Original article

Effect of intranasal nerve growth factor administration in rats with spinal cord injury

A. DE BELLIS, M.-L. ROCCO, P. BIANCHI*, L. ALOE*

"Maria Rosaria Maglione" Foundation, Naples, Italy

* Institute of Neurobiology and Molecular Medicine, National Research Council (CNR), Rome, Italy

SUMMARY: BACKGROUND AND AIMS. Nerve growth factor (NGF) is known to play a critical protective role on a number of brain neurons in mammals, including humans. However, its role in the spinal cord is still unclear. Indeed, NGF does not cross the blood-brain barrier if injected subcutaneously or intravenously, and another delivery method is therefore required. Hence the aim of this study was first to investigate whether purified NGF reaches spinal cord neurons and has any effect on the motor skills skills of rats with indued spinal cord injury, and, second, to determine its effect on NGF concentrations and NGF-receptors in injured spinal cord neurons in adult rats when administered via the nasal cavity.

MATERIALS AND **METHODS.** Spinal cord injury was surgically induced in adult rats under deep anaesthesia. Purified NGF was then administered to these rats via a single intrathecal injection during surgery and then via the nasal cavity for three consecutive weeks. Untreated controls were subjected to the same experimental protocol but were administered physiological solution in place of NGF. Treated and untreated spinal cord injury rats were subjected to locomotor tests (against healthy controls) prior to sacrifice, spinal tissue harvesting and biochemical and histological analysis, 24 hours after the final dose of intranasal NGF.

RESULTS AND CONCLUSIONS. We found that NGF administered via the nasal route did in fact reach the spinal cord, where it increased the concentration of NGF and enhanced the expression of both high- and low-affinity NGF receptors 24 hours after administration. It was evident that this daily treatment reduced the degenerative signal of injured spinal cord neurons, suggesting that administration of NGF via the nasal cavity could be a useful non-invasive method of NGF delivery into the spinal cord without apparent side effects. This finding opens new avenues for exploring the role of NGF, either alone or in combination with other identified neuroprotective molecules, in spinal cord injury.

KEY WORDS: Nasal administration route, Nerve growth factor, Spinal cord injury.

\Box INTRODUCTION

Nerve growth factor and its receptors are known to play a crucial role in the survival, differentiation and protective action of cholinergic neurons in developing and adult mammalian forebrains^(1,7,13). NGF and NGF receptors are also expressed in the spinal cord cells^(2,5,11), and it has been reported that NGF increases over 4-fold after spinal cord injury⁽¹⁴⁾, suggesting a similar function in this location.

Indeed, it has been demonstrated that NGF administration can restore synaptic inputs in axotomized spinal cord neurons^(8,20), suggesting that these neurons are receptive to NGF signals. That being said, this

Correspondence: Dr. Alberto de Bellis, "Maria Rosaria Maglione" Foundation, via Francesco Petrarca 113, 80123 Naples (NA), Italy, fax +39-(0)81-2542770, e-mail: albertodebellis@hotmail.com

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LIST OF ACRONYMS AND ABBREVIATIONS: ABC = Avidin-Biotin Complex; ANOVA = ANalysis Of VAriance; BDNF = Brain-Derived Neurotrophic Factor; BSA = Bovine Serum Albumin; CTRL = healthy ConTRoL (rats); DAB = 3,3'-DiAminoBenzidine; DOC = sodium DeOxyCholate; DTT = DiThioThreitol; ECL = Enhanced ChemiLuminescent; EDTA = EthyleneDiamineTetraacetic Acid; EGTA = Ethylene Glycol Tetraacetic Acid; EIA = Enzyme ImmunoAssay; ELISA = Enzyme-Linked Immunosorbent Assay; GAPDH = GlycerAldehyde 3-Phosphate DeHydrogenase; HCI = Hydrochloric Acid; HSD = Honestly Significant Difference; HRP = HorseRadish Peroxidase; IgG = Immunoglobulin G; IN = IntraNasal; NGF = Nerve Growth Factor; NGFRs = NGF Receptors; NT-3 = NeuroTrophin-3; PBS = Phosphate Buffered Saline; PMSF = PhenylMethaneSulfonylFluoride or PhenylMethylSulfonyl Fluoride; PVDF = PolyVinylidene DiFluoride; pTrkA = phospho Tyrosine kinase receptor A; rpm = revolutions per minute; RIPA = Radio Immune Precipitation Assay; SCI = Spinal Cord Injury; SCI/NGF = Spinal Cord Injury rats treated for three consecutive weeks with intranasal Nerve Growth Factor administration; SDS = Sodium Dodecyl Sulphate; SDS-PAGE = Sodium Dodecyl Sulphate - PolyAcrylamide Gel Electrophoresis; SEM = Standard Error of the Mean; TBS-T = Tris Buffered Saline-Tween; TrkA = Tyrosine kinase receptor A; Tris = Tris(hydroxymethyl)aminomethane.

