# GDNF selectively promotes regeneration of injury-primed sensory neurons in the lesioned spinal cord

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# GDNF selectively promotes regeneration of injury-primed sensory neurons in the lesioned spinal cord

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## Abstract

Axonal regeneration within the CNS fails due to the growth-inhibitory environment and the limited intrinsic growth capacity of injured neurons. Injury to DRG peripheral axons induces expression of growth associated genes including members of the glial-derived neurotrophic factor (GDNF) signaling pathway and "pre-conditions" the injured cells into an active growth state, enhancing growth of their centrally projecting axons. Here, we show that pre-conditioning DRG neurons prior to culturing increased neurite outgrowth, which was further enhanced by GDNF in a bell-shaped growth response curve. *In vivo*, GDNF delivered directly to DRG cell bodies facilitated the pre-conditioning effect, further enhancing axonal regeneration beyond spinal cord lesions. Consistent with the *in vitro* results, the *in vivo* effect was seen only at low GDNF concentrations. We conclude that peripheral nerve injury upregulates GDNF signaling pathway components and that exogenous GDNF treatment selectively promotes axonal growth of injury-primed sensory neurons in a concentration-dependent fashion.

## INTRODUCTION

Injuries of the central nervous system (CNS) result in little or no re-growth of injured axons and consequently no target reinnervation. One major factor responsible for this failure is the non-permissive growth environment of the CNS. Growth inhibitory molecules in the CNS prevent axonal regeneration by inducing growth cone collapse, while CNS cellular and matrix barriers block axon extension (Liu et al., 2006,Yiu and He, 2006,Busch and Silver, 2007). Attempts to make the CNS more permissive for growth have included neutralizing myelin growth inhibitors (Schnell and Schwab, 1990,Bartsch, 1996,Wang et al., 2002) and degrading the inhibitory glycoprotein matrix in the CNS with chondroitinase (Bradbury et al., 2002,Massey et al., 2006,Massey et al., 2007), as well as providing permissive substrates for growth (Ramon-Cueto and Santos-Benito, 2001,Houle et al., 2006,Barnett and Riddell, 2007). While some of these approaches increase collateral sprouting and promote modest improvements in functional recovery (Barritt et al., 2006,Ji et al., 2006,Wang et al., 2006), they have had only limited success at restoring disrupted circuits (Steinmetz et al., 2005,Bradbury and McMahon, 2006,Massey et al., 2006). Together, these data demonstrate that a permissive environment may not be sufficient for successful regeneration of injured CNS axons.

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A second major obstacle preventing successful central regeneration is the limited growth capacity of mature CNS neurons (Goldberg et al., 2002,Blackmore and Letourneau, 2006). This is in contrast to neurons of the peripheral nervous system (PNS) that can successfully regenerate. Peripheral, but not central, axonal injuries initiate changes in transcription factors and signal transduction pathways that drive axonal growth programs, (Raivich et al., 2004,Qiu et al., 2005,Seijffers et al., 2006) resulting in the expression of growth-associated proteins necessary for axon formation and elongation (Bomze et al., 2001,Bonilla et al., 2002). Because CNS neurons do not mount the same axonal injury-induced response, they are unable to initiate the intrinsic mechanisms necessary for axon formation and growth (Zhou and Snider, 2006,Rossi et al., 2007).

In some cases axons can grow within the CNS. For example, in dorsal root ganglion (DRG) neurons, which have both a central and a peripheral projecting axon, injury to the peripheral axon leads to successful regeneration, while injury to the central axon in the CNS does not. However, central axonal growth can occur following a pre-conditioning peripheral nerve injury (Neumann and Woolf, 1999). This pre-conditioning effect was first observed as an acceleration of growth in peripheral axons that had been previously injured, (McQuarrie et al., 1977,Bisby and Pollock, 1983,Hu-Tsai et al., 1994,Chong et al., 1996,Smith and Skene, 1997) and as growth of central axons into a permissive peripheral nerve environment (Chong et al., 1999). Pre-conditioning also leads to increased neurite growth of cultured DRG on permissive substrates *in vitro* (Seijffers et al., 2006), reflecting an increase in the intrinsic growth capacity of pre-conditioned neurons.

The pre-conditioning effect encompasses another component: a reduced response to myelin-containing non-permissive growth environments. Pre-conditioned DRG neurons have a decreased responsiveness to myelin *in vitro*, an effect mediated by CREB (Gao et al., 2004). *In vivo*, a peripheral pre-conditioning lesion enhances growth of centrally injured dorsal column fibers into and through spinal cord lesions (Neumann and Woolf, 1999,Neumann et al., 2002,Neumann et al., 2005). Increasing cAMP levels within the DRG mimics features of the pre-conditioning effect, enhancing CNS regeneration (Neumann et al., 2002). The response to myelin may also be reduced by the reduction in p75 expression after peripheral axonal injury, because p75 acts as a co-receptor with Nogo-R (Zhou et al., 1996,Yamashita et al., 2002,Yamashita and Tohyama, 2003).

A third mechanism for the pre-conditioning effect, in addition to increased intrinsic growth capacity and decreased responsiveness to growth suppressing cues, may be increased sensitivity to extrinsic growth promoting molecules. Specifically, alterations in neurotrophin signaling may increase the sensitivity of injured neurons to extrinsic growth promoting molecules (Perlson et al., 2005). Peripheral nerve injury increases expression of the GFRα1 membrane-bound co-receptor for the GDNF family of growth factors (Bennett et al., 1998,Bennett et al., 2000). GDNF binds to GFRα1 monomers or dimers to initiate functional coupling with RET tyrosine kinase, which elicits cellular signaling via RAS/extracellular signal-regulated kinase (ERK), phosphatidylinositol 3-kinase (PI-3K)/AKT, p38 mitogen activated protein kinase (MAPK), c-Jun N-terminal kinase (JNK) (Airaksinen and Saarma, 2002), and Src family kinases (SFK) (Tansey et al., 2000,Encinas et al., 2001). GDNF increases axon growth and expression of markers of axon growth in multiple neuronal systems after spinal cord injury (Blesch and Tuszynski, 2003). For example, GDNF activates expression of regeneration-associated genes such as βII-tubulin and GAP-43 in chronically injured rubrospinal tract neurons (Storer et al., 2003).

We hypothesize that the upregulation of the GFRα1 co-receptor by DRG neurons after peripheral axonal injury increases the sensitivity and growth responses of these cells to GDNF.

