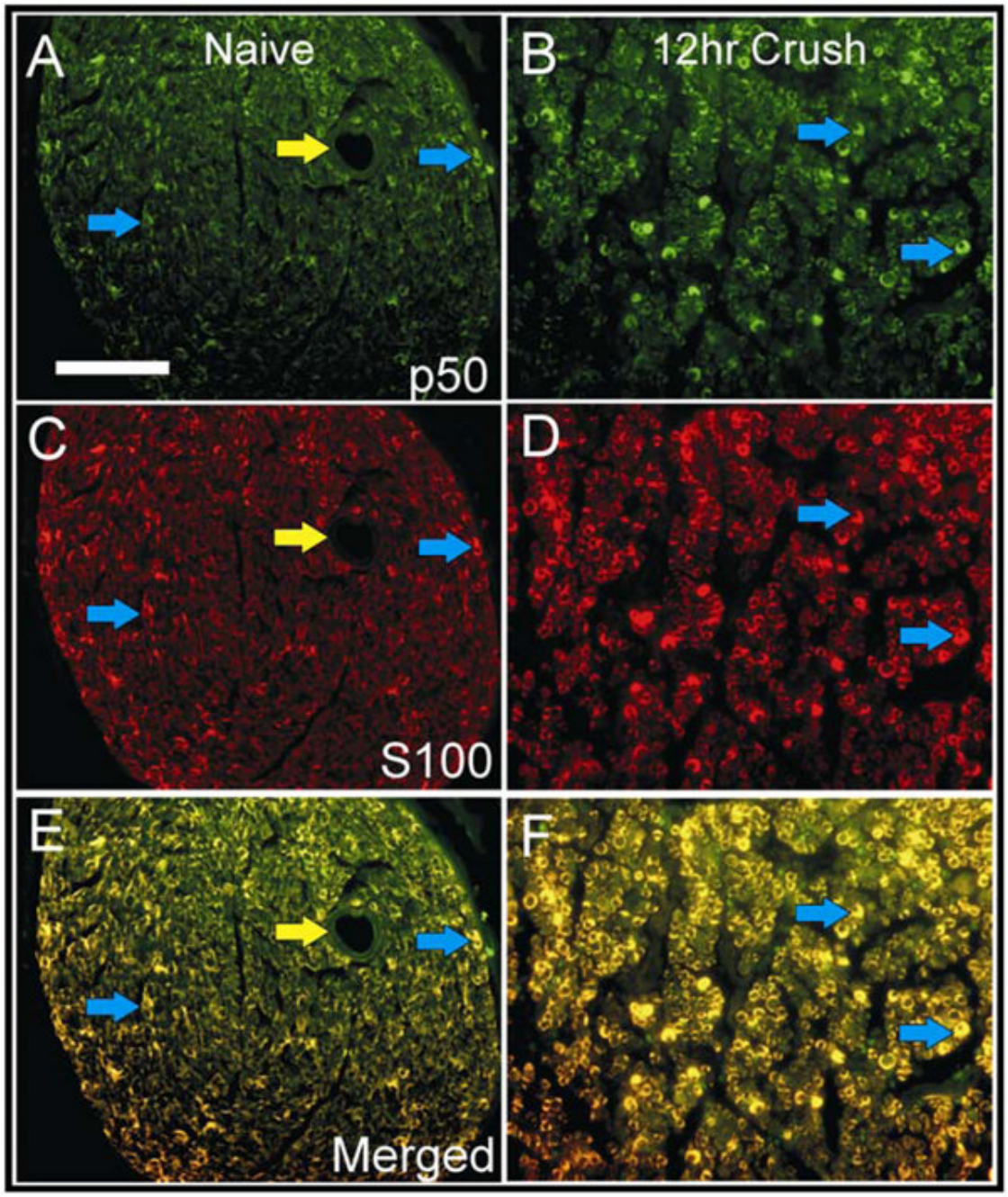

FIGURE 3.

Localization and expression of the p50 subunit of NF-κB in naive sciatic nerve and 12 hrs post-crush. Images are of transverse sciatic nerve sections. **A,** naïve animal (no surgery performed) with p50 staining. **B,** 12 hrs post-crush with p50 staining. **C,** naïve animal with S-100 (Schwann cell) staining. **D,** 12 hrs proximal segment post-crush with S-100 staining. **E,** naïve animal with S-100 and p50 image overlay. **F,** 12 hrs post-crush with S-100 and p50 image overlay. Red arrows indicate blood vessels and blue arrows indicate Schwann cells. In the crushed sciatic nerve p50 staining appeared greater in the Schwann cells, but absent in endoneurial blood vessels and axons in naïve and crushed sciatic nerves. Bar = 100 μm.