postulated neuroprotective effect on spinal cord neurons still remains controversial, and the debate is reinforced by the lack of a feasible non-invasive method for delivering NGF directly into the spinal cord.

However, findings published over the last few years have shown that large molecules, including NGF, can be administered to the central nervous system via intranasal pathways^(6,10), and other studies have shown that other neurotrophic factors delivered via IN pathways can reach spinal cord neurons^(18,19). Based on these observations, we tested the effect of IN NGF administration on the spinal cord of rats with experimentally induced SCI to determine whether intranasal administration of NGF could represent a viable and effective means of treating spinal cord lesions.

□ MATERIALS AND METHODS

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

SCI-INDUCTION SURGERY. Adult rats were deeply anaesthetized via intraperitoneal injection of 3% sodium pentobarbital (30 mg/kg). The spinal cord was exposed microsurgically at vertebral segments T8-T10. The T10 vertebral lamina was removed and the surgical lesion was performed using a Yasargil titanium aneurysm clip, without opening the dura mater. Before closing the wound, test subject animals received an intrathecal administration, in the space under the arachnoid membrane of the spinal cord, of 5 µg of NGF dissolved in 5 µl of physiological solution, while controls received 5 µl of physiological solution alone. The animals were housed in cages (2 animals per cage), under standard light/dark conditions, with food pellet and water *ad libitum*. Post-operative care included feeding twice a day, constant room temperature, manual abdominal compression until urinal reflex was established (the urinary bladder was evacuated before the operation). The mortality rate during and immediately after surgery was lower than 12%. All experimental procedures were approved by the local animal welfare committee and carried out in accordance with the guidelines for the care and use of laboratory animals published by the Italian National Institute of Health.

■ NGF PURIFICATION AND ADMINISTRATION. The NGF was purified from the adult male mouse submaxillary salivary gland, following the method described by Bocchini and Angeletti⁽³⁾, and stored at -70 °C until used.

Following induction of SCI but prior to wound closure, 8 SCI-induced rats were given a single intrathecal injection of 5 μ l of 200 μ g/ml NGF dissolved in physiological solution and administered using a 25 Hamilton syringe with 27-gauge needle. 18 further rats were treated in the same way, but also received daily intranasal administration (via spray) of 10 μ l NGF (200 μ g/ml, dissolved in physiological solution) for 3 consecutive weeks. Equal numbers of control rats were subjected to the same experimental protocols but received physiological solution alone. 18 rats were subjected neither to the surgery nor to drug/placebo administration and served as healthy controls.

The single-dose groups (intrathecal NGF) of treated rats were sacrificed 24 hours after surgery prior to tissue harvesting and immunohistochemical analysis, while the multiple-dose (intrathecal + intranasal NGF) groups were sacrificed after 3 weeks of treatment, 24 hours after their final dose.

■ LOCOMOTOR BEHAVIOR. Prior to sacrifice and tissue analysis, treated and untreated SCI-induced rats from the multiple-dose groups were subjected to locomotor tests, and their performance compared with that of healthy controls. We measured the time it took each rat to reach a food source located one metre away. The effect of NGF administration was also evaluated using a semiquantitative analysis of hind limb function, as previously described⁽¹⁶⁾.

■ TISSUE DISSECTION AND PRESERVATION. All animals were sacrificed using an overdose of 3% sodium pentobarbital (30 mg/kg). After sacrifice, control and lesioned spinal cords were careful removed and freed of their meninges, vessels, spinal roots and dorsal root ganglia. All tissue samples scheduled for NGF and NGF-receptor assay were immediately stored at -80 °C. Prior to morphological and immunohistochemical analysis, removed spinal cords of both controls and operated rats were fixed in 4% paraformaldehyde dissolved in 0.1 M phosphate buffer, pH 7.4, for 24 hours, and then left overnight in the same buffer containing 20% sucrose.