To test this, we compared the growth promoting effects of GDNF on naïve and pre-conditioned DRG neurons *in vitro* and *in vivo.*

# RESULTS

## Injury-induced changes in GDNF receptor expression in peripheral neurons

To determine if changes in the components of the GDNF signaling pathway play a role in the pre-conditioning effect, we used quantitative RT-PCR to examine changes in GFRα1, GFRα2, GFRα3, GFRα4 and RET mRNA expression in adult rat L4/5 DRGs following a sciatic nerve axotomy (the pre-conditioning injury). Sciatic nerve injury induced a 3-fold increase in GFRα1 at 3 days after injury that was maintained through 7 days (Fig 1A). GFRα2 expression decreased and GFRα3 expression increased 1.5-fold 3 and 7 days after axotomy. RET and GFRα4 expression were unaffected by the peripheral nerve injury. GFRα1 *in situ* hybridization in the DRG 3 days following peripheral nerve injury showed a strong upregulation in large diameter cell bodies (Figs. 1B, C). The increase in GFRα1 expression suggests a role for GDNF signaling in the increased growth capacity of peripherally injured neurons.

## Effects of a pre-conditioning injury on cultured DRG neurons

To establish the effects of GDNF on pre-conditioned neurons, we first determined the effects of a pre-conditioning lesion on cultured adult rat DRG neurons grown in the absence of any neurotrophic supplement on both permissive (laminin) and inhibitory (myelin) substrates (Fig. 2). On laminin, 6.3 ± 2.0% of naïve (uninjured) DRG neurons had initiated neurites after 15 hours in culture. Axonal outgrowth, assayed as length of the longest neurite was 145 ± 0.3 μm. Pre-conditioning the DRG neurons with a sciatic nerve transection one week prior to culturing increased the percentage of neurons with neurites >9-fold (62 ± 1.3%) and axonal outgrowth >3-fold to 467 ± 30 μm. (Fig. 2C).

On myelin, few naïve cells initiated neurites (2.4 ± 1.8%; Fig. 1D), and axonal outgrowth was slight (96 ± 6 μm) at 24 hours after culturing. Pre-conditioning DRG neurons increased the percentage of neurons with neurites >17-fold (41 ± 2.7%; Fig. 2D) and increased axonal growth 2-fold to 175 ± 9 μm (Fig. 1D). These data confirm the pre-conditioning effect on a permissive substrate (Seijffers et al., 2006) and establish that the effect increases growth on an inhibitory substrate (myelin). Assay times were chosen empirically to allow accurate quantification.

## Effects of GDNF on cultured DRG neurons

In naïve (non pre-conditioned) DRG neurons grown on laminin, GDNF produced a linear, dose dependent increase in cells that initiated neurites, but did not have a dose dependent effect on axonal length (Fig. 3). In contrast, the growth of naïve neurons on myelin was not markedly enhanced by GDNF (2ng/ml); the proportion of neurons that initiated a neurite was 3.2± 0.6% vs. 5.7± 1.7%, and axonal length 90.7± 3.4μm vs. 122.5±16.4μm, with and without GDNF, respectively. Growth of pre-conditioned neurons on laminin with GDNF was too extensive to quantify.

We next examined the effects of GDNF on pre-conditioned DRG neurons grown on myelin. GDNF significantly increased both the percentage of neurons that initiated neurites and neurite outgrowth (Fig. 4), with lower doses (0.1 - 2 ng/ml) being more effective than higher doses (20 and 40 ng/ml). The percentage of DRG neurons that initiated neurites increased from 39.4 ± 2.8% in untreated to 55.2 ± 2.0% in 2 ng/ml GDNF treated (Fig. 3B) and neurite outgrowth increased from 150.1 ± 10.6μm in untreated to 266.2 ± 22.5μm in 2ng /ml GDNF treated (Fig. 4C).

### Dose dependent effects of GDNF on CNS regeneration

To assess the effects of GDNF on the regeneration of dorsal column fibers in the spinal cord, three doses (0.2, 2.0, and 20 ng/day) were delivered for seven days by osmotic pump with the catheter tip positioned directly rostral to the L4 DRG beginning one day before dorsal column lesion. One month after the dorsal column injury, and in the absence of a pre-conditioning lesion or GDNF treatment, dorsal column fibers had retracted from the lesion site by an average of -0.5 ± 0.1 mm from the center of the lesion, with no evidence of sprouting in labeled fibers (Figs. 5, 6 and 7). In contrast, axons that were pre-conditioned prior to a central lesion grew into and beyond the lesion site by a mean distance of 1.4 ± 0.3 mm (Figs. 5, 6 and 7). Fiber growth was seen in both grey and white matter (Fig 5) and camera lucida tracings demonstrated fiber sprouting around the lesion site (Fig 6). Treatment of pre-conditioned DRGs with low dose GNDF (0.2 ng/day) significantly increased regeneration compared to the vehicle treatment group ($p<0.01$), whereas higher doses (2.0 and 20 ng/day) did not further enhance the pre-conditioning effect (Fig. 6). Lesioned dorsal column fibers grew 2.6 ± 0.6 mm in the 0.2 ng/day treatment group, compared to 1.1 ± 0.1 mm and 1.2 ± 0.1 mm for the 2.0 and 20 ng/day treatment groups, respectively. In some of the pre-conditioned, low dose GDNF animals, bundles of fibers could be seen 3 mm rostral to the lesion site (Figs. 6 and 7), and more sprouting was found in and around the lesion site than in vehicle or high dose GDNF treatment groups (Fig. 6). Bundles of regenerating fibers were seen exclusively within the white matter, primarily along the surface of the spinal cord (Fig 6 and 7). While significant sprouting was seen at the lesion site itself, into both grey and white matter, no bundles of regenerating fibers were ever seen in the grey matter. There was no evidence of functionally significant synapses in either the grey or white matter, rather just uncoordinated, misdirected growth.

## DISCUSSION

Cultured dorsal root ganglion (DRG) neurons provide a useful tool for studying the relative contributions of the intrinsic growth capacity and environment to regeneration. DRG neurons grow robustly on permissive substrates like laminin, but exhibit only limited growth when grown on CNS myelin (Cai et al., 2002) (Fig. 2). When DRG neurons are primed into an active growth state by a prior peripheral nerve injury (Hu-Tsai et al., 1994,Smith and Skene, 1997,Neumann and Woolf, 1999) greater growth occurs on laminin and myelin, which reflects increased growth and a reduced response to myelin inhibition (Fig. 2).

Since peripheral axonal injury increases GDNF receptor expression, we investigated the effects of GDNF on pre-conditioned DRG neurons. We hypothesized that the changes in GDNF signaling components would make pre-conditioned neurons more sensitive to the growth promoting effects of GDNF. In non-injured L4/5 DRGs, about 40% of large- and small-diameter cells express GFRα1 while 60% express RET (Bennett et al., 1998,Bennett et al., 2000). Expression of GFRα1 increases after sciatic nerve axotomy so that over 65% of DRG neurons express GFRα1. The greatest increase occurs in large diameter cells, where over 80% express GFRα1 (Bennett et al., 2000). Levels of RET, the tyrosine kinase receptor that GDNF signals through, remains unchanged after sciatic nerve axotomy (Bennett et al., 2000).

