

Dorsal column sensory axons lack TrkC and are not rescued by local neurotrophin-3 infusions following spinal cord contusion in adult rats

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Abstract

By reducing the progressive degeneration and disconnection of axons following spinal cord injury the functional outcome should improve. After direct transection of dorsal column sensory axons, neurotrophin-3 (NT-3) treatment can reduce degeneration and promote regeneration of the proximal stumps. Here, we tested in adult rats whether NT-3 infusion at the site of a moderate T9 spinal cord contusion would rescue sensory connections to the gracile nucleus in the medulla. Sensory projections were anterogradely traced bilaterally with injections of cholera toxin B (CTB) into the sciatic nerve 3 days before analysis. Seven days after the contusion plus intrathecal (subarachnoid) vehicle infusion as a control, the CTB-positive innervation of the gracile nucleus was reduced to ~25% of sham-operated rats. Intrathecal infusion of 10 µg/day of NT-3 did not affect this reduced innervation. To ensure good tissue penetration and high concentrations of NT-3 early after the injury, other rats received intraparenchymal infusions of vehicle or NT-3 near the injury site starting 2 days before until 7 days after the injury. This NT-3 treatment also did not affect the reduced innervation. This suggests that local NT-3 treatments cannot protect sensory axons from secondary degeneration after a contusive spinal cord injury. These results are likely because TrkC is not present in axons of the dorsal columns or gracile nucleus, or in other dorsal column cell types, even after the contusion. Together with published results, our data suggest that NT-3 is a peripherally – but not centrally – derived neurotrophic factor for sensory neurons.

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Introduction

Following spinal cord injury, the long projecting axons that have not been directly severed by the trauma undergo a progressive phase of degeneration from hours to days (Maxwell et al., 1997; Coleman and Perry, 2002). The timing of this secondary degeneration makes it an attractive target for therapeutic intervention, particularly after contusive injuries, which is the most common spinal cord injury in humans. Neurotrophins promote maintenance of axons during development (Huang and Reichardt, 2001; Ginty and Segal, 2002). For example, in compartmentalized cultures, sympathetic and sensory neurites degenerate after trophic factor withdrawal from their compartment despite survival of the cell bodies (Campanot, 1982a,b; Finn et al., 2000). Brain-derived neuro-

trophic factor prevents the degeneration of proximal axon stumps after transection of the neonatal rat optic nerve (Weibel et al., 1995). Furthermore, neurotrophins, including NT-3, reduce degeneration of the proximal endings of dorsal column sensory axons (Sayer et al., 2002) and acidic fibroblast growth factor reduces the die-back of corticospinal axons following spinal cord transection in adult rats (Guest et al., 1997). It is unknown whether neurotrophic factors can reduce the progressive degeneration of long-projecting axons after a contusive spinal cord injury, thereby increasing the number of axons which remain “connected”.

Protective strategies can be readily investigated in the ascending dorsal column sensory system because it is so accessible to experimental interventions, is easily labeled with anterograde tracers and has clearly defined target nuclei. This system is also functionally important for sensation as well as locomotion (Muir and Steeves, 1995; Bouyer and Rossignol, 2003), and is often affected in humans with spinal cord injuries.

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The large proprioceptive and mechanoceptive sensory dorsal root ganglion (DRG) neurons have large, myelinated axons and form the ascending projections through the dorsal column to the gracile and cuneate nuclei in the medulla. They express the NT-3 receptor TrkC and are directly responsive to NT-3 (McMahon et al., 1994; Wright and Snider, 1995; Bergman et al., 1996). NT-3 reduces DRG cell death during development and after peripheral nerve injury (Ernfors et al., 1994; Fariñas et al., 1994; Klein et al., 1994; Lefcort et al., 1996; Ljungberg et al., 1999; Wright et al., 2002), proximal central sensory fiber degeneration after transection (Sayer et al., 2002) and promotes their regeneration in the adult after spinal cord injury (Nakahara et al., 1996; Oudega and Hagg, 1999; Zhang et al., 1998; Bradbury et al., 1999; Ramer et al., 2000, 2001, 2002; Bloch et al., 2001; Romero et al., 2001; Lu et al., 2004). TrkC is also present in glial cells of spinal cord white matter and transected fibers rostral to the injury site after lateral funiculi hemisections (Frisén et al., 1993). This raised the possibility that NT-3 might also play an indirect protective role, e.g., through oligodendrocyte progenitors or oligodendrocytes which are known to respond to NT-3 (Barres et al., 1993; Cohen et al., 1996; Kumar et al., 1998; Kahn et al., 1999; Saini et al., 2004) as oligodendrocytes reportedly play an important role in supporting and maintaining axons (Lappe-Siefke et al., 2003; Wilkins et al., 2003; Edgar and Garbern, 2004).

As mentioned, NT-3 can protect against degenerative swelling of transected sensory dorsal column axons and can induce their regeneration. Therefore, we hypothesized that local 7-day NT-3 treatment would reduce the secondary axonal degeneration and disconnection from the gracile nuclei in the medulla that occurs after a contusive spinal cord injury. Because NT-3 failed to do so, we next determined whether the dorsal column axons or cells at the injury site could respond to NT-3, by determining whether they had TrkC protein.

