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RESEARCH ARTICLE

EFFECT OF INTRANASAL NGF ADMINISTRATION IN INJURED SPINAL CORD AND LEPTIN LEVELS IN ADULT RATS

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Abstract

Spinal cord injury alters a number of endogenous biological signals known to be involved in the modulation of neurotrophic and neuroprotective events. Nerve growth factor (NGF) is a neurotrophic factor expressed in neuronal and non-neuronal tissues including spinal cord, and increases after spinal cord injury. Recent findings revealed that leptin, an adipocyte-derived cytokine (adipokine), enhance neuronal survival and exert neuroprotective action, and played an important role in nociceptive behavior induced by nerve injury. Whether NGF affects the expression of leptin in injured spinal cord has not been investigated. The present study was designed to evaluate: (*i*) whether intranasal NGF administration reached the spinal cord of the rat, (*ii*) if NGF affects the expression of leptin in the spinal cord and adipose tissue, and (*iii*) whether intranasal NGF affects the behavioral and spinal cord neuronal deficits induced by spinal cord injury. The result showed that intranasal NGF enhances the expression of (*i*) NGF and NGF-receptors (TrkA and $p75^{NTR}$) in injured spinal cord exerting behavioral and neuroprotective action, and (*ii*) leptin in injured spinal cord and in subcutaneous (white) and interscapular (brown) adipose tissue. Altogether, the present data demonstrate the efficacy of intranasal administration of NGF, and suggest a link between the neurotrophin NGF and the adipokine leptin that may be therapeutically explored in injured spinal cord.

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Key words: adipose tissue, leptin, NGF, $p75^{NTR}$, TrkA, spinal cord injury

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Introduction

The nerve growth factor (NGF) and its receptors, TrkA and $p75^{NTR}$, are known to play a critical role in the survival, and protective action in a number of nerve cells of the peripheral and central nervous systems (1-3). NGF and receptors are also expressed in the spinal cord (4,5), but the functional significance of these NGF signals in spinal cord injury (SCI) is not clear. Spinal cord injury has been shown to alter, not only NGF expression, but also other endogenous biological signals, including leptin, an adipocyte-secreted cytokine (adipokine) (6-9), which has been shown to enhance neuronal survival *in vitro* and *in vivo* and reduce experimentally induced neuronal damages (10-12). It has also been reported that SCI can be associated with obesity and altered circulating levels of various adipokine including leptin (6,7,13,14), while NGF administration can restore synaptic inputs in axotomized spinal cord motoneurons (15-17). However, whether a correlation exists between NGF signals and leptin in SCI has not been investigated.

Other findings have shown that large molecules, including NGF can be delivered to the central nervous system (18-20), including spinal cord (21), via olfactory pathways reaching brain neurons through the anatomical connections, naso-lacrimal duct and brain ventricles. These observations suggested investigating the effect of intranasal (IN) NGF administration on spinal cord injury, leptin levels in the spinal cord and adipose tissue in adult rats with SCI. The results showed that this non-invasive method of NGF administration can reach the spinal cord, reduce the deficit of locomotor activity, induced by SCI, and enhances the presence of leptin, in both spinal cord and adipose tissue.

Materials and Methods

2-month old male Sprague-Dawley rats raised in our animal facilities were used for this study. The animals were housed in polypropylene cages (2 animal per cage) under standard light/dark conditions with food pellets and water *at libitum*.

Surgery. Animals (n=22) were deeply anesthetized with intraperitoneal injection of 3% pentobarbital sodium (30mg/kg). The spinal cord was exposed at the level of the vertebral segments T8-T10. The T10 vertebral lamina and the dura matter were removed, and a surgical lesion was done to the half portion of the spinal cord. Before closing the wounded tissues, the animal received one intrathecal administration of 5ug of NGF dissolved in 5ul of physiological solution. The first intrathecal injection of 5ul of NGF was followed by 10ul of daily intranasal (IN) NGF administration of (200ug/ml, dissolved in physiological solution), for 3 consecutive weeks. An equal number of rats received IN administration of physiological solution and served as controls (CTRL).