Consistent with the upregulation of GFRα1, GDNF facilitates growth in pre-conditioned sensory neurons. The growth promoting effects of GDNF show a bell-shaped dose-dependency, with the highest and lowest concentrations of GDNF producing least growth. These data are consistent with the hypothesis that neurites grow up concentration gradients of GDNF, slowing when concentrations exceed a certain threshold. Synthesis of GDNF in Schwann cells increases in the distal stump after peripheral nerve injury and returns to baseline upon reinnervation, and may help to establish such gradients (Fu and Gordon, 1997). GDNF doses far in excess of those used in the current study prevent expression of many of the markers of peripheral axonal injury in DRG cells, including a reduction of IB4 and $P2X_3$ expression,

and increased ATF3 and galanin expression (Wang et al., 2003,Pezet et al., 2006). This suggests that low concentrations of GDNF may increase some of the effects of injury (i.e. stimulation of growth), while higher concentrations may be growth inhibitory.

GDNF treatment at the injury site in the spinal cord produces only limited regeneration in descending axons (Dolbeare and Houle, 2003) while treatment of ascending sensory tracts with high doses (12 μg/day/28 days) produces no growth past the lesion (Bradbury et al., 1999). Grafting GDNF-secreting fibroblasts into the lesion site enhances growth of axons into the graft and promotes remyelination by attracting Schwann cells, but the regrowing fibers fail to exit the graft (Blesch and Tuszynski, 2003). We find that that low doses of GDNF facilitate both the sprouting and regeneration of pre-conditioned central axons of sciatic afferents following dorsal column lesions. The major difference between this study and earlier studies that failed to show an effect of GDNF on spinal cord regeneration are: (i) we pre-conditioned DRG neurons to increase GFRα1 expression, (ii) we used lower dose of GDNF, and (iii) we applied treatment directly to the DRG, rather than at the lesion site. However, even though pre-conditioning enhanced DRG central axon growth and this was further enhanced by GDNF, the growth was misdirected and disorganized. Although bundles of fibers grew across and beyond the lesion, they never followed their original projection path and instead, tended to run along the surface of the spinal cord. Therefore these data suggest that enhancing intrinsic growth pathways is not enough; the environment rostral to the lesion remains non-permissive and inhibitory to growth. This may reflect the presence of proteoglycans or other inhibitory factors (Busch and Silver, 2007).

Several studies have combined pre-conditioning DRG neurons with additional treatments looking for additive effects. Lu et al (2004) preconditioned DRG neurons by injecting cAMP, applied a bone marrow stromal cell graft into the injury site, and administered NT-3 to the injured spinal cord. Some regeneration beyond the injury site was seen, but there were only a few regenerating axons. This may be because the receptor for NT-3, TrkC, is down regulated by peripheral nerve injury (Bergman et al., 1999). A role for IL-6 in producing increased growth after a conditioning injury has been proposed; the conditioning effect is absent in IL-6 null mice and growth of naïve DRG neurons *in vitro* in the presence of NGF is stimulated by IL-6 (Cafferty et al., 2004). Whether addition of IL6 has any facilitating effect on the pre-conditioning phenomenon has not been examined. Combining olfactory ensheathing cell bridges with a pre-conditioning lesion has been tried, but no regeneration was noted beyond the injury site (Andrews and Stelzner, 2004). It would be interesting to test if combining GDNF treatment of DRG cell bodies with chondroitinase-ABC treatment at the lesion site to disrupt inhibitory CSPG accumulation could further increased sprouting and regeneration or affect the trajectory of regenerating axons (Busch and Silver, 2007).

Treatment strategies that lead to the re-establishment of disrupted circuits within the injured spinal cord will need to enhance the intrinsic growth capacity of the injured neurons and reduce the inhibitory environment, while avoiding aberrant or dysfunctional sprouting either of the injured or non-injured neurons. Although low dose GDNF significantly facilitates the pre-conditioning effect, this growth-priming effect still fails to produce sufficient growth towards the brainstem along appropriate tracts. Nevertheless, this finding provides further insight into how to enhance growth by acting on systems that are themselves induced by the injury and indicates that once central neurons have been primed for growth, the axons will need to be guided by cues in a permissive environment to their targets.

# EXPERIMENTAL METHODS

## Experimental animals and injury production

Adult male Sprague-Dawley rats, 190-210g, were obtained from Charles River Laboratories and housed in a light/dark cycle of 12hr/12hr. All procedures were performed in accordance with the Massachusetts General Hospital animal care regulations. Injuries were done under isoflurane anesthesia (4% induction, 2.5% maintenance). Axotomy was produced by exposing the left sciatic nerve at the mid-thigh level, ligating with 3-0 suture and transecting the nerve distal to the ligature. After injury, muscle and skin were closed in layers and animals allowed to recover. Preconditioning peripheral axotomies were performed seven days prior to harvesting DRGs for cell culture or dorsal column lesions. For dorsal column lesions, a partial laminectomy was performed at the spinal T8 level, the dura mater opened, and a transection of the dorsal columns performed from the dorsal root entry zone down to the central canal with microscissors (Neumann and Woolf, 1999). Muscle and skin were closed in layers and animals allowed to recover.

## Quantitative RT-PCR

Extraction of RNA was performed using Trizol (Invitrogen). Quantitative real-time PCR was performed using the Sybr green detection system with primer sets designed on Primer Express. Specific PCR product amplification was confirmed using the dissociation protocol. Transcript regulation was determined using the relative standard curve method (Applied Biosystems). Relative loading was determined prior to RT with RNA spectrophotometry followed by gel electrophoresis and post RT by amplification of glyceraldehyde-3-phosphate dehydrogenase. For each time point 3 samples of pooled tissue from 2 rats were analyzed. Expression levels are defined as fold change relative to naïve levels.

## In situ hybridization

Fresh frozen DRGs were cut at 14 μm, postfixed and acetylated. Riboprobes were obtained by i*n vitro* transcription of cDNA and labeled with digoxigenin (Dig-labeling kit, Roche). Sections were hybridized with 200 ng/ml of sense or antisense probes in a prehybridization mix (5 × SSC, 50% formamide, 2 × Denhardt's, 500 μg/ml herring sperm DNA, 250 μg/ml yeast tRNA), hybridized with 200 ng/ml of sense or antisense probes in prehybridization mix, incubated with anti-Dig-alkaline phosphatase 1:1000 (Roche) in 0.1M maleic acid buffer, developed with NBT/BCIP/levamisole (Roche). Sections were embedded in glycerol/gelatin.