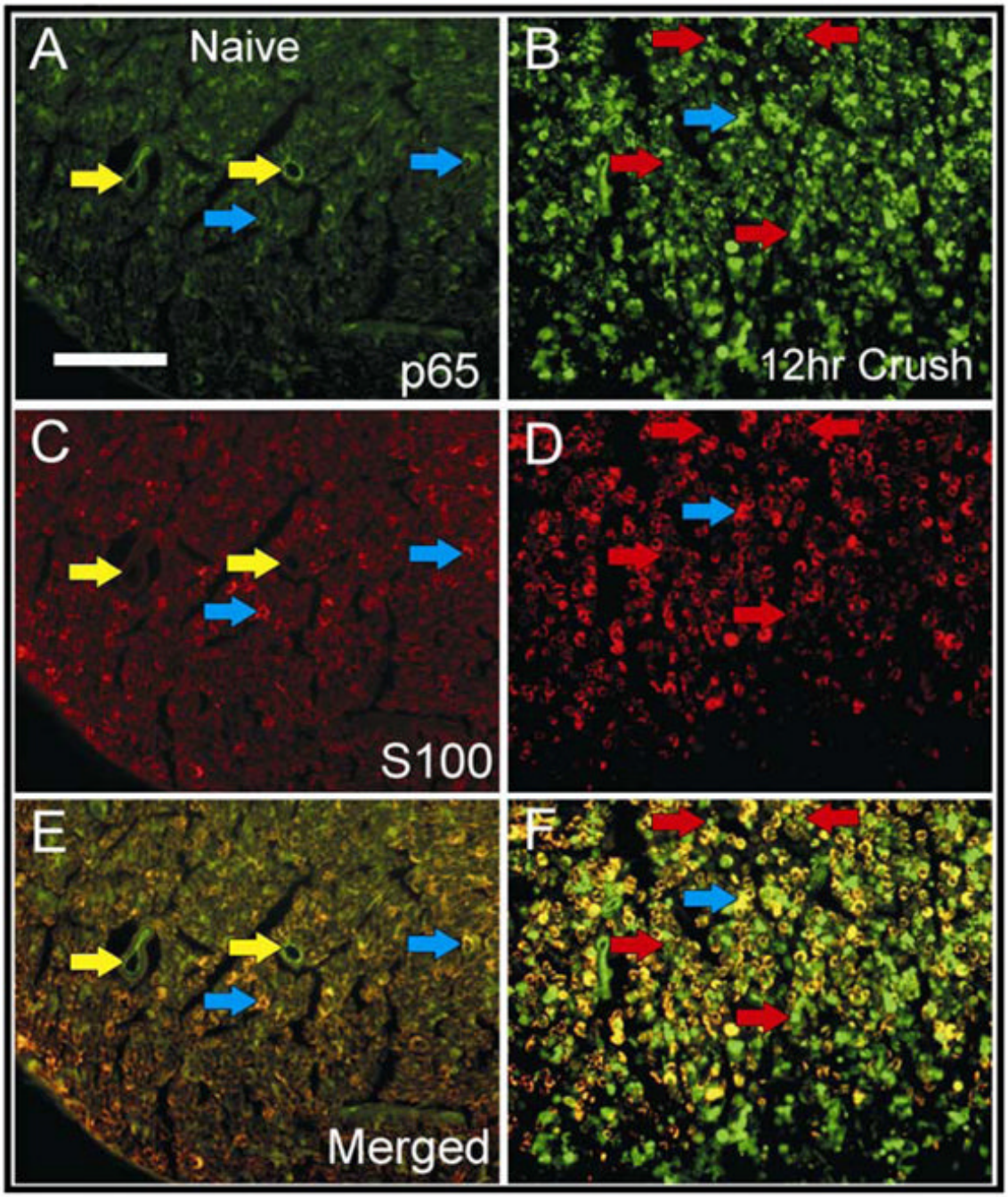

FIGURE 4.

Localization and expression of the p65 subunit of NF-κB in naive sciatic nerve and 12 hrs post-crush. Images are of transverse sciatic nerve sections. **A,** naïve animal (no surgery performed) with p65 staining. **B,** 12 hrs post-crush with p65 staining. **C,** naïve animal with S-100 (Schwann cell) staining. **D,** 12 hrs post-crush with S-100 staining. **E,** naïve animal with S-100 and p65 image overlay. **F,** 12 hrs post-crush with S-100 and p65 image overlay. Yellow arrows indicate blood vessels, red arrows indicate axons, and blue arrows indicate Schwann cells. In the crushed sciatic nerve p65 staining appeared greater in the Schwann cells, and unchanged in the blood vessels. Staining was present in the axons of only the crushed nerve. Bar = 100 μm.