Materials and methods

Animals and experimental design

A total of 41 young adult 8- to 9-week-old female Sprague–Dawley rats (Harlan, Indianapolis, IN) weighing 180–200 g were used. In the first study, we assessed the ability of intrathecal (subarachnoid) infusions of 10 $\mu\text{g}/\text{day}$ of NT-3 at T9 ($n=7$) for 7 days compared to infusion of vehicle as a control ($n=11$) on sensory innervation of the gracile nucleus after a moderate contusion at T9 (Fig. 1A). This dose has been shown to be effective for reducing degeneration of the proximal stumps of sensory axons following spinal cord transection (Sayer et al., 2002). The second group of animals received infusions into the dorsolateral white matter starting 2 days prior to contusion at T9 until 7 days post-injury (NT-3, $n=6$ and vehicle, $n=6$; Fig. 1B). As an additional control eleven rats received laminectomies only as a sham operation ($n=7$ and 4 for experimental groups 1 and 2, respectively). All rats received bilateral CTB injections into the sciatic nerve 4 days after the spinal cord injury and were processed 3 days after the injection, i.e., 7 days after the injury. The extent of remaining CTB-labeled fibers in the gracile

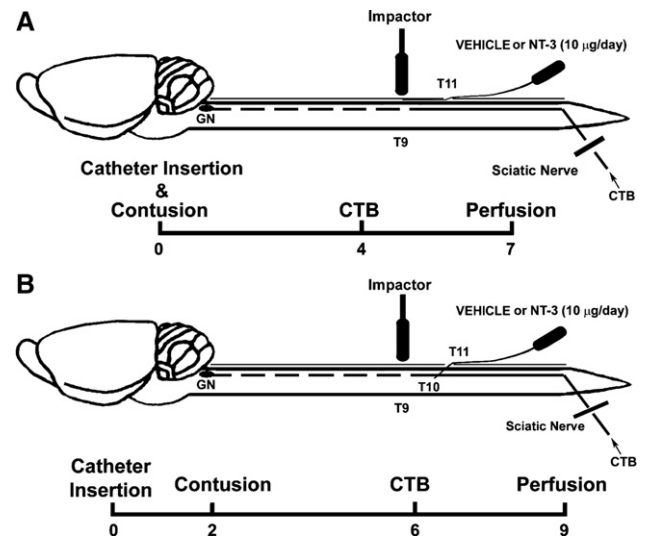


Fig. 1. Schematic representation of the 2 experimental groups. (A) At the time of a T9 contusion the tip of an intrathecal catheter filled with vehicle or NT-3 was placed over the injury site under the dura-arachnoid from spinal level T11. Four days later animals received bilateral injections of CTB into the crushed sciatic nerves to label the sensory terminal fibers in the gracile nuclei (GN). They were then perfused and analyzed 3 days later. (B) In a second group of rats, the catheters were inserted into the lateral white matter at spinal level T10 2 days before the contusions were performed at T9.

nucleus was assessed to determine the ability of NT-3 to promote axon integrity. We have previously reported that this is an effective strategy to study axonal protection and that the estrus cycle did not affect the outcome (Baker and Hagg, 2005). All experiments were conducted in accordance with the National Institutes of Health guidelines and were approved by the University of Louisville IACUC. A one-way analysis of variance followed by Tukey's post hoc test was used to determine the significance between sham, vehicle and NT-3 groups. *T*-tests were used to assess whether significant differences existed in force, displacement or CGRP-positive fiber area between the vehicle and NT-3 groups. Differences were considered significant at $p<0.05$.

Chronic infusions

Thin, flexible catheters were prepared by heating PE-60 polyethylene tubing (Becton Dickinson and Company, Sparks, MD) and manually pulling them to an outer diameter of 100–125 μm over 1 cm. The thick end of the catheter was affixed to the flow moderator of an Alzet osmotic pump (model: 2001; 1 $\mu\text{l}/\text{h}$ for intrathecal infusions or model: 2002; 0.5 $\mu\text{l}/\text{h}$ for intraparenchymal infusions; Durect Corporation, Carpinteria, CA) with Krazy glue (Elmer's Products, Columbus, OH). The patency of the catheter was assessed by flushing the line with 70% ethanol and left to dry. The catheter and attached moderator were gas sterilized and on the day prior to surgery filled with either sterile vehicle (PBS containing 1 mg/ml of rat serum albumin (Sigma-Aldrich Company; St. Louis, MO) plus 25 $\mu\text{g}/\text{ml}$ of gentamicin sulfate antibiotic (Boehringer Ingelheim, St. Joseph, MO)) or vehicle containing 10 $\mu\text{g}/\text{day}$ NT-3.

Animals were anesthetized with an intramuscular injection of 3.3 ml/kg of a mixture containing 25 mg/ml ketamine hydrochloride (Abbott Laboratories, North Chicago, IL), 1.2 mg/ml acepromazine maleate (The Butler Company, Columbus, OH) and 0.25 mg/ml xylazine (Butler) in 0.9% saline. Their backs were shaved and cleansed with betadine. Lacrilube ophthalmic ointment (Allergen, Irvine, CA) was placed on their eyes to prevent drying and 50 mg/kg of gentamicin antibiotic (Boehringer Ingelheim, St. Joseph, MO) was administered intramuscularly to reduce the risk of postoperative infection.

In animals with intrathecal infusions at T9, laminectomies were performed above spinal level T11 and a small hole made in the dura with the tip of a 30-gauge needle. The catheter was placed into the cerebrospinal fluid under the dura-arachnoid and its tip pushed rostral to spinal level T10 prior to the contusion. Following the contusion, the tip was moved more rostrally to a position immediately dorsal to the site of injury at T9. For intraparenchymal infusions, the tip of the catheter was inserted in the lateral white matter caudal to the site of injury at T10 by gently pushing the line into the dorsolateral funiculus of the spinal cord with microforceps. The catheter was held in place with the forceps until it was secured by suturing to the back muscles and applying a small drop of Krazy Glue to affix it to the spinous process. Fibrin glue (Tisseel VH, Baxter Healthcare Corporation, Glendale, CA), was then placed over the hole in the dura at T11 to prevent leakage of the infused reagents. In both cases, ~2–3 mm of the ~2-cm-long flow moderator remained to be inserted into the pump and was slowly inserted all the way once the catheters were in place. This ensured that blood or tissue did not clog the catheter. The pumps were placed under the skin and 0.1 mg of gentamicin sulfate in 0.9% saline injected with the pump under the skin. Fig. 1 provides a schematic representation of the surgical procedures and timelines used in these studies.