## Cell culture

Primary adult dissociated DRG neuron cultures were prepared by dissecting DRGs into HBSS (Cellgro) and 10mM HEPES, followed by digestion with 5mg/ml collagenase A and 1mg/ml dispase II (Roche) prior to treatment with 0.25% trypsin (GibcoBRL). Triturated cells were centrifuged through 15% BSA and then a Percoll (Sigma) gradient prior to culturing in Neurobasal media (GibcoBRL) containing 2% (vol/vol) B27 supplement (GibcoBRL), 50ug/ml Pen-Strep, 10μM Ara-C, 200mM L-glutamine, and GDNF where indicated. The BSA ad Percol gradients are designed to produce highly enriched sensory neuron cultures; Ara-C prevents extensive growth of dividing cells (Grothe and Unsicker, 1987,Lewis et al., 1999).

For CNS myelin isolation, adult rats were euthanized using $CO_2$. Spinal cords were removed and washed in PBS at 4°C. Twenty grams of tissue was homogenized in 20 mM HEPES buffer with 15 mM EDTA, 0.25 M sucrose, aprotinin 10 μg/ml, leupeptin 10μg/ml, pepstatin 1μg/ml, and PMSF (174 μg/ml). The suspension was centrifuged through a sucrose gradient (0.85 to 10 mM sucrose in HEPES buffer) at 80,000g for at least 4 hrs at 4°C. The myelin layer was collected and washed in HEPES buffer with protease inhibitors to remove the sucrose, re-

homogenized and centrifuged at 170,000g for 1 hr at 4°C. The resulting pellet was resuspended in HEPES and protein concentration determined with the Bradford assay. The day before each culture experiment, 150μl of sterile water containing 0.15μg of myelin was pipetted into each well of the culture plates and allowed to evaporate dry.

### Neurite outgrowth assays

Cells were fixed with 4% paraformaldehyde for 20 minutes, washed with PBS, incubated with monoclonal antibody to βIII Tubulin 1:1000 (Sigma) and visualized using Alex Fluor 488 (Molecular Probes). All *in vitro* neuron growth data given were the average 3 separate culture experiments (error SEM between experiments). Each experiment consisted of counting at least three distinct culture wells (8 well plates, 0.89cm/0.89cm, Nunc). For well analysis, 9 non-overlapping pictures were taken at low power (4×) in a 3 × 3 configuration across the well. All images were analyzed blind to growth conditions. A representative culture experiment (GDNF dose on myelin) consisted of 530 ± 17 neurons counted. Neurite outgrowth was determined by measuring the length of the longest neurite per cell using Image J software (NIH). A cell was defined as having initiated a neurite if it extended a neurite twice the diameter of the cell body.

### In vivo GDNF treatments

For *in vivo* treatments, GDNF (Sigma) was prepared in 0.1% BSA/NaCl. Osmotic pumps (Alzet Model 2001) and attached catheters were filled with GDNF and primed overnight at 37°C. Six days post axotomy and one day prior to dorsal column lesion, pumps were implanted with the catheter tip positioned just superior to the L4 DRG, at the vertebral L4/5 junction. Animals showing any neurological deficit after pump implantation were removed from the study.

### Anterograde labeling of dorsal column fibers and camera lucida drawings

Four weeks after dorsal column lesions, the left sciatic nerve was exposed and 3μl of choleragenoid conjugated horseradish peroxidase solution (BHRP; List Biological Laboratories) was injected (1% dissolved in sterile water). Three days after injection, animals were perfused with 500 ml of saline followed by 750 ml of 1% paraformaldehyde and 1.25% gluteraldehyde in 0.1M phosphate buffer. The entire spinal cord was removed, cryoprotected in 30% sucrose overnight, and cut into 3 cm sections centered on the site of dorsal column lesion. These segments were frozen in OCT, serially sectioned at 30μm, mounted on slides, and stored at -20°C. Sections were processed for peroxidase activity using tetramethylbenzidine (TMB; Sigma) as substrate and sodium nitroferriyanide as a stabilizing agent (Mesulam and Mufson, 1980). Slides were allowed to air dry overnight and coverslipped.

Camera lucida drawings of spinal cords were produced in Adobe Photoshop. For each spinal cord, six longitudinal images (sometimes a montage of several images when growth was extensive; e.g. Fig. 6) taken at least 30μm apart were obtained. BHRP labeled fibers were traced for each section individually and then merged with other sections from the same spinal cord. Extent of regeneration was determined by measuring the maximum distance of BHRP labeled fiber growth from the center of the dorsal column lesion.

### Statistical analysis

An unpaired, two-tailed t test was used for significance testing of RT-PCR results and neurite initiation and outgrowth in pre-conditioning *in vitro* experiments. One way ANOVA was used for significance testing of neurite initiation and outgrowth *in vitro* after GDNF treatment, and length of maximum regeneration after dorsal column lesion *in vivo*. Post hoc comparisons were done using Tukey's multiple comparison test. Data are reported as means ± standard deviation (SD) or standard error of the mean (SEM) as indicated.

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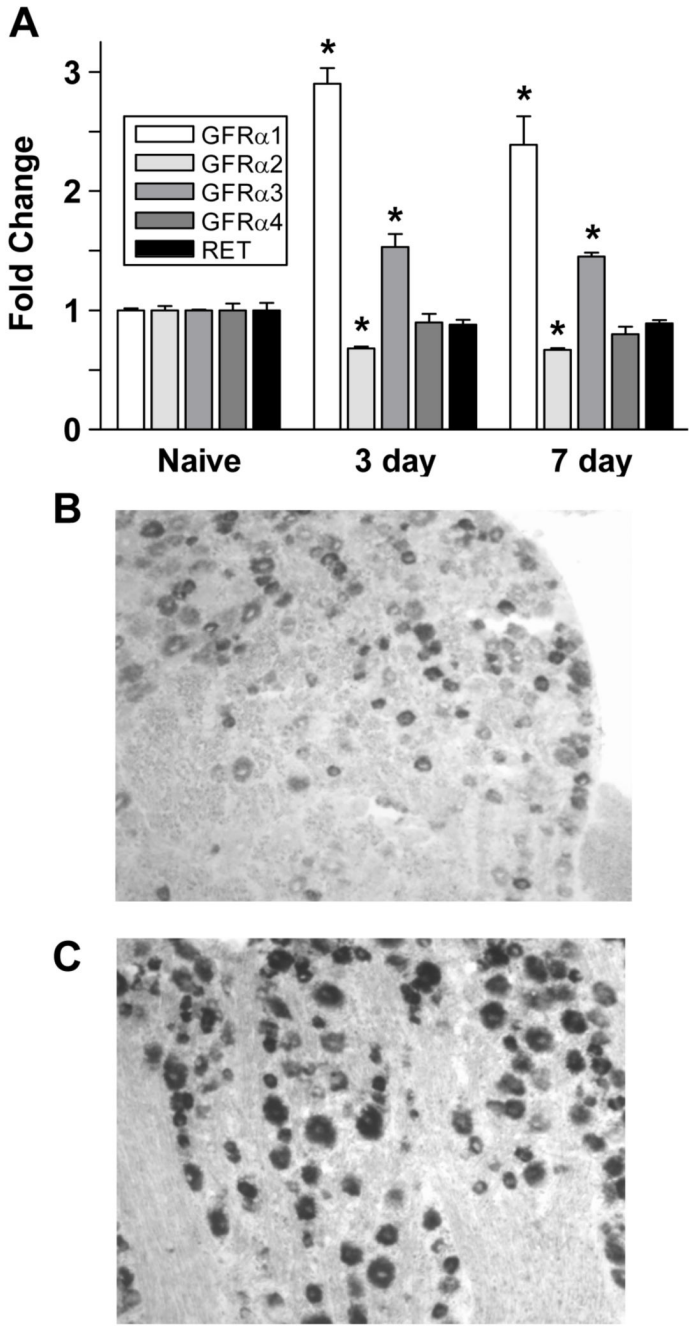