Post-operative period included feeding care twice a day, constant room temperature, manual abdominal compression until urinal reflex was established and urinary bladder evacuated for operation. The mortality rate during and immediately after surgery was less than 10%. All experimental procedures were approved by the local animal committee and carried out in accordance with the Guidelines for the care and use of Laboratory Animals published by National Institutes of Health, USA.

NGF administration

The NGF used for this experiment was purified from adult male mouse submaxillary salivary gland following the method described by Bocchini and Angeletti (22). Once purified, aliquots of NGF were dissolved in physiological solution (0.9% NaCl) at concentration 200 μg/ml and stored at –70°C until used for topical nasal administration.

Locomotor behavior. To test the locomotor activity and the effect of NGF, we measured the time that each animals need to reach a food source distant one meter. The effect of NGF administration was evaluated using a semiquantatative analysis for the hind limb function as described (23).

Biochemical analyses. Animals were sacrificed with an overdose of 3% pentobarbital sodium (30mg/Kg) and injured and non-injured spinal cord, as well as subcutaneous adipose tissue and brown adipose tissue (from interscapular area), carefully removed and immediately stored at -80°C for NGF, NGF receptor and leptin determinations.

NGF determination. Tissues were homogenized with ultrasonication in Radio Immune Precipitation Assay (RIPA) buffer (10 mM tris-HCl, pH7.4; 100 mM NaCl; 1 mM EDTA; 1 mM EGTA; 1% Triton X-100; 10% glycerol; 0.1% SDS; 2 mM Na-3VO4; 20 mM Na4P2O7; 1 mM NaF; 2 mg/ml aprotinin; 1mM PMSF; 1μg/ml leupeptin), centrifuged at 4°C for 20 min at 13000 rpm, then supernatant was recovered and used for NGF, determination as suggested by the instructions provided by the manufacturers. (NGF ELISA kits, Emax Immuno Assay System, were purchased from Promega (Madison, WI, USA). Assays were performed in duplicate and the data are expressed as concentration of growth factors pg/μg of total proteins.

Western blot analysis of leptin. Briefly, tissues were homogenized with ultrasonication in RIPA buffer (50 mM tris-HCl, pH7.4; 150 mM NaCl; 5 mM EDTA;; 1% Triton X-100; 0.1% SDS; 0,5% DOC (Sodium deoxycholate; 1mM PMSF; 1μg/ml leupeptin), centrifuged at 4° C for 20 min at 13000 rpm, then supernatant was storage at -20°C. Samples (30μg of total protein) were dissolved in loading buffer (0.1 M Tris–HCl buffer, pH 6.8, containing 0.2 M dithiothreitol, DTT, 4% sodium-dodecil-phosphate, SDS, 20% glycerol, and 0.1% bromophenol blue), separated by 8% or 12% SDS-PAGE, and electrophoretically transferred to PVDF membrane overnight. The membranes were incubated for 1 hr at room temperature with blocking buffer constituted by 5% BSA (for TrkA) or non-fat dry milk (for GAPDH) in TBS-T (10 mM Tris, pH 7.5, 100 mM NaCl, and 0.1% Tween-20). Membranes were washed three times for 10 minutes each at room temperature in TBS-T followed by incubation at 4°C with primary antibodies overnight polyclonal rabbit anti-TrkA 1:1000 (Santa Cruz biotechnology, CA, USA), polyclonal rabbit anti-leptin 1:1000 (Santa Cruz biotechnology, CA, USA). Membranes were washed three times for 10 minutes each at room temperature in TBS-T and incubated for 1 hour with horseradish peroxidase-conjugated anti-rabbit IgG 1:4000 or horseradish

peroxidase-conjugated anti-mouse IgG as the secondary antibody (Cell Signaling Technology, MA, USA) at room temperature. The blots were developed with an ECL chemiluminescent horseradish peroxidase (HRP) substrate as the chromophore (Millipore, MA, USA). The public Image J Software was used to evaluate band density, which was expressed as arbitrary units of grey level. The Image J program determines the optical density of the bands using a grey scale shareholding operation. The optical density of polyclonal rabbit anti-GAPDH 1:4000 (Santa Cruz biotechnology, CA, USA) bands was used as a normalizing factor. For each gel blot, the normalized values were then expressed as percentage of relative normalized controls and used for statistical evaluation.