Spinal cord contusions and postoperative care

A laminectomy was performed at spinal cord level T9 and the dura left intact. The impactor force of the IH impactor (Precision Systems Instrumentation, Lexington, KY; Scheff et al., 2003) was set at 150 kdyn and a contusion delivered to the T9 spinal cord (dura intact). The impactor probe was placed 3 mm above the cord and laminectomies were sufficiently large enough to allow unobstructed access during the impact. Sham-injured animals received laminectomies at spinal levels T9 and T11 only. Following contusion and implantation of the catheter, the layers of muscle and skin were sutured separately and Bacitracin zinc antibiotic ointment (Altana Inc., Melville, NY) applied to the incision area. All animals were injected subcutaneously with 10 ml of lactated ringer's solution with 5% dextrose and placed in fresh cages set on a thermal blanket overnight before being returned to the animal care facility. Bladders were manually expressed twice daily throughout the experiment.

The IH impactor force applied to the cord and the extent of cord displacement correlate with histopathological and beha-

vioral outcomes in mice (Ghasemlou et al., 2005). In this study, no significant differences were observed between any of the groups. The groups infused intrathecally with vehicle- and NT-3 had a mean (\pm standard error of the mean; SEM) force of 154 ± 2 kdyn and 164 ± 5 kdyn ($p=0.10$), respectively and a cord displacement of 789 ± 37 μ m and 799 ± 44 μ m, respectively ($p=0.87$). In the intraparenchymal infusion groups the impactor force for the vehicle- and NT-3-infused groups were 158 ± 5 kdyn and 155 ± 4 kdyn, respectively ($p=0.64$) and the displacement 799 ± 31 μ m and 776 ± 30 μ m, respectively ($p=0.59$).

CTB labeling of sensory fibers

Rats were again anesthetized 4 days after the contusion or sham operation and received a prophylactic intramuscular gentamicin injection as described above. After shaving the outer lower hindlimbs and cleaning them with betadine, an incision was made along the dorsal surface at mid-thigh level and the muscles separated to expose the sciatic nerve. A suture was loosely tied around the nerve and the sciatic nerve crushed at mid-thigh level distal to the tail branch using fine forceps. A small incision was made in the epineurium proximal to the bifurcation of the sciatic nerve into the tibial and fibular branches, and the needle of a Hamilton syringe was gently inserted along the center of the nerve with its tip just distal to the crush site. Injuring the sciatic nerve ensures tracing of all dorsal column sensory fibers with CTB (Oudega et al., 1994; Tong et al., 1999). After tightening the suture around the nerve 2 μ l of 1% CTB (List Biologicals, Campbell, CA) was slowly injected over 2 min and left in place for another 3 min before being slowly retracted and the suture further tightened. Following CTB injections, the upper hindlimb muscles and skin were sutured, Bacitracin applied to the incision area and 10 ml of lactated Ringer's solution with 5% dextrose injected subcutaneously. The hindlimbs were sprayed with Bitter Mist spray (Butler) to prevent autophagy.

Spinal cord and medulla tissue processing

At the end of the treatments, all rats were anesthetized with 4 ml/kg of the ketamine anesthetic mixture and perfused with 100 ml of 0.1 M PBS following by 200 ml of 4% paraformaldehyde (PFA, pH=7.4) in 0.1 M phosphate buffer (PB). Spinal cords and medulla were removed and attached with 30-gauge needles to hard flat dental wax (SPI Supplies, West Chester, PA) to reduce curvatures of the spinal cords and postfixed overnight at 4 °C. The tissue was then cryoprotected for 1 day in 30% sucrose before being sectioned in the horizontal (longitudinal; spinal cord) or sagittal (medulla) orientation at 40 μ m, using a freezing sliding microtome. Sectioned tissue was stored in anatomical order in phosphate-based Millonig's buffer with 0.06% sodium azide at 4 °C prior to immunohistochemical processing.

Every fourth section through the gracile nuclei and the lumbar spinal cord was selected for CTB immunostaining. Sections were rinsed in 0.1 M PBS and endogenous peroxidases

quenched in a 10% methanol, 3% hydrogen peroxide solution, and then blocked in PBS containing 5% normal goat serum, 0.3% Triton X-100 (Sigma-Aldrich) for 30 min, followed by incubation in rabbit anti-CTB IgG (1:80,000; List Biologicals) in serum, 0.3% Triton X-100 in PBS overnight at 4 °C. To amplify the antibody reaction, biotinylated goat anti-rabbit IgG (1:300; Vector Laboratories, Burlingame, CA) in serum-containing PBS was used, followed by avidin–biotin complex (1:600; ABC Elite; Vector) in PBS. Peroxidase activity was visualized by the addition of 0.04% 3,3'-diaminobenzidine (DAB; Sigma-Aldrich) and intensified with 0.06% nickel ammonium sulfate and 1% hydrogen peroxide in 0.1 M PB. After development in DAB, the sections were rinsed 3 times in 0.1 M PB and mounted on gelatin-coated slides in the same solution and allowed to dry overnight. On the following day, the sections were dehydrated in a series of ethanol solutions followed by xylene and coverslipped in Entellan (Electron Microscopy Sciences, Gibbstown, NJ). The *in vivo* biological activity of NT-3 was assessed in the rats that received intraparenchymal infusions. Therefore, horizontal (longitudinal) tissue sections through the injured thoracic cord of intraparenchymally infused rats were processed for CGRP staining to visualize the local small diameter sensory afferents, as previously described (Chemicon AB1971; Hagg et al., 2005). To visualize TrkC protein, sections through the cervical and thoracic spinal cord, gracile nucleus, DRG, glabrous skin of the foot and muscle of contused rats were processed for immunostaining with mouse monoclonal TrkC antibody 2B7 (1:1000; a gift from Dr. Uri Saragovi, McGill University). This antibody was raised against peptide ESTDNFILFDEVSPTPPI corresponding to a portion of the human TrkC extracellular domain and can detect mouse TrkC by immunostaining (Esteban et al., 2006). The sequence recognized in mouse TrkC is ESTDFDFESDASPTPPI and is 100% homologous to that of rat TrkC.