■ NGF ASSAY. To determine the concentration of NGF, tissues were homogenized by ultrasonication in RIPA buffer (10 mM tris-HCl, pH 7.4; 100 mM NaCl; 1 mM EDTA; 1 mM EGTA; 1% Triton X-100; 10% glycerol; 0.1% SDS; 2 mM Na₃VO₄; 20 mM Na₄P₂O₇; 1 mM NaF; 2 µg/ml aprotinin; 1 mM PMSF; 1 µg/ml leupeptin), then centrifuged for 20 min at 13,000 rpm at 4 °C. The supernatant was then recovered and subjected to NGF assay, according to the instructions provided by the manufacturers. (NGF ELISA kits, Emax ImmunoAssay System, purchased from Promega, Madison, Wisconsin, USA). Assays were performed in duplicate and the data are expressed as concentration of growth factors pg/µg of total proteins.

■ WESTERN BLOT ANALYSIS. Tissues were homogenized by ultrasonication in RIPA buffer (50 mM tris-HCl, pH 7.4; 150 mM NaCl; 5 mM EDTA; 1% Triton X-100; 0.1% SDS; 0.5% DOC (Sodium deoxycholate; 1 mM PMSF; 1 µg/ml leupeptin), then centrifuged for 20 min at 13,000 rpm at 4 °C. The supernatant was then stored at -20 °C.

Samples (30 µg of total protein) were dissolved in loading buffer (0.1 M tris-HCl buffer, pH 6.8, containing 0.2 M DTT, 4% 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 and pTrkA) or non-fat dry milk (for p75, GAPDH) in TBS-T (10 mM Tris, pH 7.5, 100 mM NaCl, and 0.1% Tween-20). Membranes were washed three times in TBS-T for 10 minutes each at room temperature, followed by overnight incubation at 4 °C with the primary antibodies polyclonal rabbit anti-TrkA 1:1,000 (Santa Cruz Biotechnology, California, USA), monoclonal mouse anti-p75 1:1,000 (Santa Cruz Biotechnology, California, USA), and monoclonal mouse anti-pTrkA 1:1,000 (Santa Cruz Biotechnology, California, USA). Membranes were washed three times in TBS-T for 10 minutes each at room temperature, and incubated for 1 hour with horseradish peroxidase-conjugated antirabbit IgG 1:4,000, or horseradish peroxidaseconjugated anti-mouse IgG as the secondary antibody (Cell Signaling Technology, Massachusetts, USA) at room temperature.

Blots were developed with an ECL HRP substrate as the chromophore (Millipore, Massachusetts, USA). Public ImageJ software was used to evaluate band density, which was expressed as arbitrary units on a grey scale, which the ImageJ software uses as a measure of the optical density of the bands. The optical density of polyclonal rabbit anti-GAPDH 1:4,000 (Santa Cruz Biotechnology, California, 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 subjected to statistical evaluation. At least 8 spinal cord horns per experimental group, taken from different rats, were tested, and experiments were conducted in triplicate. Statistical evaluations were performed using the GraphPad Prism package for Windows, and data were expressed as means \pm SEM. MORPHOLOGICAL ANALYSIS AND IMMUNOHISTO-CHEMISTRY. Spinal cord sections were immunostained with NGF-receptor antibodies (Santa Cruz Biotechnology, California, USA). Briefly, sections were incubated for 1 hour in PBS containing 10% of horse or goat serum, and then overnight at 4 °C with polyclonal rabbit anti-TrkA 1:100 (Santa Cruz Biotechnology, California, USA), monoclonal mouse anti-p75 1:100 (Santa Cruz Biotechnology, California, USA). After washing, sections were exposed to biotinylated anti-mouse or anti-rabbit IgG 1:300 (anti-IgG and avidin-conjugated horseradish peroxidase complex), purchased from Vector Laboratories (California, USA), with 2% goat or horse serum, depending on the secondary antibody produced by the animal, for 2 hours at room temperature. Immunoperoxidase staining was then performed for 2 hours at room temperature using an ABC (1:100) solution and Vectastain Elite Kit (Vector Laboratories, California, USA). All sections studied were subjected to all procedures simultaneously to minimize any variations in immunohistochemical staining.