Histological and immunohistochemical analyses. The animals were deeply anesthetized and perfused through the ascending aorta, first with physiological solution and then phosphate buffer 0.1M, pH 7.4 followed by 300 ml of buffered 4/% paraformaldhehyde. The spinal cord was removed, post-fixed overnight with the same fixative and then in phosphate buffer containing 20% of sucrose. Coded twenty-micron thick sections were then cut with a cryostat and stained with toluidine blue for histological observation or immunostained for localization of NGF receptor and leptin.

For immunohistochemical staining, spinal cord sections sectioned with a cryostat, at 20 micron thick, followed by exposure to 0.1M PBS containing 10% of horse or goat serum for 1 hour, then incubated overnight at 4°C with monoclonal mouse anti-p75 1:100, rabbit anti-leptin (Santa Cruz, CA, USA). After washing, sections, after having been washed, were then exposed to biotinylated anti-mouse or anti-rabbit IgG 1:300 (anti-IgG and avidin-conjugated horseradish peroxidase complex) were purchased by Vector Laboratories (Burlingame, CA, USA) with 2% of goat or horse serum, depending on the animal in which the secondary antibody was produced, for 2 hours at room temperature, and then to immunoperoxidase staining was performed using an ABC (1:100) (Avidin-Biotin complex solution, Vectastain Elite Kit (Vector Laboratories, CA, USA) for 2 hours at room temperature. All the sections studied passed through all procedures simultaneously to minimize any difference from immunohistochemical staining itself. Signals were visualized by DAB (3,3'-diaminobenzidine) as a chromate. Sections incubated identically with normal IgG was used as negative controls. Stained sections were visualized using a Zeiss Axiophot microscope equipped with a 40X objective with the aid of a computerized image analysis system. Sections (n=10) of each NGF-treated and NGF-untreated rats were used for a quantitative analysis to count the number of immunostained spinal cord cells in 8 fields of 4 section of 4 different rat per experimental group as previously described (24).

Statistical Analysis

Statistical evaluations were performed using the Stat View package for Windows and data were expressed as mean±SEM. A *post-hoc* comparison within logical sets of means was performed using Tukey's test. A p-value of less than 0.05 was considered significant. ELISA and western blot (n=6 for each experimental group) were evaluated by one-way ANOVA.Post-hoc comparisons were performed using the Tukey's HSD test. A p-value less than 0.05 were considered significant.

Results

Figure 1A shows an anesthetized rat after spinal cord injury that was induced in the gray matter. The lesion of the spinal cord causes neuronal damages and inflammatory cell infiltration, including mast cells increase (Fig.1C). Figure 1D reports the locomotor activity of CTRL rats, rat with SCI, and rats with SCI treated for 3 weeks with IN NGF administration (SCI/NGF). The non-operated rats need 8 sec to reach the food source, rats with SCI treated with NGF 12 sec, whereas rats with SCI but untreated with NGF needs more that 30 sec.

Intranasal NGF reaches the injured spinal cord

The levels of NGF in the injured spinal cord increases after IN NGF administration (SCI plus NGF) compared to controls and operated untreated rats (Fig. 2A). NGF administration can bypass the blood-brain barrier and can reach injured spinal cord neurons. Quantitative evaluation indicates that the increase is statistically significant (Fig. 2D).

Structural analyses

Since the low-affinity NGF $p75^{NTR}$ is implicated in mediating survival of injured motoneurons after axotomy (22), we studied the expression of this receptor. Immunohistochemical analysis performed on section of spinal cord of the three rat groups revealed that $p75^{NTR}$ neurons are more numerous in the injured spinal cord of rats treated with NGF (SCI plus NGF), compared to the injured spinal cord of NGF untreated rats (SCI). In spinal cord sections immunostained with $p75^{NTR}$, the number of $p75^{NTR}$-positive neurons are markedly reduced as compared to controls, while IN NGF administration reduced the SCI, though not completely (Fig. 3). Nonetheless, quantitative evaluation indicated that the differences are statistical significant. This observation suggests that large molecular size, like NGF, can reach spinal cord neurons via olfactory pathways (21) and can exert neuroprotective action.