Quantification and statistical analyses

Sensory fibers arising from the hindleg terminate densely within the dorsal horn of lumbar segments L1–L6 (Swett and Woolf, 1985). CTB labeling of these processes in the lumbar spinal cord was visually assessed as an indication of the success of the CTB injections. All rats were found to have similarly dense and intense levels of bilateral CTB labeling.

As previously described (Baker and Hagg, 2005), images of every fourth section through both right and left gracile nuclei were taken with a Leica DMIRE2 inverted microscope with an attached Spot RT_{KE} digital camera and associated Spot software for Windows (Version 4.0.8; Diagnostic Instruments Inc., Sterling Heights, MI) and saved in Adobe Photoshop 6.0. Images were analyzed using Scion Image software (Scion Corporation, www.scioncorp.com). The density slice feature of SCION Image is used to manually set the detection threshold and thus highlight the CTB-labeled fibers in the entire gracile nucleus. To ensure greater accuracy, this is done 3 times per section, and the average total area of fibers (not the tissue area) is calculated for each section. The mean terminal fiber areas of

all sections were summed to determine the total for both sides of each animal and are represented as the percentage of sham values. The density of CGRP-positive sensory afferents in the rats with intraparenchymal infusions was measured 1 mm caudal to the injury in horizontal sections through the central region of the cord where differences are readily detectable (Hagg et al., 2005). To determine the diameter of the catheter hole in the spinal cord, sections through the dorsal quarter of the cord were viewed using differential interference contrast microscopy.

Results

Intrathecal infusion of NT-3 at the injury site does not protect sensory axons from degeneration

In control animals that received laminectomies only (sham-operated), a dense CTB-immunoreactive fiber plexus within the gracile nuclei was seen (Fig. 2A) with a pattern and density similar to that which we have previously reported in normal rats without a sham operation (Baker and Hagg, 2005). The density of the fiber plexus was significantly reduced 7 days after the spinal contusion throughout the entire nuclei (Fig. 2B). Fibers innervating more ventral regions of the gracile nuclei appeared to be more severely affected. In rats that had received an intrathecal infusion of NT-3 at the thoracic injury site (Fig. 2C), the extent and location of sensory terminal fiber loss were similar to that seen with vehicle infusions. In all cases the immunostaining in spared individual fibers was intense suggesting that CTB transport was not affected by the injury or NT-3 treatment. In the lumbar cord, the density or staining intensity of the CTB-labeled primary afferent plexus was not noticeably affected by the T9 contusion or NT-3 infusion (Figs. 2D–F). Quantification of the sensory fibers in sham-operated animals revealed a mean (\pm SEM) CTB-immunoreactive terminal fiber area within both gracile nuclei of 0.99 ± 0.05 mm² ($n=7$). Following spinal cord contusion plus intrathecal infusion of vehicle ($n=11$), the labeling was reduced to $26 \pm 7\%$ ($p<0.001$; Fig. 3A). In animals with an intrathecal infusion of 10 μ g/day of NT-3 the terminal fiber area was reduced to $23 \pm 6\%$ ($n=7$; $p<0.001$) compared to sham-operated rats, which is not different than those infused with vehicle ($p=0.95$). Others have shown a decreased variability when mice with displacements more than 100 μ m from the average are removed from the dataset (Ghasemlou et al., 2005). Aberrant force values might also be an indicator of abnormal contusions. When we remove the rats with displacement values more than 100 or 150 μ m away from the average, or remove the rats with forces more than 15 kdyn away from the intended 150 kdyn ($>10\%$), the vehicle- and NT-3-infused groups remain not statistically different ($p=0.86$, 0.42 , and 0.74 , respectively).

Intraparenchymal infusions of NT-3 started 2 days prior to injury does not promote axonal integrity

One possible explanation for the inability of NT-3 to protect sensory axons in the first experiment was that NT-3 did not