**Fig 1.**

Changes in GDNF signaling components following peripheral nerve injury. (A) RT-PCR results demonstrate an increase in GFRα1 and GFRα3 mRNA at 3 and 7 days following sciatic nerve axotomy in L4/5 DRGs. GFRα2 mRNA expression decreases, whereas GFRα4 and RET expression is unchanged. *In situ* hybridization in naïve (B) and injured (C) DRGs 3 days after peripheral nerve injury demonstrates an increase in expression of GFRα1 mRNA in injured cell bodies. Data are presented as mean ± SD. Significance vs naïve (uninjured) DRG expression (defined as 1). * = $p < 0.05$.

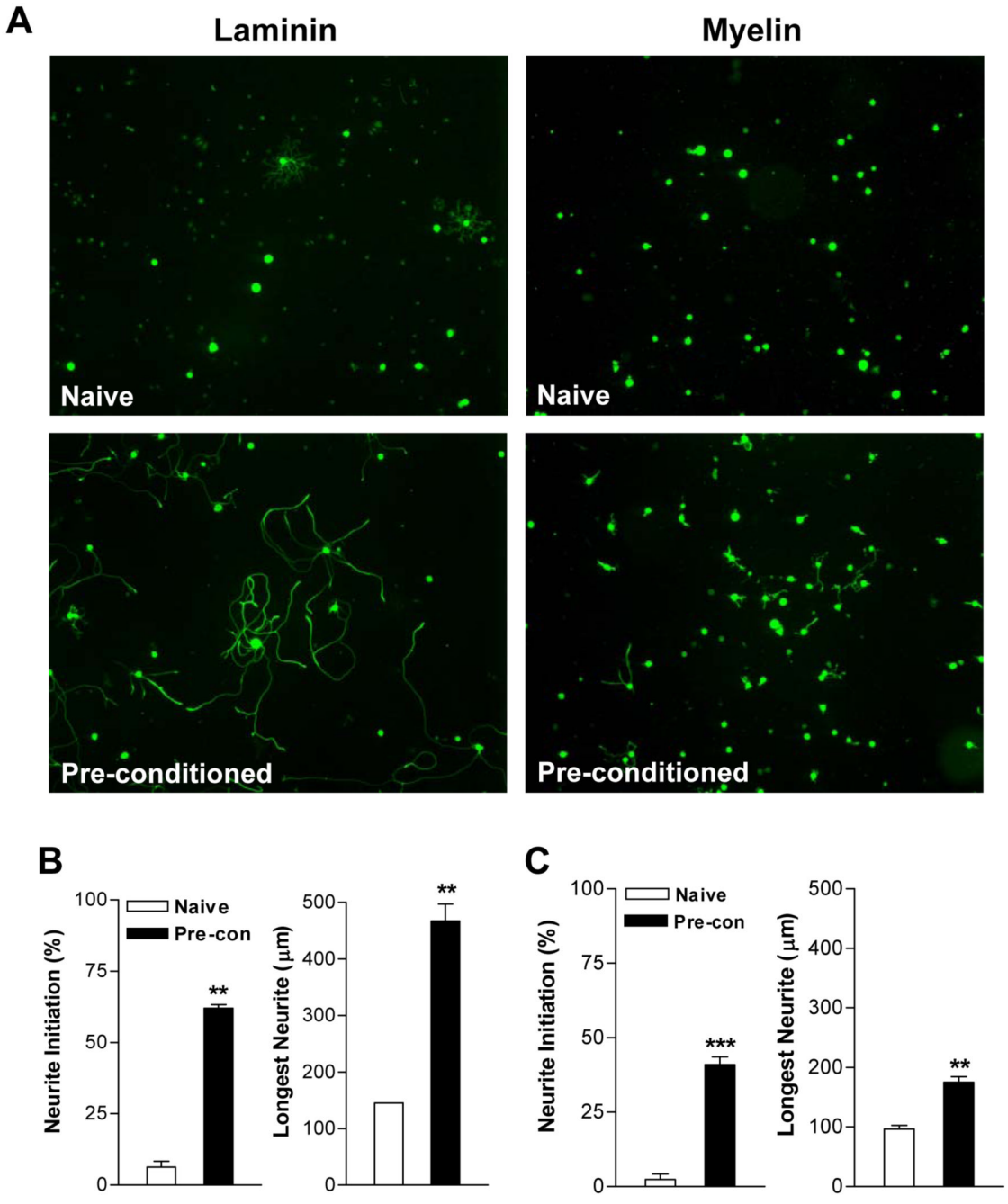


**Fig 2.**

Pre-conditioning effect on adult dissociated DRG neurons in culture without neurotrophic support. (A) Representative photomicrographs of naïve and pre-conditioned dissociated DRG neurons grown on the permissive substrate laminin and the non-permissive substrate myelin (B) without neurotrophic support. A pre-conditioning injury to the peripheral axon (the sciatic nerve) one week prior to culturing increases the percentage of neurons that initiated neurites and axonal outgrowth both on laminin (B) and myelin (C). Data are means ± SEM. Significance vs naïve (uninjured) DRG neurons. ** = $p < 0.01$, *** = $p < 0.001$.