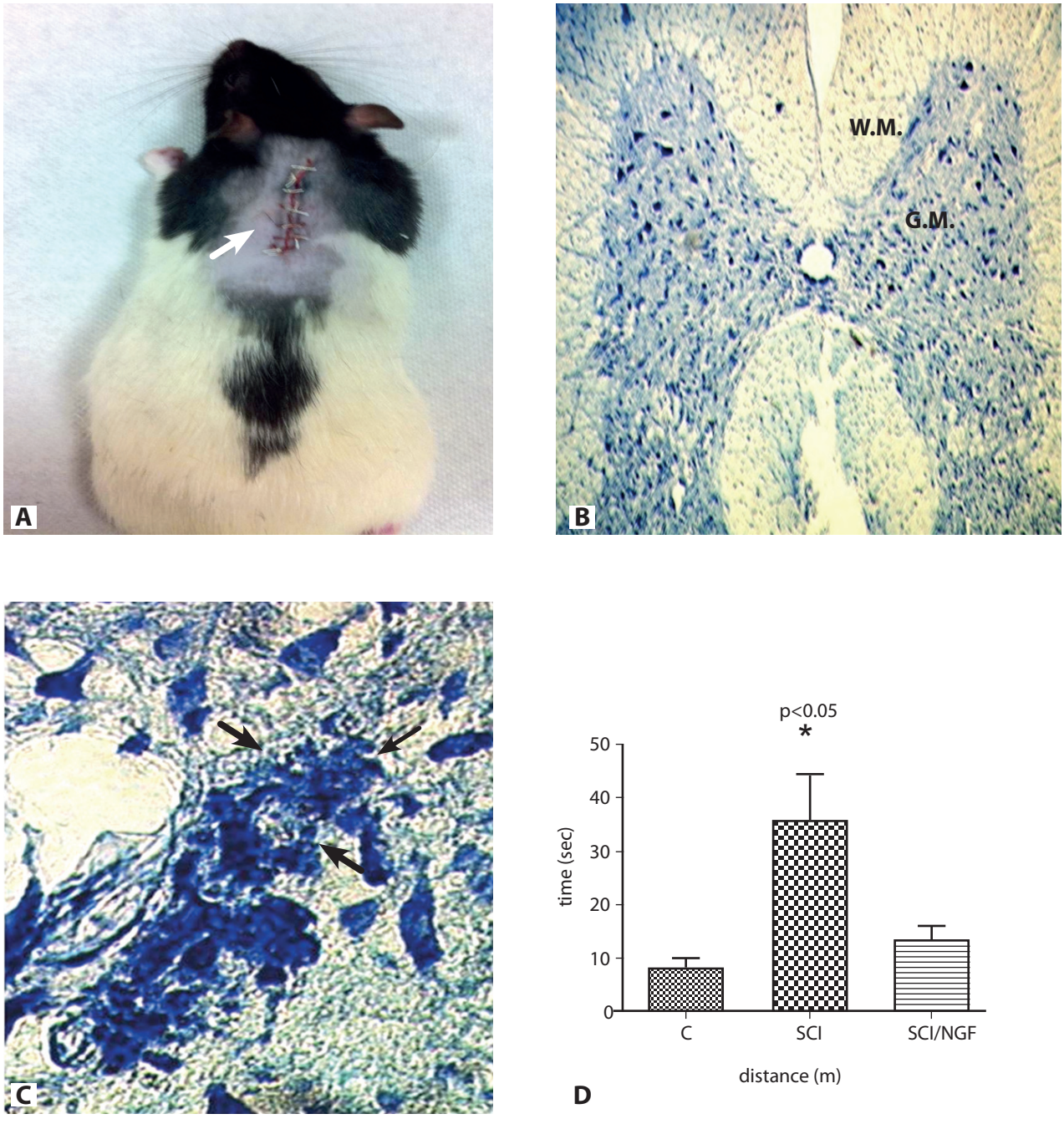


Figure 1. Illustration of anesthetized rat with lesion in the dorsal region (arrow) after spinal cord surgical injury in the dorsal horn (**A**). A representative section of the spinal cord showing nerve cells in the gray matter (GM) and glial cells in the white matter (WM.); the lesion was induced in the GM region (**B**). This lesion causes neuronal damages inflammatory cell infiltration (**C**), including increase of mast cells. The results of locomotor activity of CTRL, rat with SCI, and rats with SCI treated for 3 weeks with NGF administration (SCI/NGF), evaluated as the time needed to cover a distance of 1 meter (**D**). Note that the non-operated rats need 8 sec to reach the food source, rats with SCI treated with NGF 12 sec and rats with SCI but untreated with NGF over 30 sec. Magnification, B: X-35; C: X-310.