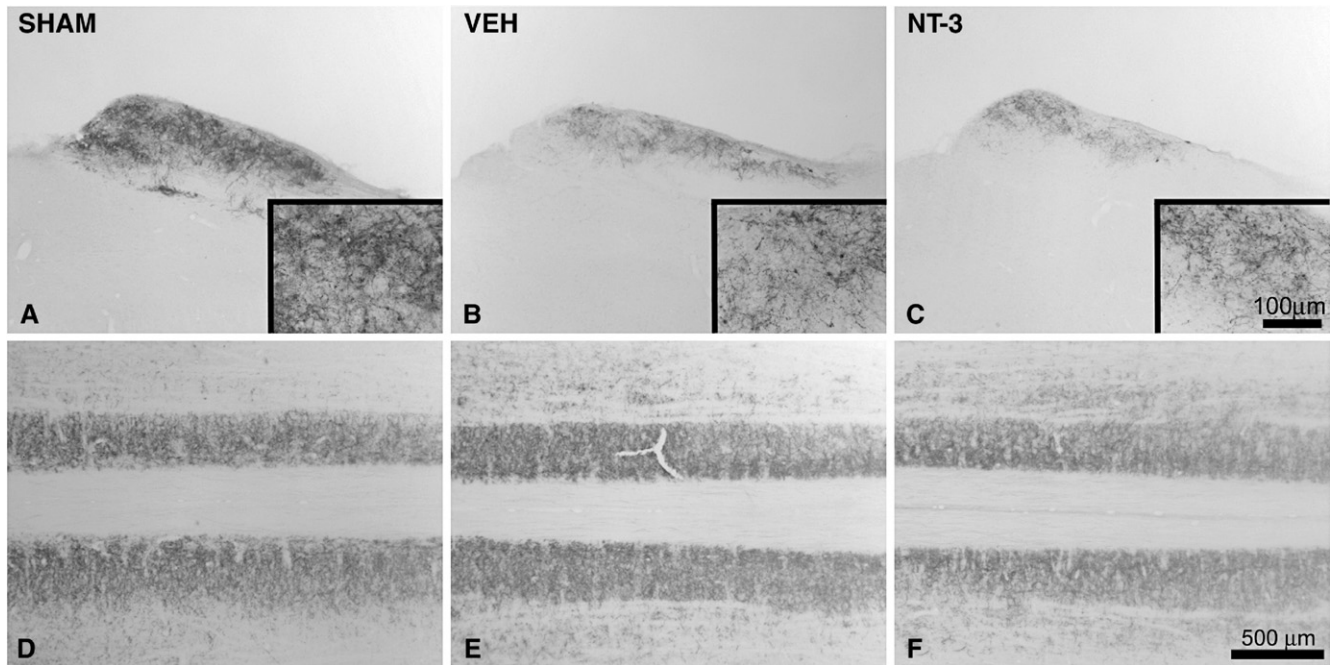


Fig. 2. NT-3 infusion at the lesion site does not affect the loss of sensory terminals in the gracile nuclei after a spinal cord contusion. (A) A representative parasagittal section through the gracile nucleus shows a normal dense plexus of CTB-positive sensory fibers in animals with sham surgeries. After spinal contusions, the extent of this CTB-positive terminal plexus is markedly reduced to a similar extent in both intrathecal vehicle (B)- and NT-3-infused animals (C). Insets are higher magnification views of the fibers in the gracile nucleus. Intrathecal NT-3 infusions started 2 days before the lesion resulted in a similar loss of fibers (not shown). The photomicrographs represent the section with the maximal area of CTB-positive fibers from rats that had terminal fiber area values (see Fig. 3) around the mean of their respective group. Panels (D–F) are representative sections of the sensory afferents within the lumbar dorsal horn from the same animals. The left-side of photomicrographs represents the rostral direction.

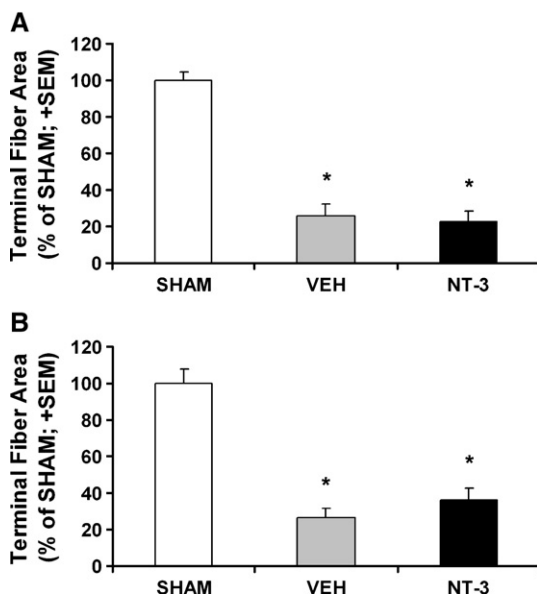


Fig. 3. NT-3 infusion at the lesion site does not affect the reduction of the sensory terminal fiber area within the gracile nuclei after spinal cord contusion. (A) Spinal contusions caused a significant reduction in the CTB-labeled terminal fiber area in both vehicle- and NT-3-infused groups ($*p < 0.001$) as compared to sham-operated rats. Intrathecal infusion of NT-3 starting at the time of injury did not change the extent of the CTB-positive terminal fiber area in the gracile nuclei compared to vehicle infusions (values are presented as a percentage of sham animals and represent combined data from both sides of the animals). (B) Intraparenchymal infusion of NT-3 starting 2 days before the injury also did not affect the outcome compared to the vehicle control animals ($p > 0.05$).

reach sufficiently high concentrations around the axons quick enough to prevent their disconnection or could not diffuse into the spinal cord from the intrathecal infusion site. Therefore, the tip of the catheter was placed 2 days before the contusion at spinal level T10 within the dorsolateral funiculus just lateral to the dorsal columns where the ascending sensory axons are located (Fig. 1B) and the infusions started. The density and staining intensity of the CTB-labeled plexus in the lumbar cord were not noticeably affected by the vehicle or NT-3 infusions. Rats with vehicle infusions into the cord ($n=6$) after the spinal contusion had a significant reduction in the CTB-positive terminal fiber area in the gracile nucleus to $27 \pm 5\%$ ($p < 0.001$; Fig. 3B) of sham-operated rats ($n=4$). In animals with NT-3 infusions ($n=6$) $36 \pm 6\%$ of the CTB-positive terminal fiber area remained, which was significantly less than sham animals ($p < 0.001$) but not different from that of vehicle-infused animals ($p = 0.49$). When we remove the rats with displacement values more than 100 or 150 μm away from the average, or remove the rats with forces more than 15 kdyn away from the intended 150 kdyn, the vehicle- and NT-3-infused groups remain not statistically different ($p = 0.58, 0.36, \text{ and } 0.70$, respectively).