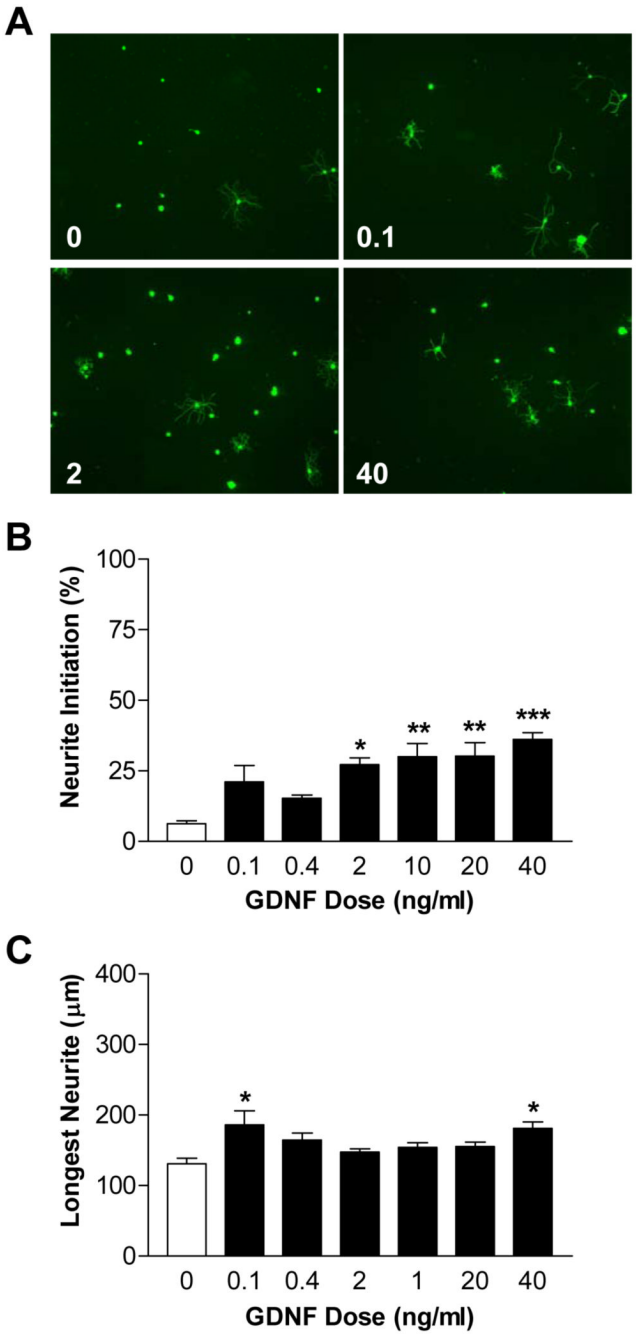


**Fig 3.**

Effects of GDNF on naïve dissociated DRG neuron growth when cultured on the permissive substrate laminin. (A) Representative photomicrographs of naïve dissociated DRG neurons grown on laminin at four concentrations of GDNF (0, 0.1, 2, 40 ng/ml). (B) Proportion of DRG neurons initiating neurites at differing concentrations of GDNF when cultured on laminin. The dose-response curve to GDNF is linear with low doses of GDNF producing low levels of neurite initiation and higher levels producing more neurites (0.1-40 ng/ml). (C) Treatment with GDNF had only minimal effects on axonal growth of naïve DRG neurons grown on laminin at any concentration assayed (0.1-40 ng/ml). Data are means ± SEM. Significance vs no GDNF treatment. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

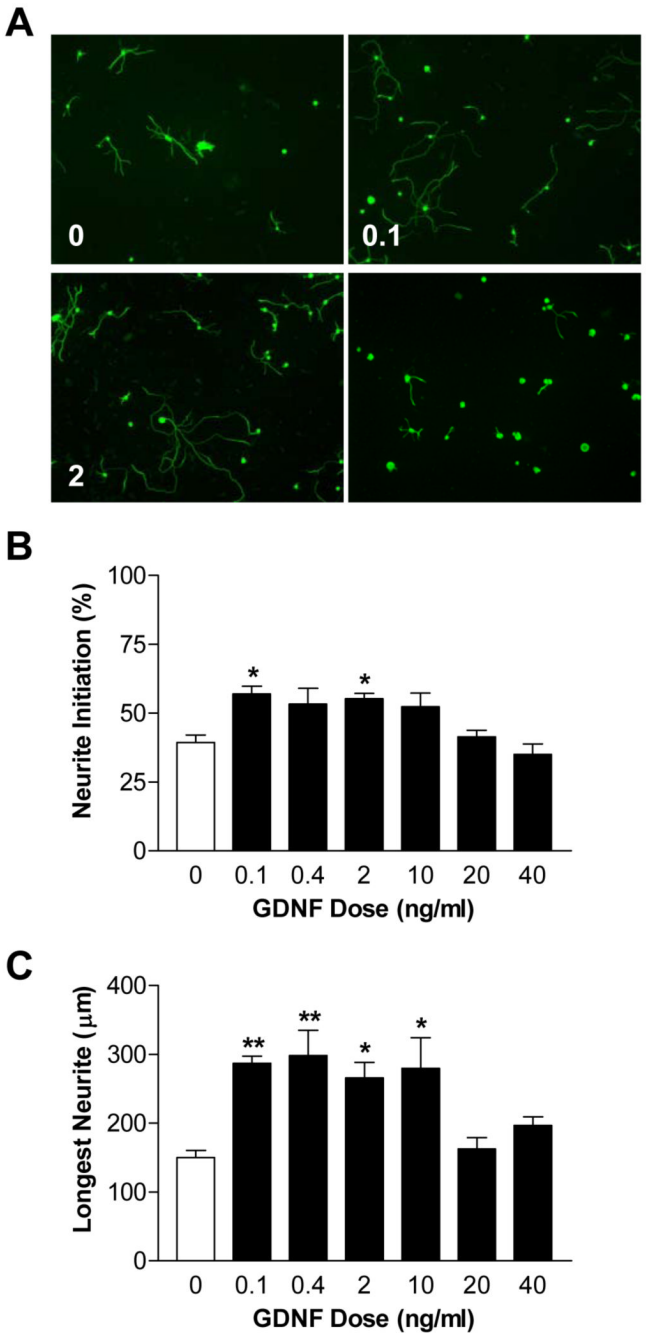


**Fig 4.**

Effects of GDNF concentration on pre-conditioned, dissociated DRG neurons cultured on myelin. (A) Representative photomicrographs of pre-conditioned dissociated DRG neurons grown on myelin at four concentrations of GDNF (0, 0.1, 2, 40 ng/ml). (B) Percentage of cells initiating neurites at differing concentrations of GDNF when cells were cultured on myelin. The dose-response curve to GDNF is bell-shaped with lower doses of GDNF producing higher levels of growth (0.1-10 ng/ml). Higher concentrations of GDNF (20-40 ng/ml) had no effect on neurite initiation relative to untreated control cells. (C) Length of axonal growth at differing concentrations of GDNF when neurons were cultured on myelin. Lower doses of GDNF (0.1-10 ng/ml) produced longer neurites than higher doses (20-40 ng/ml), which had no effect

compared to no GDNF treatment. Data are means ± SEM. Significance vs no GDNF treatment. * = $p < 0.05$, ** = $p < 0.01$.