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Signals were visualized using DAB as a chromate. Sections incubated with normal IgG following an identical procedure were 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 NGF-treated and untreated SCI rats were subjected to quantitative analysis in which the number of immunostained spinal cord cells in 8 fields of 4 sections from 4 different rats per experimental group were counted⁽⁹⁾.

■ STATISTICAL ANALYSIS. Statistical analysis was performed using the StatView package for Windows, and data were expressed as mean \pm 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.

Western blot (n = 6 for each experimental group), ELISA and EIA (n = 10 for each experimental group) analyses were evaluated by one-way ANOVA. Posthoc comparisons were performed using Tukey's HSD test. P-values of less than 0.05 were considered significant.

RESULTS

□ SINGLE-DOSE (INTRATHECAL NGF) SCI. The biohistochemical analyses revealed no differences between treated single-dose SCI rats and controls (data not shown).

□ MULTIPLE-DOSE (INTRATHECAL + INTRANASAL NGF).

- IN NGF improves locomotor capacity in SCI rats. To study whether intranasal administration is associated with the protection of damaged spinal cords, the locomotor performance of rats treated with daily IN NGF administration for three weeks was tested. It was found that the NGF-treated rats displayed the ability to move their legs and reach a food source placed at a distance of one metre far more quickly than their untreated SCI counterparts. As shown in Figure 1, the non-operated rats reached the food source in a mean of 8 s, and SCI rats treated with NGF took 12 s, whereas untreated rats with SCI needed over 30 s to reach the food (Figure 1 A).
- IN NGF administration reaches the spinal cord. After the 3-week experimental protocol and 24



Figure 2. A. The effect of a single dose of intrathecal NGF followed by 3 weeks of daily intranasal NGF on the NGF protein concentrations in the spinal cord, measured via ELISA. B. A representative section of spinal cord immunostained with anti-NGF antibodies. NGF immunoreactivity is mainly localized in the grey matter (GM) region, including neurons, less so in the white matter (WM) (magnification: 30x). C. The result of Western blot analysis of pTrkA, in the injured spinal cord 24 hours after final intranasal NGF administration (SCI/NGF), compared to spinal cord injury untreated rats (SCI). D. Similar findings for p75. Note the enhanced expression of these two NGF receptors after intranasal NGF administration.

hours after their final dose, multiple-dose (treated, untreated and healthy controls) rats were sacrificed as above. We first investigated whether IN NGF administration reaches the injured spinal cord.

- *NGF proteins*. The NGF protein concentrations, measured via ELISA, in the spinal cord of treated rats were significantly increased after IN NGF administration (p < 0.05) (Figura 2 A).
- *NGF location*. Immunohistochemical analysis carried out on spinal cord sections revealed that NGF is mainly localized in cells of the grey matter (Figure 2 B) and neurons.
- *NGF receptors*. The results of Western blot analysis indicate that both high-affinity TrkA (Figure 2 C), and low-affinity p75 (Figure 2 D) NGF-receptors are up-regulated after IN NGF administration, suggesting that nasally adminis-

tered NGF can reach spinal cord neurons and remain functionally active.

- *IN NGF increases NGF and pTrkA*. We then assessed the levels of NGF and TrkA expression to determine whether the locomotor improvement was associated with changes in NGF and pTrkA, the active form of the high affinity NGF receptor. Immunoenzyme assay and Western blot analysis confirmed that the levels of NGF and pTrkA proteins in the injured spinal cord of rats treated with IN NGF administration for 3 weeks (SCI/NGF) are greater (p < 0.05) than those found in the spinal cord of control and NGF-untreated SCI rats (Figura 1 B-C).
- Intranasal NGF exerts a neuroprotective effect in the spinal cord. To investigate the effect of three-week daily IN NGF administration, spinal cord

sections immunostained with TrkA were immunostained with NGF receptors (TrkA) to evaluate the morphological conditions and responsiveness of spinal cord neurons. Qualitative and quantitative analyses of spinal cord sections of indicated that the number of TrkA positive neurons decreases in rats with SCI, while NGF administration protects these neurons, though not completely. These differences were found to be statistically significant (p < 0.05) (Figure 3 A-D). Similar results were observed in spinal cord sections immunostained with p75 (Figures 3 E-H). Thus, SCI causes a decrease in the number of p75-positive spinal cord neurons, as compared to control, while IN NGF administration protects a significant number of these cells.