The hole of the 100- μm diameter intraparenchymal catheter was difficult to see at the cord surface after perfusion of the rats and withdrawal of the catheter. This is probably caused by collapse of the white matter tissue that had been divided by the catheter after its withdrawal, and further shrinkage due to the postfixation. In all but 3 rats, horizontal sections showed catheter holes in the dorsolateral funiculus, the intended

location and did not appear to cause direct damage to the dorsal column (Fig. 4A). In two rats, the catheter hole was positioned in the dorsal horn and in one rat inadvertently in the middle of the dorsal column (Fig. 4B). In all but one rat, the diameter of the hole was between 60 and 120 μm , with no difference between the vehicle- and NT-3-infused groups. The catheter that had been placed in the middle of the dorsal column caused a hole of at most 350 μm in diameter with more than half of dorsal column tissue spared and did not extend deeper than the dorsal third of the dorsal column. The diameter of the catheter tract was not correlated to the CTB-positive fiber density in the gracile nucleus. In fact, among the vehicle group, the rat with the catheter in the middle of the dorsal column had the most CTB-labeled fibers in the gracile nucleus. This is consistent with the fact that the dorsal column region where tissue loss was evident is occupied by sensory axons from spinal cord levels caudal to the CTB-injected sciatic nerve (Tracey, 1995). The lack of extensive damage to the spinal cord after catheter placement into the white matter is consistent with our previous study (Hagg et al., 2005).

The NT-3-infused rats exhibited transient flexion (20–30 s) of the body towards the side in which the catheter was inserted once the flow moderators were fully inserted into the osmotic pumps during the surgery. This response was not observed in animals with vehicle-filled pumps. The body flexion might be

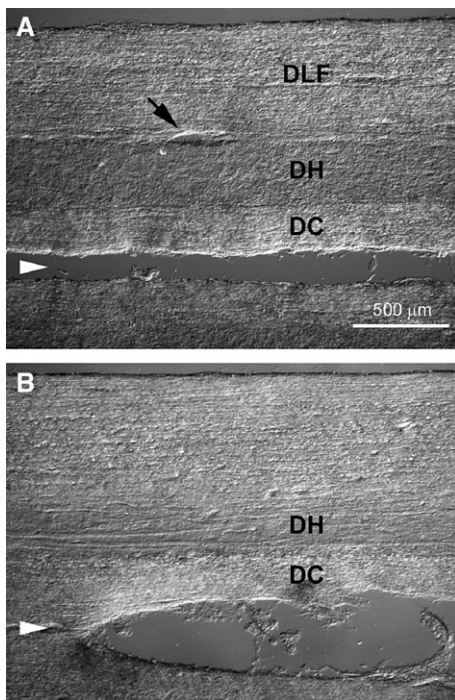


Fig. 4. When placed in the dorsolateral funiculus, the intraparenchymal catheter causes minimal damage and does not damage the dorsal column. (A) A horizontal thoracic section viewed by differential interference contrast microscopy shows a catheter tract hole (arrow) of approximately 100 μm (the diameter of the catheter) in the dorsolateral funiculus (DLF). The space in the midline is an artifact caused by histological processing. DC=dorsal column, DH=dorsal horn, arrowhead=spinal cord midline. (B) A section through the rat with the catheter inadvertently placed in the middle of the dorsal column shows bilateral damage to the medial parts of the dorsal column. Scale bar=500 μm .

mediated by thoracic motor neurons, as they express TrkC (Frisén et al., 1992, 1993). Moreover, a two-fold increase in the density of CGRP-positive sensory afferents 1 mm caudal to the injury was seen in the NT-3-infused rats ($7873 \pm 1952 \mu\text{m}^2$; $\pm\text{SEM}$) compared to vehicle-infused animals ($3428 \pm 792 \mu\text{m}^2$; measured in a $1.1 \times 10^6 \mu\text{m}^2$ rectangular area; $p < 0.05$). This suggests that NT-3 was biologically active and diffused at least 1 mm throughout the cord. We have previously seen effects of NT-3 over distances up to 5 mm from the intraspinal infusion site (Oudega and Hagg, 1999). Furthermore, others have observed diffusion of fluorescently tagged GDNF (a larger protein) 5–6 mm into the spinal cord after intrathecal infusions (Iannotti et al., 2004).

TrkC protein is present in peripheral but not central sensory projections

To gain insight into the cause of the inability of local NT-3 treatment to protect the dorsal column sensory axons we performed immunostaining for TrkC receptor in the dorsal column. No immunoreactive axons or cell bodies were detected in sections through the cervical dorsal columns of contused rats (Fig. 5A). These cervical sections contain the surviving ascending sensory axons from caudal to the injury in the medial part of the column and the uninjured axons that enter the cord rostral to the thoracic injury in the lateral part of the column (Tracey, 1995). TrkC immunostaining was present in neurons of the adjacent dorsal horn gray matter (Fig. 5A) and in ventral motor neurons (not shown). TrkC staining was also absent from the gracile nucleus which contains the sensory terminals but in the same sections was detectable in neuronal cell bodies in more ventral regions of the medulla (not shown). TrkC was also absent from the dorsal column in thoracic sections just caudal to the injury site (Fig. 5B), where it might have accumulated in injured axonal stumps due to ongoing transport (as it does for CTB). This was the same in rats infused with either vehicle or NT-3. TrkC was seen in some apparently injured axons of the lateral funiculus (Fig. 5C; as in Frisén et al., 1993). TrkC staining was clearly detectable in DRG neuronal cell bodies (Fig. 5D), as well as in structures of the skin and muscle (Figs. 5E and F), resembling mechanoreceptive innervation of touch domes and proprioceptive innervation of muscle spindles, respectively (LeMaster et al., 1999; Taylor et al., 2005).