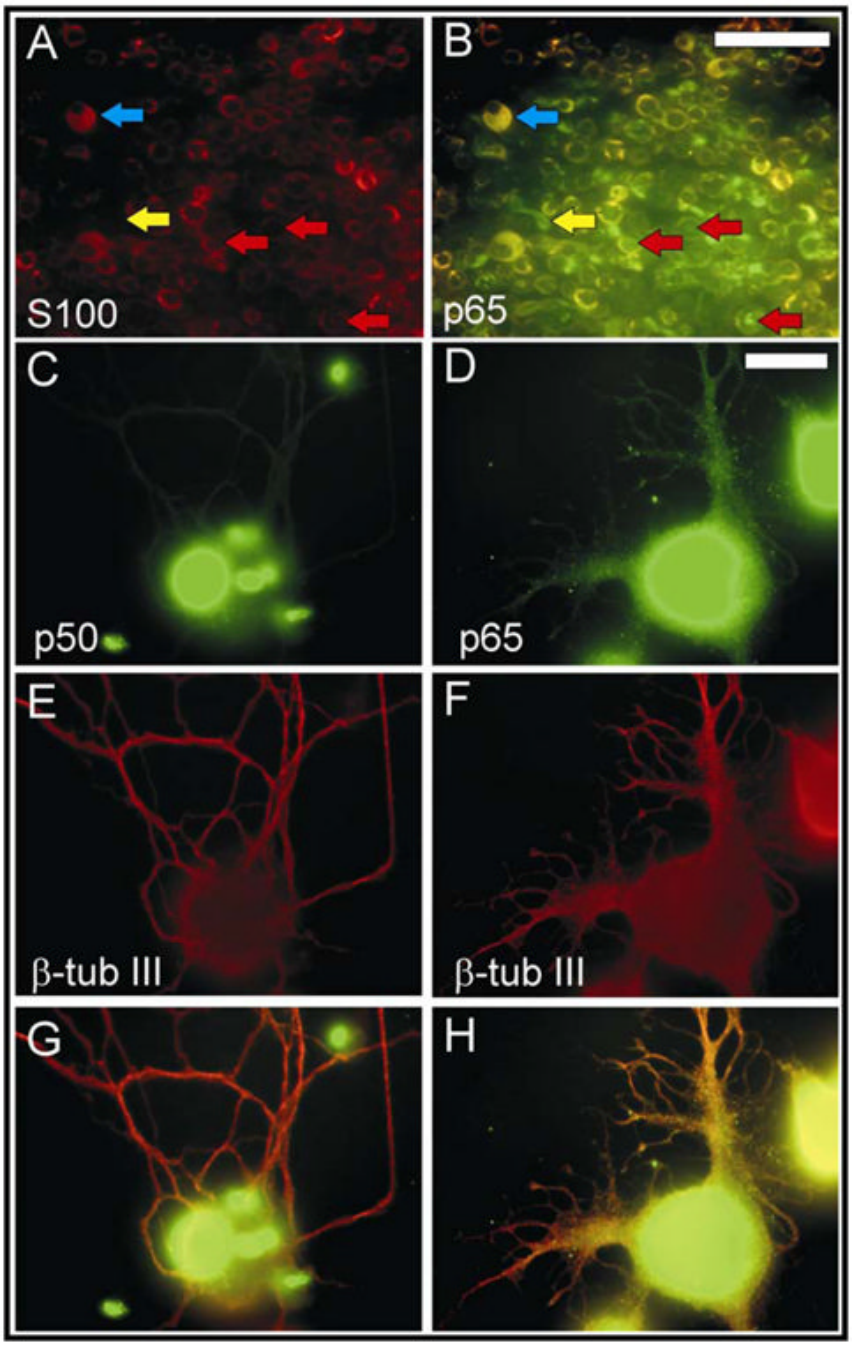

FIGURE 5.

High power images of p65 expression and axonal localization in the sciatic nerve at the crush site following 12 h sciatic nerve crush. Images are of transverse sciatic nerve sections. Exposures were imaged at x40 magnification. **A**, S-100 Schwann cell stain in crushed sciatic nerve. **B,** S-100 and p65 image overlay in crushed sciatic nerve (same field of view as **A**). Blue arrows indicate positive Schwann cell staining. Yellow arrows indicate positive blood vessel staining. Red Arrows indicate positive staining within the axon. Bar = 50 μm. **C-H:** Neurite expression of the NF-κB subunits p50 and p65 in cultured DRG neurons *in vitro*. **C,** p50 staining. **D,** p65 staining. **E,** β-tubulin (III) staining (in the same sample as C), **F,**β-tubulin (III) staining (in the same sample as D). **G,** p50 and β-tubulin (III) overlay. **H,** p65 and β-tubulin

(III) overlay. **A&B**: Following 12 hr sciatic nerve crush the NF-κB subunit p65 was present in the axons at the site of injury. **C-H**: The NF-κB subunit p65 was expressed within the neurites of neurotrophin treated, dissociated DRG neurons *in vitro,* while p50 was not. Bar = 30 μm.