□ **DISCUSSION**

Following on from recent studies showing that NGF and NGFRs are expressed by spinal cord cells^(5,11), and that spinal cord injury significantly alters the expression of these NGF signals⁽¹⁴⁾, we studied the effect of intranasal NGF administration to confirm that NGF is able to reach the injured spinal cord neurons of SCI rats via this route, which is less invasive and would therefore be of greater clinical utility. Treated rats were given a three-week course of daily intranasal NGF, and showed increased NGF concentrations in the grey matter of the spinal cord with respect to NGF-untreated. Improved locomotor capacity was also detected, and biochemical and structural analysis confirmed that this treatment regime reduced the degenerative signals from the spinal cord neurons induced by SCI.

Furthermore, we showed that IN NGF administration enhances not only the level of NGF, but also the expression of both NGF-receptors, p75 and TrkA, suggesting that intranasally administered NGF reaches the spinal cord in a biologically active form, and is likely to be involved in the neuroprotective action observed. Hence, to confirm the existence of an interaction between exogenous NGF and enhanced up-regulation of TrkA, and to gain additional information about the functional significance of IN NGF administration, we measured the expression of phosphorylated TrkA. The results showed that the active form of the high-affinity NGF receptor, pTrkA, is upregulated by IN NGF administration, indicating that intranasally administered NGF may exert a potential functional role in the protection of damaged spinal cord neurons.

NGF is expressed by microglia, astrocytes, Schwann and leptomeningeal cells⁽¹⁴⁾ and also mast cells (our unpublished observations). The fact that the amount of endogenous NGF released by these cells is unable to protect damaged neurons^(4,15,17) suggests that its levels may not be sufficient, and that exogenous supplies might be required. However, brain targeting studies revealing the existence of a nose-brain pathway have opened the possibility of delivering neurotrophic factors, such as NGF, into the brain and even into the spinal cord via the nasal route, which not only enables us to more directly investigate the role of NGF in spinal cord neurons, but also suggests possible future treatment strategies. Indeed, expanding upon previous findings, our results show that exogenous supplementation of NGF delivered via IN pathways⁽²⁾ and/or via ocular administration⁽¹²⁾, could represent a useful experimental strategy for delivering NGF to the spinal cord for further exploration of the role NGF in SCI. However, as other studies have shown altered levels of BDNF and NT-3, in addition to NGF, at different survival periods following spinal cord injury⁽²¹⁾, it appears that different but concomitant neurotrophins may be involved in protection from spinal cord injury, presumably through a variety of overlapping mechanisms. Hence, further studies are required, in particular to determine whether prolonged IN NGF administration, alone or in combination with other specific active neurotrophic molecules, is able to reduce and/or delay degenerative events occurring subsequent to spinal cord injury, and how long any such effect will last.

Figure 3. Immunohistochemical analysis of TrkA and p75 expression in the injured and non-injured spinal cords. Representative spinal cord sections, immunostained with TrkA, of control (A), SCI untreated (B) and SCI NGF-treated (C) rats, after 3 weeks of daily intranasal NGF administration. Note the reduced number of TrkA immunostained neurons after SCI (B), compared to control spinal cord neurons, and the protective action after NGF administration (C), indicated by the arrows. Quantitative evaluation indicated that these differences are statistically significant, as reported in D (magnification figures 3 A-C: 350x). E-G show a representative spinal cord section immunostained with p75 NGF receptors. The number of p75 positive neurons is markedly reduced (F) with respect to controls (E), and the protective action after IN-NGF administration (G) is indicated with arrows. Quantitative evaluation indicated that these differences are statistically significant, as reported in H (magnification figures 3 E-G: 140x).



\Box CONCLUSION

Since its discovery, NGF has been indicated as a potential pharmacological tool for a number of neurological disorders of the peripheral and central nervous system, but the difficulty in delivering it directly to the spinal cord, through the lack of a feasible non-invasive strategy, has proved an obstacle from both research and therapeutic perspectives. However, our study demonstrates not only a link between the this neurtrophic factor and injured spinal cord neurons, implying a potential preclinical and/or clinical use of NGF, but also that it can be delivered in an active form via the nasal cavity.

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