Discussion

The current results unexpectedly show that NT-3 does not protect ascending sensory axons from degeneration when applied at the lesion site, even when administered starting 2 days prior to the injury and directly into the tissue. TrkC was not present in axons or cells of the normal or injured dorsal columns. These results suggest that NT-3 cannot stimulate axons en passant and cannot have indirect axon protective actions through local cells in the injured dorsal columns.

We set out to test whether infusion of NT-3 at the injury site would reduce the degeneration of sensory axons of the sciatic

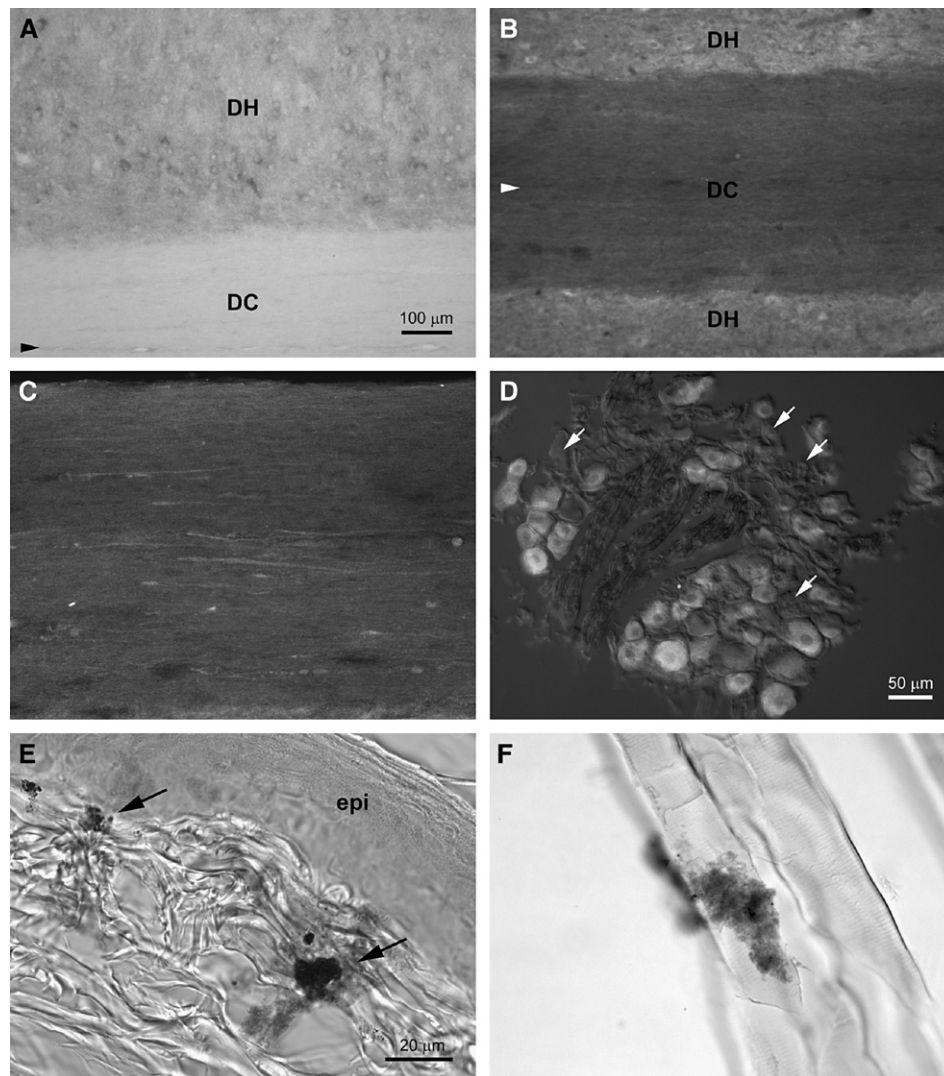


Fig. 5. TrkC immunostaining is absent from the dorsal column, but present in the peripheral projections of the same sensory neurons. (A) A horizontal section through the dorsal column (DC) of the cervical thoracic spinal cord shows a lack of TrkC staining. Surviving sensory axons from below the thoracic level course medially and uninjured axons from higher levels are located more lateral within the dorsal column (Tracey, 1995). Left=rostral, arrowhead=spinal cord midline. Note the TrkC-positive neurons in the adjacent dorsal horn (DH), evidence that the staining worked. (B) TrkC immunofluorescence staining was also absent from the dorsal column just caudal to the injury site, whereas some apparently injured fibers in the lateral funiculus contained TrkC staining (C). (D) TrkC was clearly detectable in DRG neurons, here shown by immunofluorescence merged with a differential interference contrast to identify the unstained neurons (some indicated by arrows). (E) Peripheral sensory projections to mechanoreceptors in the glabrous skin of the foot (arrows) and to muscle spindles (F) also contain TrkC staining. Differential interference contrast microscopy is used to identify the different layers and the individual muscle fibers. epi=Epidermis. Scale bar in A for A–C=100 μm , D=50 μm , E for E and F=20 μm .