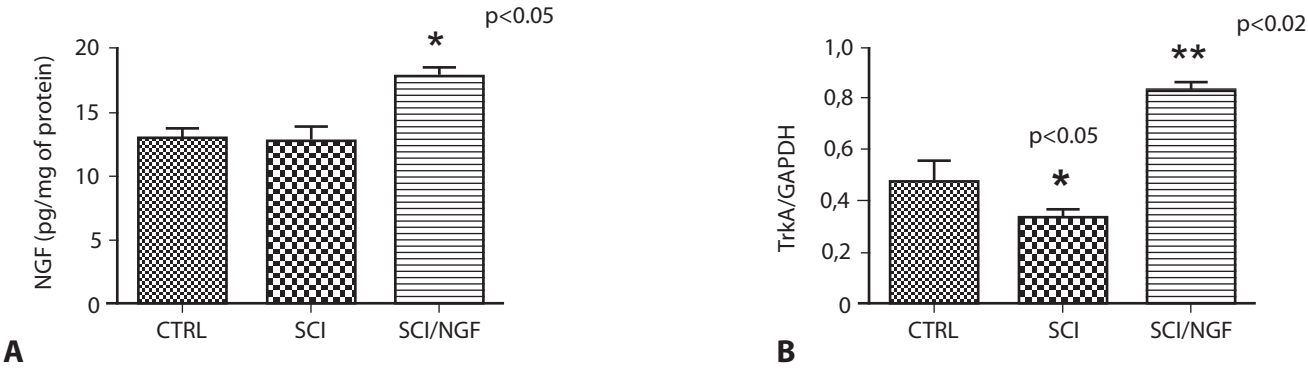


Figure 2. Levels of NGF in the spinal cord of rats untreated and treated with IN NGF (**A**). The concentration of NGF in injured spinal cord treated for 3 consecutive weeks with IN NGF administration (SCI/NGF) increase, compared to control (**CTRL**) and operated NGF untreated rats (SCI), p<0.05 compared and untreated injured spinal cord (SCI). Expression of TrkA protein evaluated with western blot in the spinal cord of in injured spinal cord treated for 3 consecutive weeks with IN NGF administration (SCI/NGF) (**B**). In the injured NGF-untreated spinal cord, TrkA decreases after IN NGF administration, p<0.05.