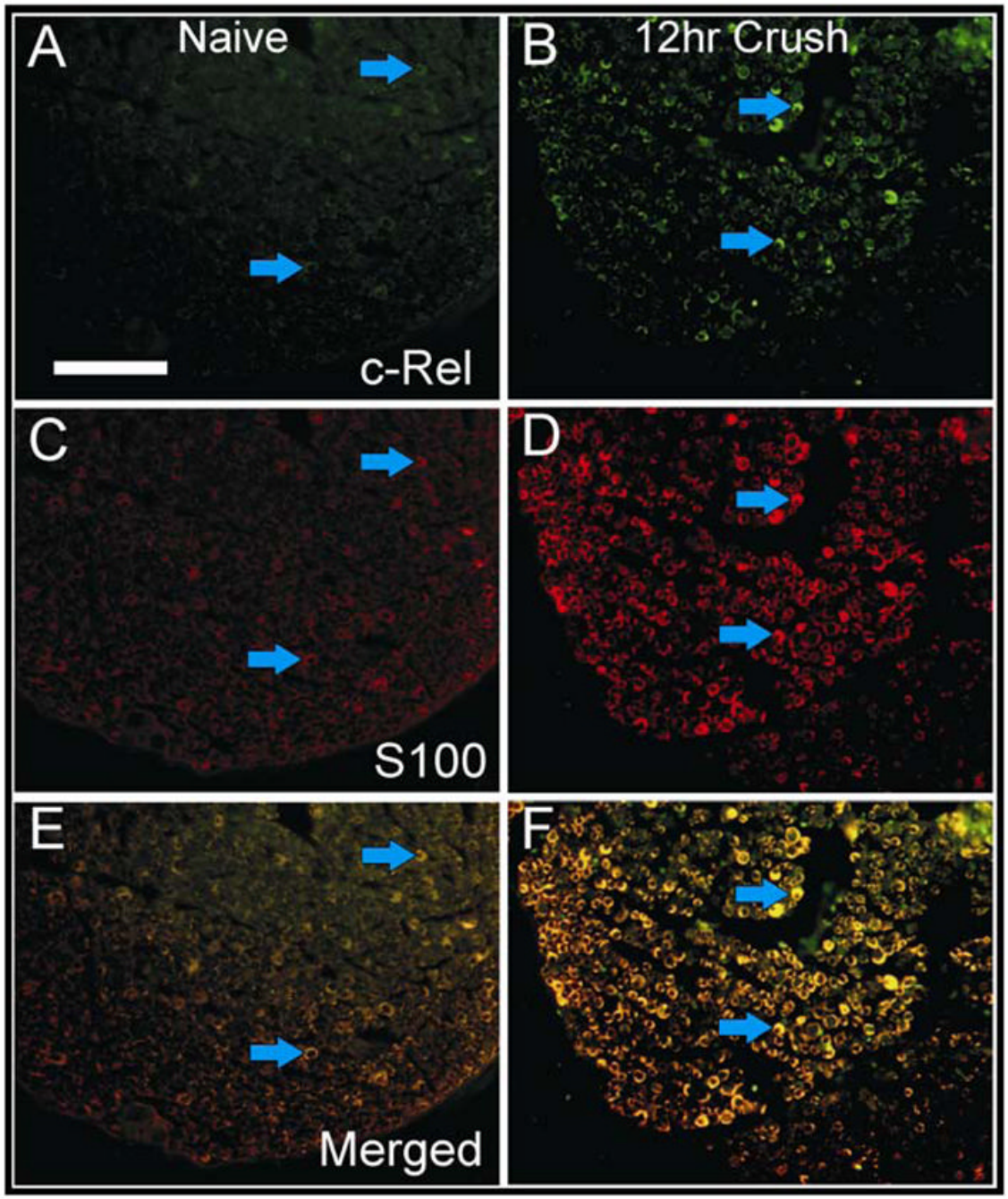

FIGURE 6.

Localization and expression of the c-Rel subunit of NF-κB in naive sciatic nerve and 12 hrs post-crush. Images are of transverse sciatic nerve sections. **A,** naïve animal (no surgery performed) with c-Rel staining. **B,** 12 hrs post-crush with c-Rel staining. **C,** naïve animal with S-100 (Schwann cell) staining. **D,** 12 hrs post-crush with S-100 staining. **E,** naïve animal with S-100 and c-Rel image overlay. **F,** 12 hrs post-crush with S-100 and c-Rel image overlay. Blue arrows indicate S-100 and/or c-Rel staining in the Schwann cells. In the crushed sciatic nerve c-Rel staining appeared greater in the Schwann cells. Bar = 100 μm.