nerve to the gracile nucleus after a spinal cord contusion. If successful, local application of axon-protective therapeutics may reduce the extent of functional loss following spinal cord injuries and mainly affect the injury site thus reducing the potential for side effects in the non-injured CNS regions and in the rest of the body. Our study also addresses a fundamental question about an untested biological effect of a neurotrophic factor in an *in vivo* setting. However, NT-3 even when infused directly into the spinal cord and started before the injury, did not affect the injury-induced reduction in the terminal fiber plexus in the gracile nucleus. This is unexpected, as NT-3 can prevent the death of DRG neurons *in vitro*, during development and after peripheral nerve transections (Ernfors et al., 1994; Fariñas et al., 1994; Klein et al., 1994; Lefcort et al., 1996; Ljungberg et

al., 1999; Wright et al., 2002) and the degeneration of the proximal stumps of these axons after spinal cord transection with the same dose of NT-3 as used here (Sayer et al., 2002). It is likely that biologically active NT-3 diffused throughout the spinal cord as rats infused with NT-3 showed acute flexing of the body towards the side of infusion and an increase in CGRP-positive sensory afferents after 7 days (as in Hagg et al., 2005). NT-3 is known to readily diffuse through CNS tissue following a single intracerebroventricular injection (Yan et al., 1994) and to reduce corticospinal tract sprouting close to an intraspinal infusion site (Hagg et al., 2005). NT-3 retains at least 60% of its original activity within Alzet pumps at body temperature for 14 days (Hagg et al., 2005). It is unknown whether NT-3 would have beneficial effects on the function or synaptic density of the

sensory system which might become evident at later times after the injury. However, our study was aimed at first determining whether axons and their projections to the gracile nucleus can be rescued by NT-3 during the acute and sub-acute phase. Treatments that provide a robust rescue effect for dorsal column axons would likely enhance sensory function as could be assessed by behavioral and electrophysiological methods.

The most likely explanation for the current results is our finding that TrkC receptors are not present on the dorsal column sensory axons, whether uninjured or not, regardless of treatment. TrkC was also not present on the axon terminals within the gracile nucleus. TrkC is present in the central dorsal horn terminals of developing mice (Genc et al., 2004) but we did not observe this in adult rats. On the other hand, we detected clear TrkC staining in the DRG neuronal cell bodies as expected (Chen et al., 1996) and in their peripheral terminals innervating mechanoreceptors and muscle spindles (Genc et al., 2004; Sieber-Blum et al., 2004). This raises the possibility that NT-3 is not normally derived from the CNS by these dorsal column axons. Non-radioactive *in situ* hybridization shows a complete lack of signal for NT-3 mRNA in the gracile or cuneate nuclei in the medulla (Allen Brain Atlas, www.brain-map.org). NT-3 immunostaining is also absent from the gracile and cuneate nuclei in archived sections from a previously published study (Zhou and Rush, 1994; X.F. Zhou, personal communication). In contrast, NT-3 is known to be produced in the skin (Cai et al., 1999) and muscle (Fernyhough et al., 1998), can be transported from the periphery to DRG neurons (Curtis et al., 1998) and NT-3 immunostaining is present in those neurons (Chen et al., 1996). Furthermore, overexpression of NT-3 in the developing muscle of NT-3 knockouts increases the survival of DRG neurons and the density of centrally projecting sensory axons (Wright et al., 1997). Thus, NT-3 appears to be a peripherally and not centrally derived neurotrophic factor for these sensory neurons.

Others have provided evidence that functional neurotrophin receptors are present along neurites or axons in primary unmyelinated neuronal cultures (Gallo and Letourneau, 1998; Kryl et al., 1999). Furthermore, NGF injected into the midthoracic spinal cord is retrogradely transported to the lumbar DRGs in adult rats (Richardson and Riopelle, 1984). This suggests that the unmyelinated TrkA-positive axons in the dorsal columns can bind and endocytose NGF and therefore have functional receptors along their axon shafts. Whether functional receptors are present along any myelinated axons in the adult spinal cord remains to be determined.

Our current results also suggest that glial and other cells in the injured dorsal columns cannot be induced by NT-3 infusions to indirectly protect the axons. Oligodendrocytes might play an axon-protective role, as axonopathy is often observed in demyelinating diseases (Lappe-Siefke et al., 2003; Wilkins et al., 2003; Edgar and Garbern, 2004). Furthermore, NT-3 can promote oligodendrocyte precursor proliferation and oligodendrocyte survival (Barres et al., 1993; Cohen et al., 1996; Kumar et al., 1998; Kahn et al., 1999; Saini et al., 2004). Other NT-3-responsive cells in the injured spinal cord could include microglia and macrophages (Condorelli et al., 1995; Elkabes

et al., 1998; Kahn et al., 1999; Barouch et al., 2001; Asami et al., 2006). However, these dorsal column cells are apparently unable to be activated by NT-3 to rescue the contused axons, again most likely because they do not produce TrkC protein. Moreover, TrkC mRNA in the surrounding tissues is down-regulated as early as 1 day and up to 7 days following injury (Liebl et al., 2001).

In conclusion, the findings of this study suggest that sensory axons in the dorsal column cannot be protected by local application of neurotrophins such as NT-3 because they do not have TrkC receptors, possibly because NT-3 is not normally derived from CNS sources. Local application of a neurotrophic agent at the injury site might be preferred in a clinical setting as this would lower the risk of systemic side effects or of effects on non-injured systems in the rest of the CNS. It will be important to determine which neurotrophic factor receptors are present along sensory dorsal column axons and other long projecting systems, and to test whether the appropriate ligands can prevent their disconnection after injury. If such axons do not have transmembrane neurotrophic receptors along their length, this would suggest that other strategies for axon protection would need to be developed that are not based on neurotrophic factors.

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