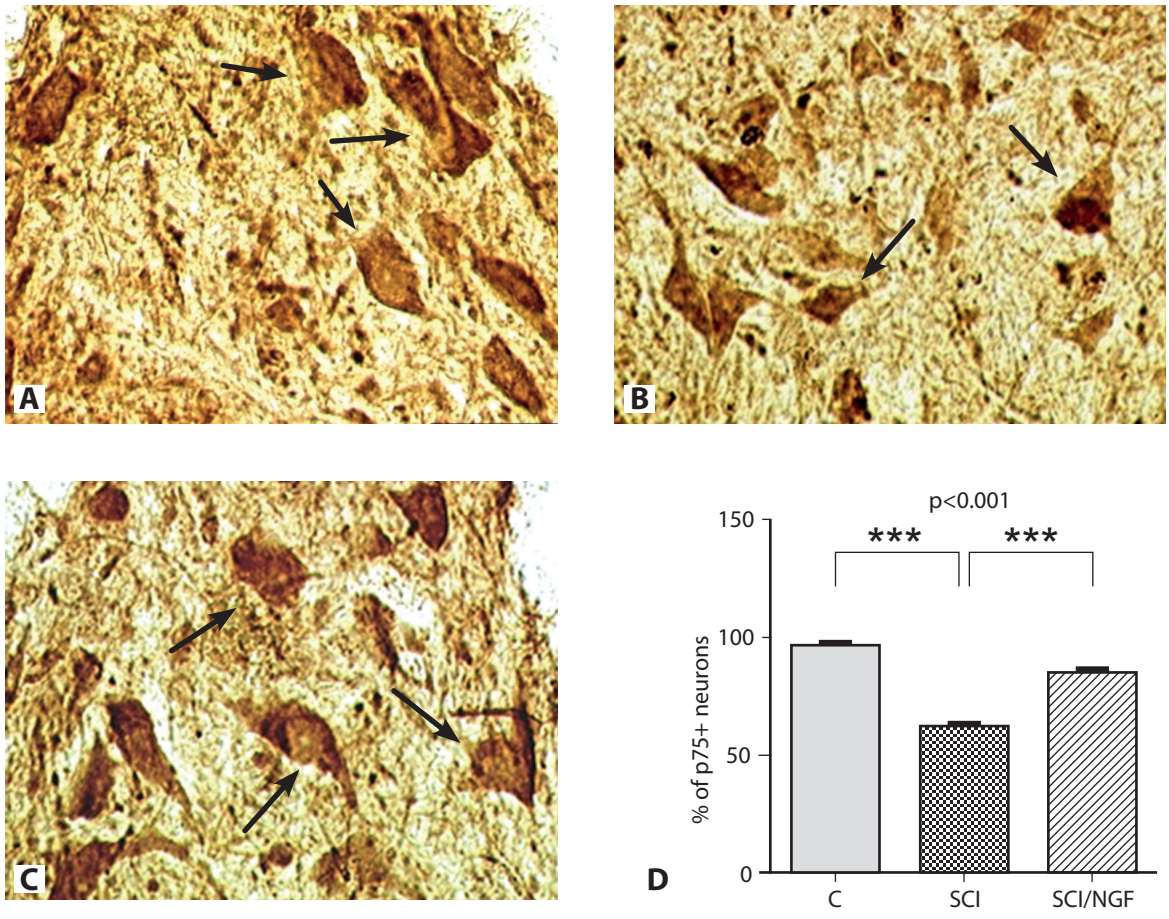


Figure 3. Representative spinal cord section of control (**A**), injured untreated (**B**) and injured NGF-treated (**C**) rats, 3 weeks after daily nasal NGF administration, and immunostained with the low-affinity NGF receptor, $p75^{NTR}$**.** Note the loss of this NGF-receptor in neurons of the gray matter after SCI, (**B**), compared to control spinal cord neurons, and the protective action after NGF administration, indicated by arrows. Quantitative evaluation indicated that these differences are statistically significant (**D**). Magnification, A-C: X 310.

Western blot analysis. NGF administration enhanced the expression of the high-affinity NGF TrkA receptor compared to control rats and untreated SCI (Fig. 3B).

Spinal cord injury had no effect on NGF levels in blood and adipose tissues, while NGF administration enhances the concentration of NGF in spinal cord and adipose tissue (Fig. 4).

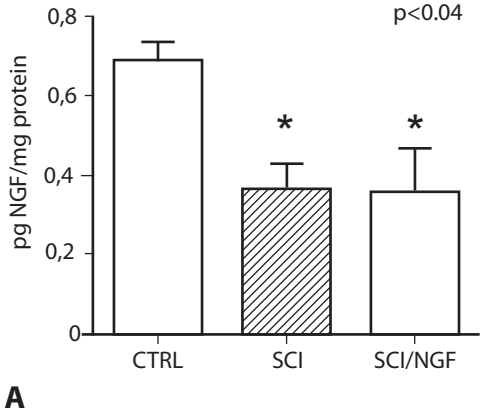
Intranasal NGF administration alters leptin levels in spinal cord

Because a number of studies indicate that SCI is associated with obesity, we evaluated the presence of leptin in the spinal cord and in subcutaneous adipose tissue. Immunohistochemical and Western blot analysis indicated that the levels of leptin is enhanced by SCI, and NGF administration reduced this increase, though the level of leptin remains above the control levels (Fig. 5).