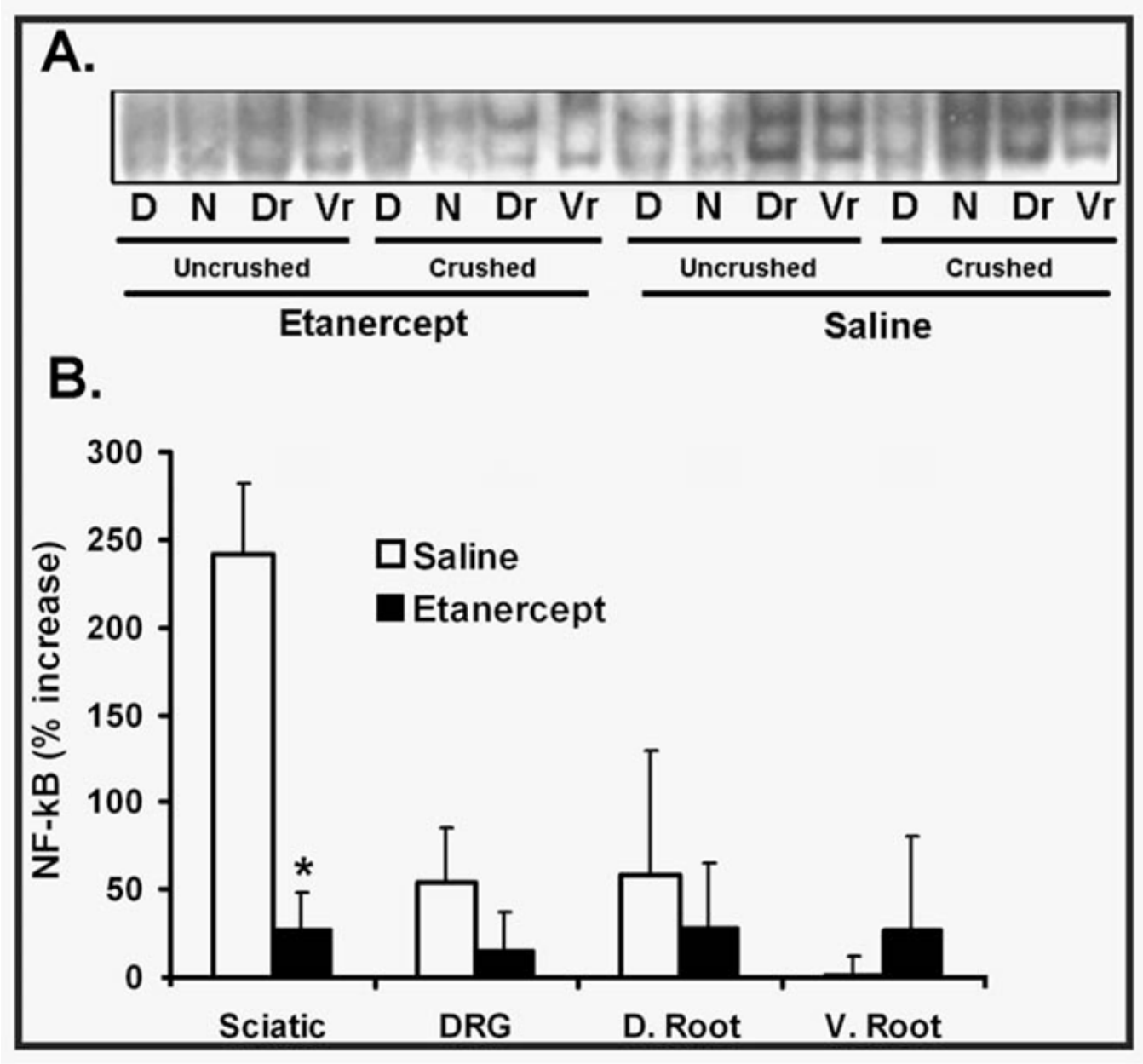

FIGURE 7.

Inhibition of TNF-α by etanercept inhibits nerve crush-induced elevation in NF-κB activity in sciatic nerve. Adult male Sprague-Dawley rats were injected s.c. with either 500 μg etanercept in 1 ml saline, or saline alone, then immediately anesthetized for surgery. Sciatic nerves were exposed and a 3 mm section at mid-thigh was crushed as described. After 3 days, animals were killed and whole L4-L6 DRGs (D), a 1 cm section of sciatic nerve centered on the crush site (N), and L4-L6 dorsal root (Dr) and ventral root (Vr) were removed and immediately processed for NF-κB EMSA as described in methods. Upper panel (**A**) shows a representative autoradiograph exposure demonstrating 2 major NF-κB binding bands. Lower panel (**B**) is results of quantification of band density. Data are means +/− SEM (n=3); *P< 0.01.