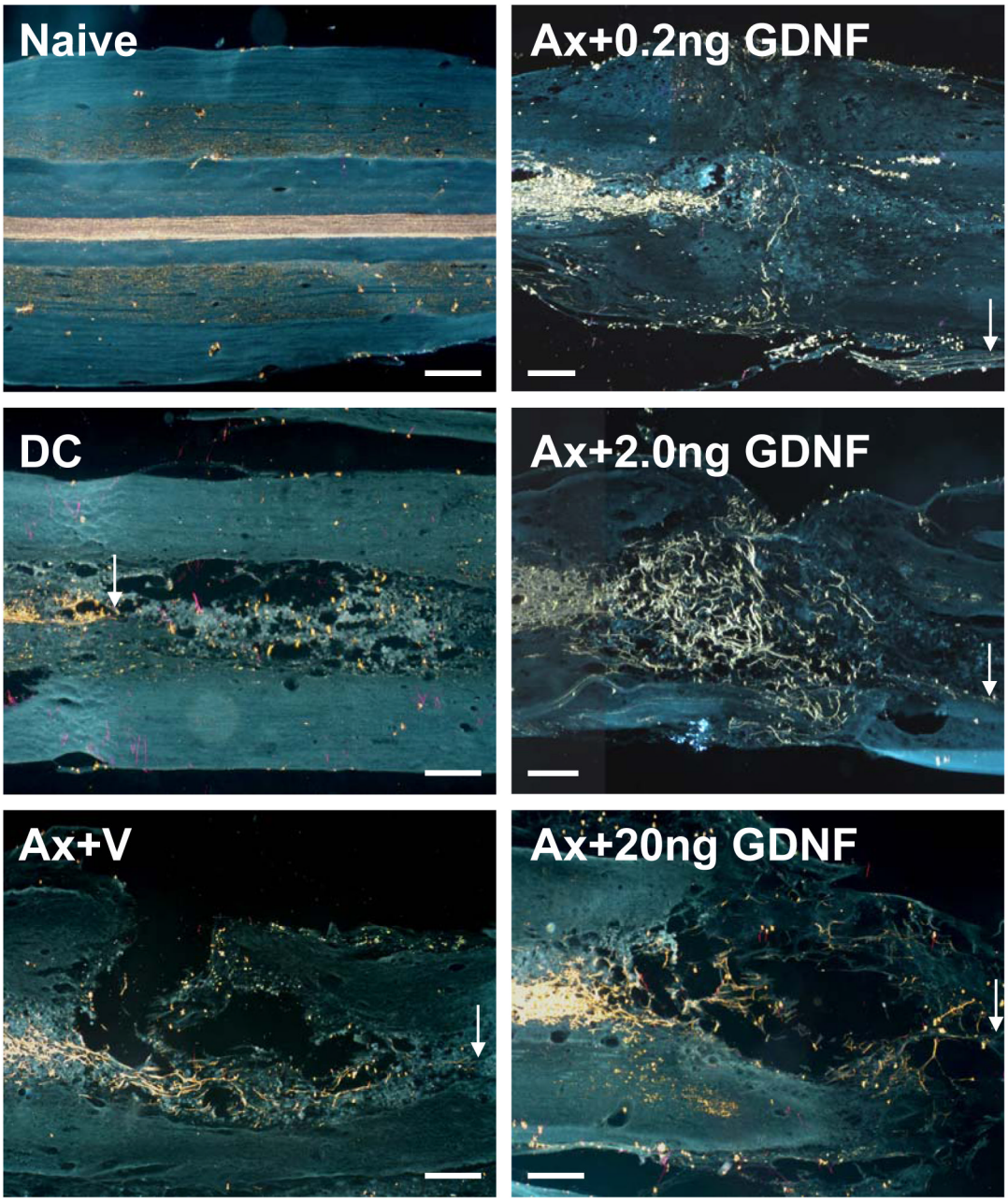


**Fig 5.**

Representative photomicrographs demonstrating BHRP tracing of the dorsal column pathway in naive (uninjured), or one month after dorsal column injury in: dorsal column lesioned only (DC), pre-conditioned (axotomized; Ax) + vehicle (V), Ax + GDNF (at 0.2, 2.0, and 20 ng per day). Arrows indicates farthest regrowth for each image. Scale bars = 250 μm.