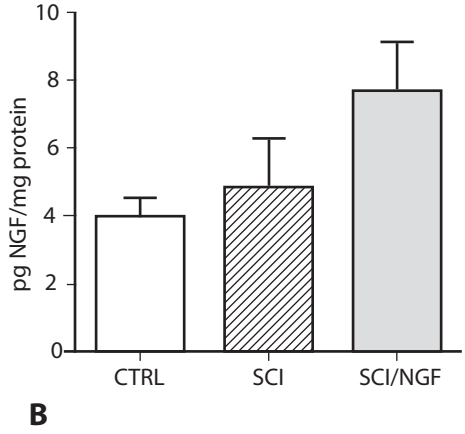
Discussion

Leptin is an adipokine exerting its actions via endocrine and paracrine pathway, playing a well-known key regulatory action on food intake and energy expenditure, also inflammation and immunity (6,7). Recent studies have demonstrated that leptin, like NGF, can affect neuronal survival *in vitro* and *in vivo* and reduce experimentally induced neuronal damages (10,12) and that NGF is expressed and released by adipose tissues (25,26). However, whether there is any functional relationship between these two endogenous signals in SCI has not been investigated.

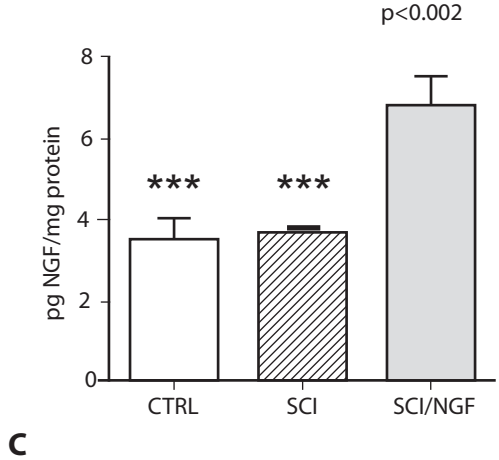
The present study was designed to evaluate: (*i*) whether IN NGF administration reached the spinal cord, (*ii*) if NGF affects the expression of leptin in the spinal cord and adipose tissue, and (*iii*) whether NGF affected the behavioral and spinal cord injury induced by SCI.



A



B



C

Figure 4. shows the levels of NGF in the serum (**A**), subcutaneous adipose tissue (**B**) and brown adipose tissue (**C**) in control rats and injured spinal cord untreated (SC) and treated while IN NGF administration enhances the levels of NGF in both subcutaneous adipose tissue (**B**) and brown adipose tissue (**C**) compared to controls. p<0.05.