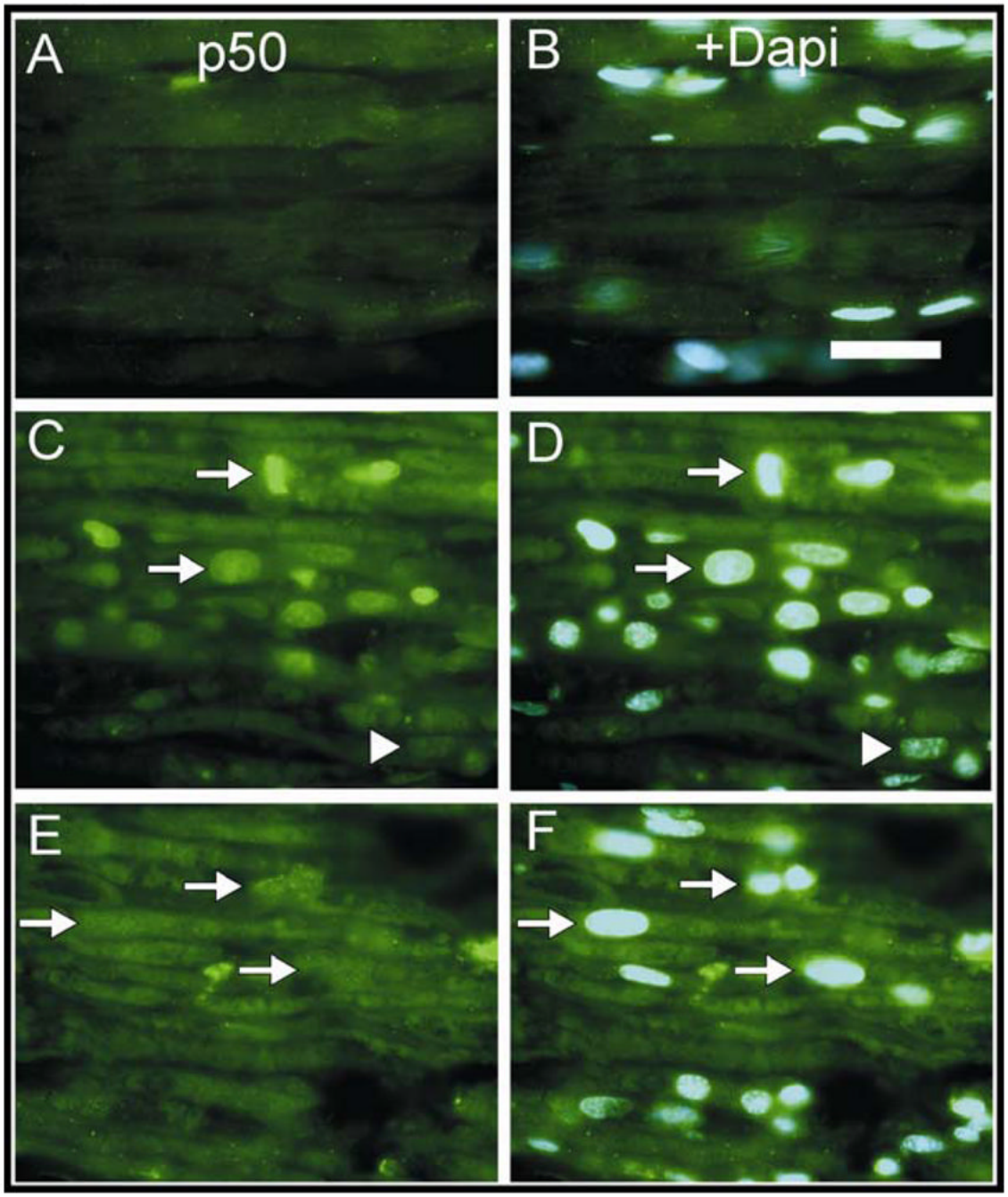

FIGURE 8.

Localization and expression of the p50 subunit of NF-κB in the sciatic nerve 3 days post-crush and treated with 500μg etanercept. Longitudinal images are from just proximal to the site of the crush at x63 magnification. **A,** naïve animal (no surgery performed) with p50 staining. **B,** naïve animal with p50 and DAPI image overlay. **C,** vehicle (saline) treated with p50 staining. **D,** vehicle treated with p50 and DAPI overlay. **E,** treated with 500 μg etanercept (TNF-α blocker); p50 staining. **F,** 500μg etanercept treated with p50 and DAPI overlay. Arrows show positive p50 nuclear staining, arrowheads show unusual DAPI staining and low nuclear p50 staining. Bar = 30 μm. The cytoplasmic and nuclear expression of p50 is significantly increased

at the crush site after 3 days, in vehicle treated animals. Treatment with 500 μg of the TNFα blocker reduced the crush-induced increase in nuclear expression of p50.