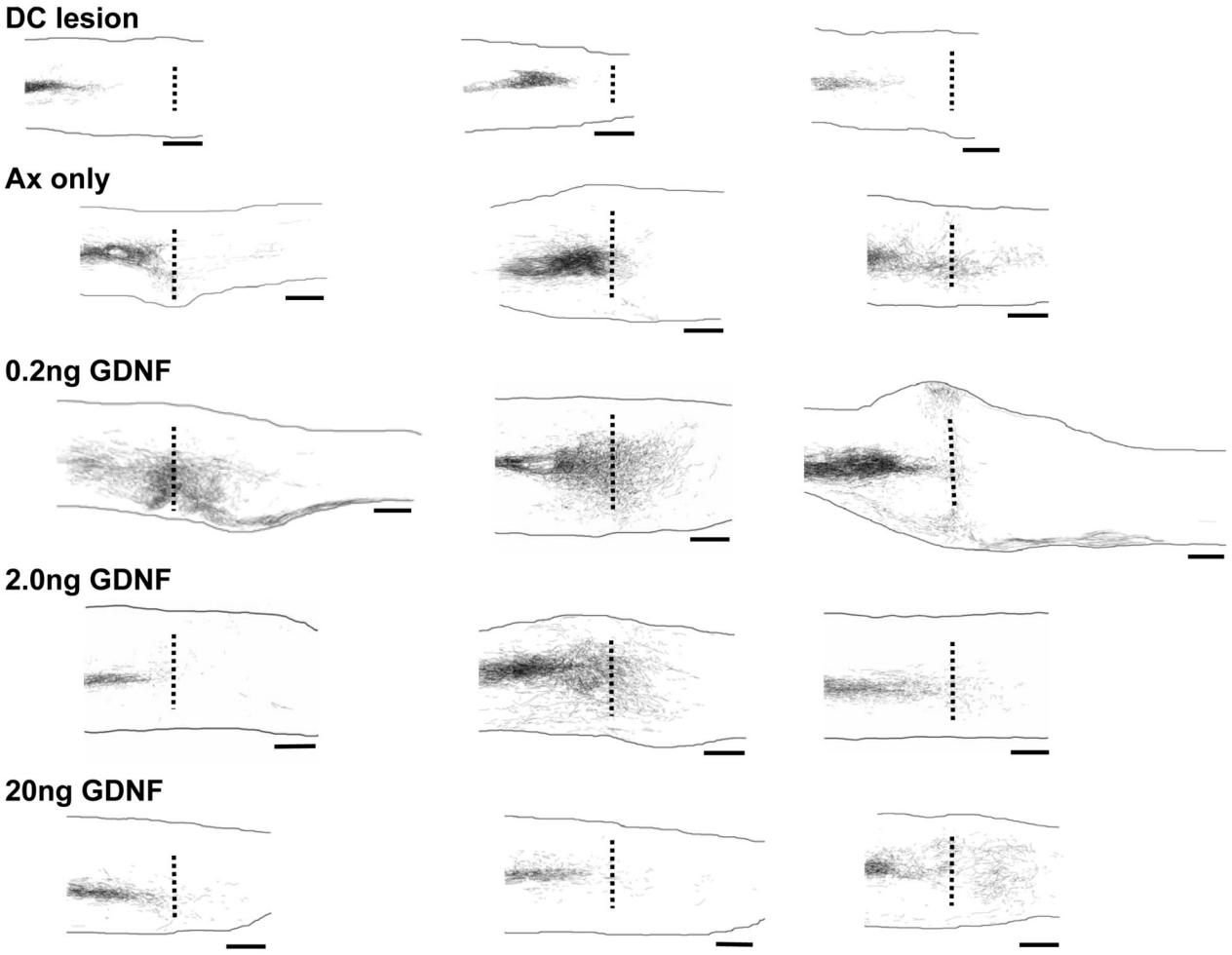


**Fig 6.**

Camera lucida drawings of BHRP labeled dorsal column axons one month after injury. Each figure represents six images, taken at least 30 μm apart, and then merged. GDNF treatment increases the distance of axonal regeneration and qualitatively increased the amount of sprouting in and around the lesion site (compare Ax+0.2ng GDNF vs Ax only). Note the bundles of fibers growing along the surface of the spinal cord in the Ax+0.2ng GDNF treatment group. Dashed line indicates lesion center. Scale bars = 600μm for each.

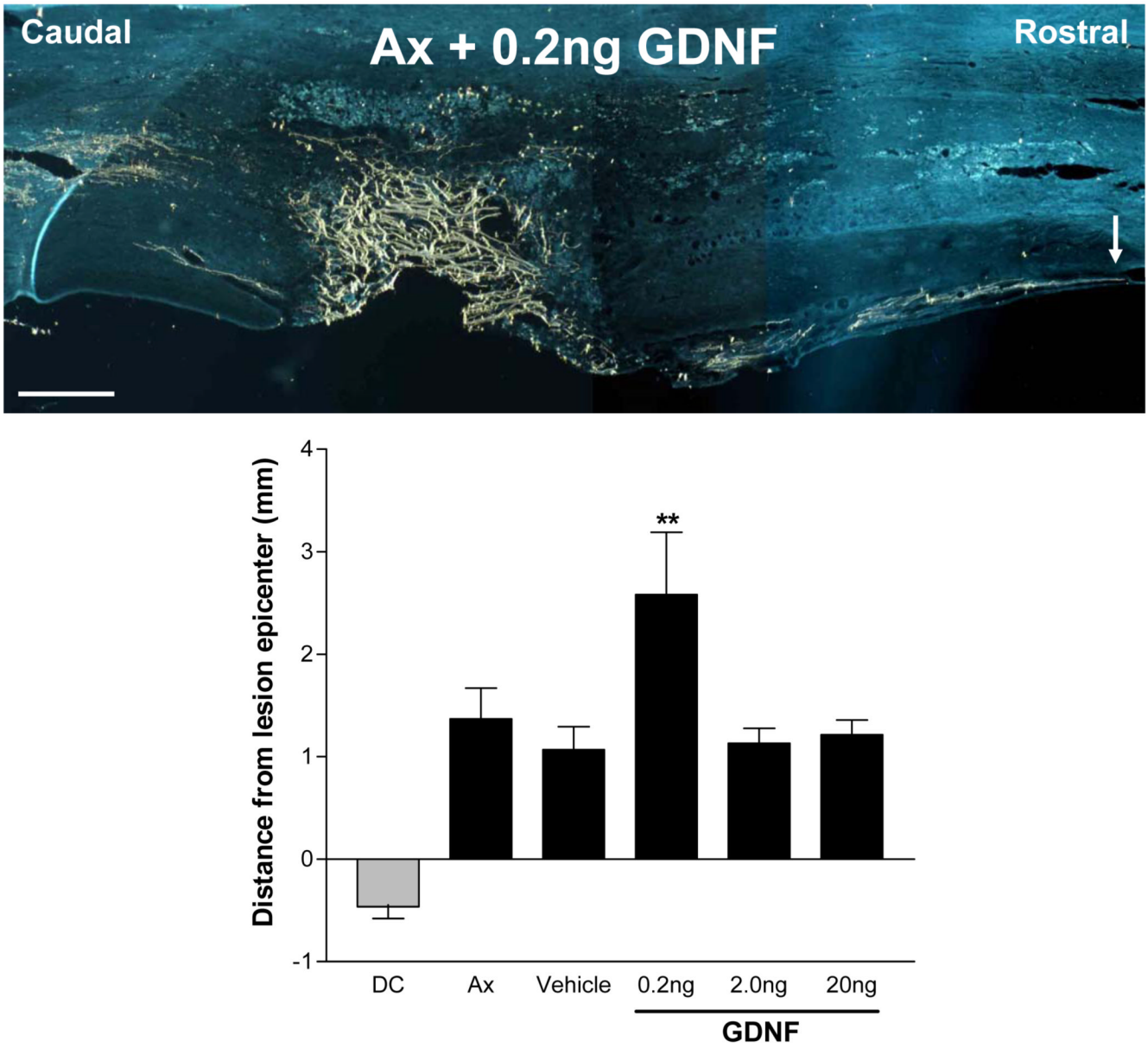


**Fig 7.**

Low dose GDNF enhances regeneration *in vivo* after a dorsal column lesion in pre-axotomized animals. Top, montage of several images showing regrowth of dorsal column axons into and beyond the lesion. Arrow indicates length of maximum regrowth. Scale bar = 350 μm. Bottom, quantification of regrowth *in vivo*. Low dose GDNF (0.2ng/day/7 days) increased axonal regeneration of pre-conditioned neurons greater than higher doses of GDNF (2.0 or 20ng/day/7 days). Data are means ± SEM. ** = $p < 0.01$ vs vehicle treated.