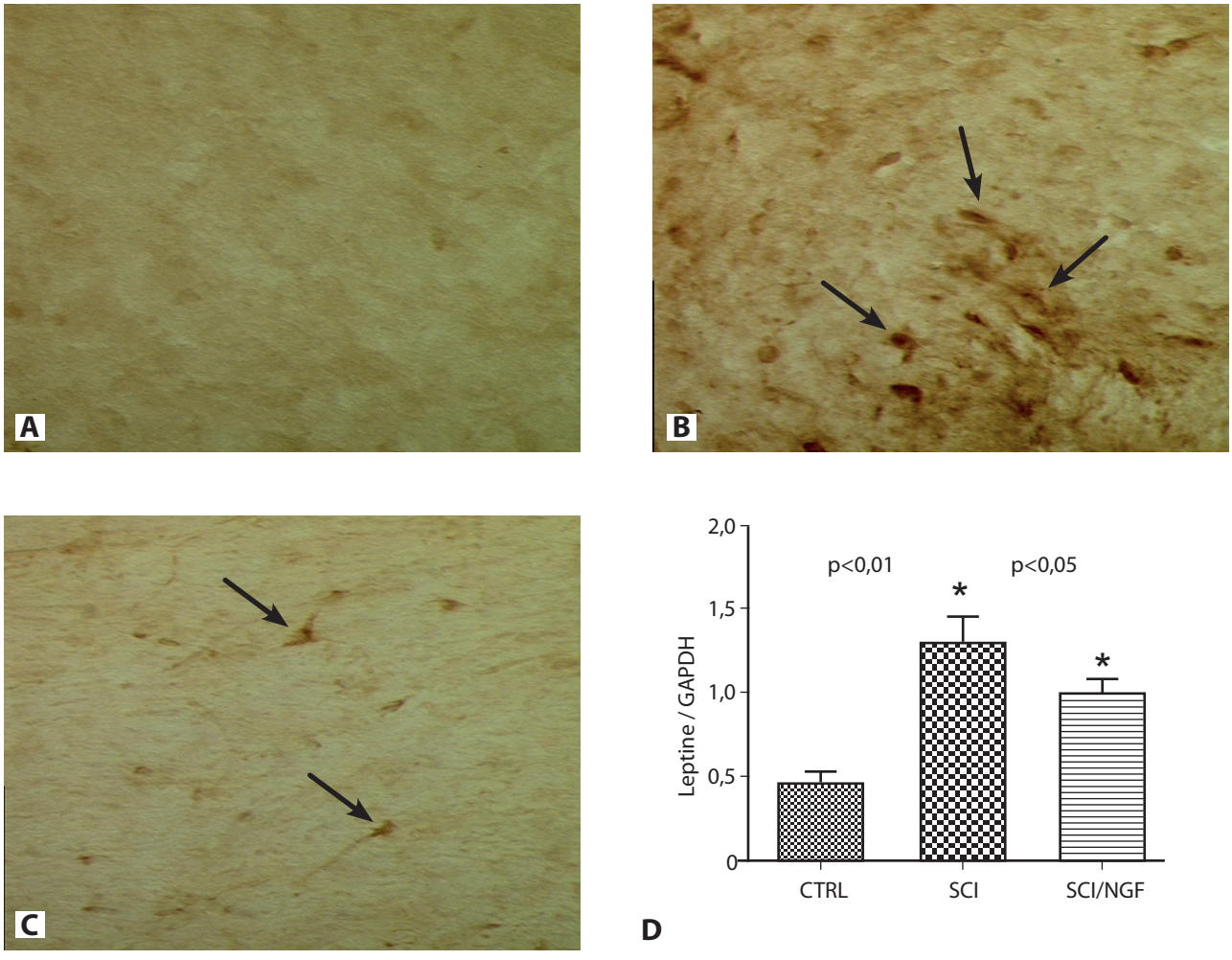


Figure 5. Leptin immunoassayed representative sections of rat's spinal cord of CTRL (**A**), injured NGF untreated (**B**) and injured NGF-treated injured spinal cord for three weeks (**C**). Note the absence of leptin immonostaining in control spinal cord (A), the marked increase after SCI (**B**) compared to control and NGF-treated (**D**). Magnification, A-C: X 170. Western blot analysis of control, untreated injured spinal cord (SCI) and NGF treated injured spinal cord (ISC/NGF). *p<0.01 and p<0.05.

Regarding the first question we found that IN NGF administration reaches the injured spinal cord neurons and enhances the expression of TrkA NGF receptor and $p75^{NTR}$. Since these receptors regulate the biological activity of the NGF (2,27), this observation suggests that the nasal NGF administration reaches the spinal cord as functional active molecule. The fact that three consecutive weeks of NGF administration protects the degenerating neurons in injured spinal cord supports this hypothesis.

Because leptin promotes neuronal activity (10-12), and has been indicated as a possible mediator of therapeutic target for degenerating brain neurons (26-30), we tested whether NGF administration affects the expression of leptin. It was found that IN NGF administration enhances the level of leptin in the injured spinal cord compared to controls, suggesting a possible bidirectional functional activity between NGF and leptin.

A critical question raised by these observations regards the source of NGF and the role of NGF on leptin level. Since a number of non-adipose cells produces leptin (4,6,26,29) and a number of non-neuronal cells produces NGF (31-35), the possibility that different cell types produce and release both NGF and leptin and thus in tandem exert a protective action on spinal cord cells cannot be excluded. Indeed, data reporting that combination of different molecules can enhance neuroprotection and functional recovery following SCI support this hypothesis.

In summary, the present pilot study provided the first convinced evidence that injured spinal cord neurons respond to

nasal NGF by enhancing the expression of both NGF/receptors and leptin and that administration of NGF via olfactory pathways, most likely similar to ocular administration (38). Whether prolonged nasal NGF administration, alone or in combination with leptin, reduces and/or delay neurodegeneration in the spinal cord and whether the effect is long lasting need to be investigated. Since NGF has been used for certain human disorders, it might be of interest further experimental studies to investigate the potential benefits of IN NGF in injured spinal cord neurons and in the absence of invasive and undesired side effects, not only in animal models.

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