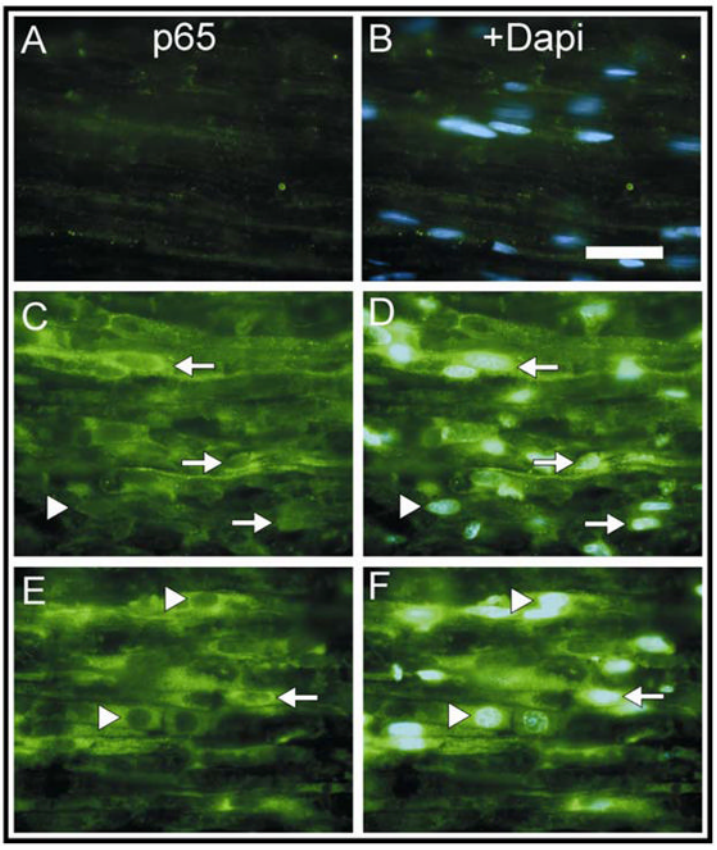

FIGURE 9.

Localization and expression of the p65 subunit of NF-κB in the sciatic nerve 3 days post-crush and treated with 500μg etanercept. Images are from just proximal to the site of the crush at x63 magnification. **A,** naïve animal (no surgery performed) with p65 staining. **B,** naïve animal with p65 and DAPI image overlay. **C,** vehicle (saline) treated with p65 staining. **D,** vehicle treated with p65 and DAPI overlay. **E,** treated with 500μg etanercept; p65 staining. **F,** 500μg etanercept treated with p65 and DAPI overlay. Arrows show positive p65 nuclear staining, arrowhead shows absent or low nuclear p65 staining. Bar = 30 μm. The cytoplasmic and nuclear expression of p65 is increased at the crush site after 3 days, in vehicle treated animals. Treatment with 500 μg of the TNF-α blocker only partially lowered the nuclear expression of p65 with no apparent effect on total p65 staining.

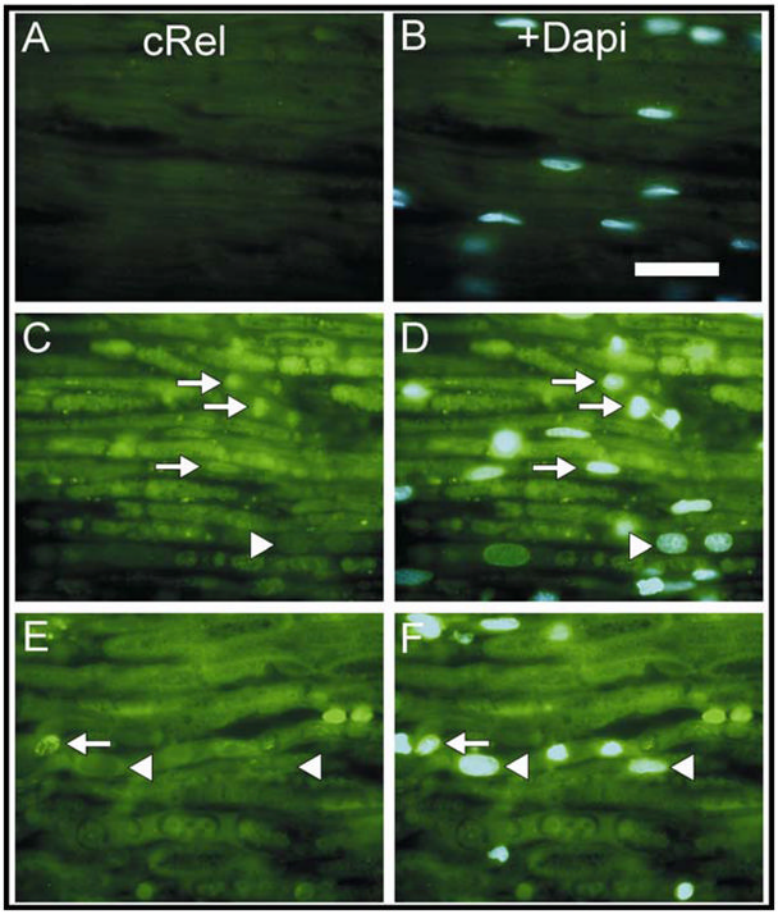

FIGURE 10.

Localization and expression of the c-Rel subunit of NF-κB in the sciatic nerve 3 days post-crush and treated with 500 μg etanercept. Images are from just proximal to the site of the crush at x63 magnification. **A,** naïve animal (no surgery performed) with c-Rel staining. **B,** naïve animal with c-Rel and DAPI image overlay. **C,** vehicle (saline) treated with c-Rel staining. **D,** vehicle treated with c-Rel and DAPI overlay. **E,** treated with 500 μg etanercept (TNF-α blocker); c-Rel staining. **F,** 500μg etanercept treated with c-Rel and DAPI overlay. Arrows show positive c-Rel nuclear staining, arrowhead shows low nuclear c-Rel staining. Bar =30 μm. The cytoplasmic and nuclear expression of c-Rel is significantly increased at the crush site after 3 days, in vehicle treated animals. Treatment with 500 μg of the TNF-α blocker did not appear to significantly alter the nuclear or cytoplasmic expression of c-Rel.

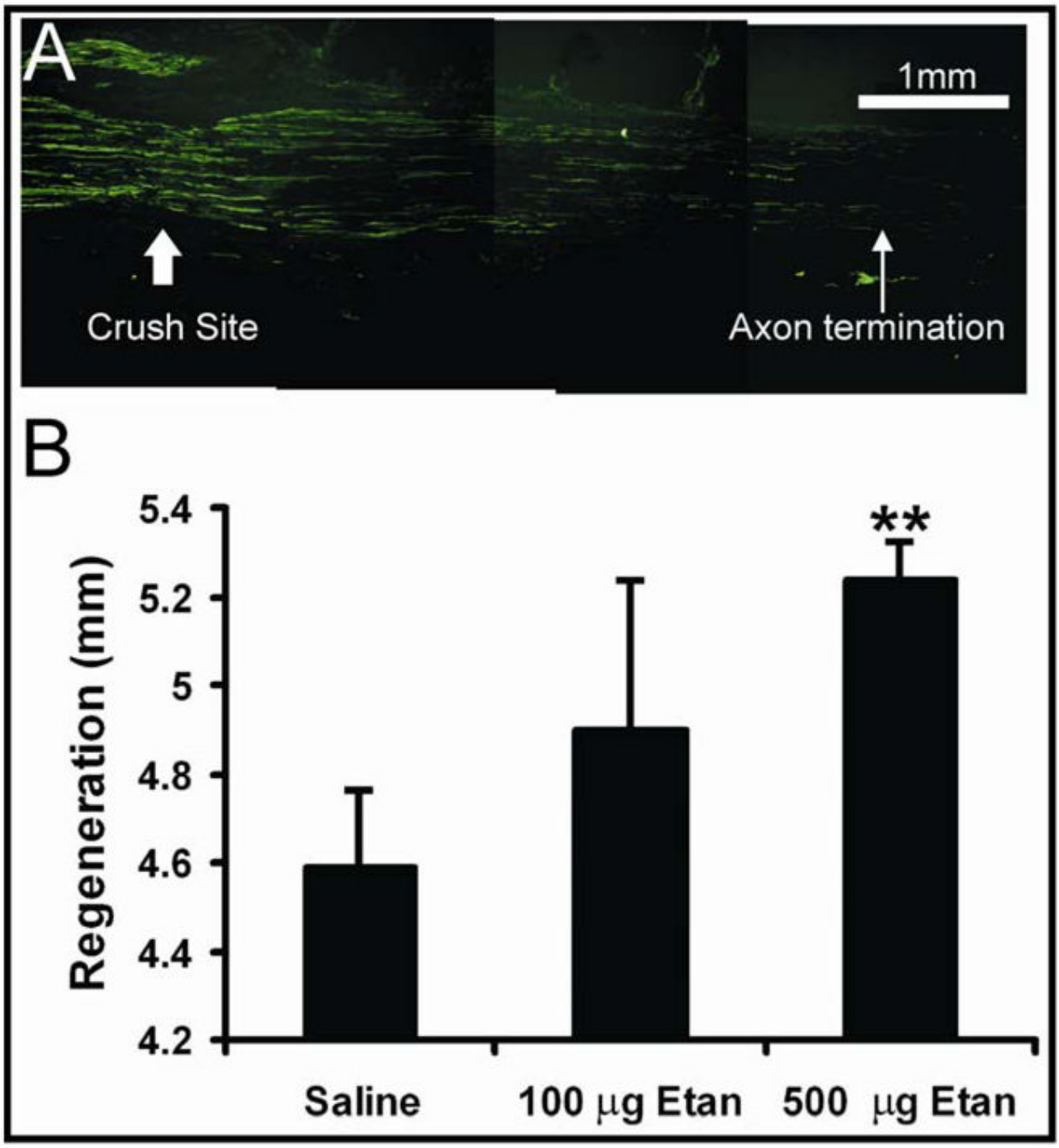

FIGURE 11.

Inhibition of TNFα signaling by etanercept increases sciatic nerve regeneration distance following nerve crush. **A**, Images of longitudinal sciatic nerve section showing regeneration of axons 3 days post sciatic nerve crush in a saline treated animal. Distance of axon regeneration was measured from the crush site to the furthest point the longest regenerating axon had extended, using immunofluorescent probing for GAP43 (2.5× magnification). **B**, Adult male Sprague-Dawley rats were injected s.c. with either 100 μg or 500 μg etanercept in 1 ml of saline, or 1 ml saline alone. After 2 hours, rats were subjected to sciatic nerve crush as described in methods. Three days later, rats were killed and perfuse-fixed, and sciatic nerves were removed and longitudinally sectioned. Nerves were stained for GAP-43, a neuron-specific

growth associated protein (longitudinal sections shown at top), and axon regeneration distance determined (bottom). Values are means ± SE (n=3–6); **p<0.05 vs. saline-injected controls.