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Functional repair after dorsal root rhizotomy using nerve conduits and neurotrophic molecules

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Abstract

Functional recovery after large excision of dorsal roots is absent because of both the limited regeneration capacity of the transected root, and the inability of regenerating sensory fibers to traverse the dorsal root entry zone. In this study, bioresorbable guidance conduits were used to repair 6-mm dorsal root lesion gaps in rats, while neurotrophin-encoding adenoviruses were used to elicit regeneration into the spinal cord. Polyester conduits with or without microfilament bundles were implanted between the transected ends of lumbar dorsal roots. Four weeks later, adenoviruses encoding NGF or GFP were injected into the spinal cord along the entry zone of the damaged dorsal roots. Eight weeks after injury, nerve regeneration was observed through both types of implants, but those containing microfilaments supported more robust regeneration of calcitonin gene-related peptide (CGRP)-positive nociceptive axons. NGF overexpression induced extensive regeneration of CGRP(+) fibers into the spinal cord from implants showing nerve repair. Animals that received conduits containing microfilaments combined with spinal NGF virus injections showed the greatest recovery in nociceptive function, approaching a normal level by 7–8 weeks. This recovery was reversed by recutting the dorsal root through the centre of the conduit, demonstrating that regeneration through the implant, and not sprouting of intact spinal fibers, restored sensory function. This study demonstrates that a combination of PNS guidance conduits and CNS neurotrophin therapy can promote regeneration and restoration of sensory function after severe dorsal root injury.

Introduction

Spinal nerve root avulsion occurs frequently in brachial plexus injury. It causes impairment of sensory and motor function, and is considered to have a very poor prognosis. In recent years, encouraging results have been reported for surgical repair of ventral root avulsion by reimplantation of injured ventral roots into the spinal cord, both in experimental animals and in humans (Bertelli *et al*., 1994; Liu *et al*., 1998; Carlstedt *et al*., 2000). Dorsal root injury, however, remains a clinical challenge, especially in severe cases that involve root avulsion, traumatic injury or surgery that results in large nerve excision. Two major problems are associated with regeneration of sensory axons after severe dorsal root injury. First, these axons show little or no regeneration across large (> 4 mm) excision gaps, even in the presence of a bridging implant. Second, the regenerative barrier present at the dorsal root entry zone (DREZ, the transitional zone between the peripheral and central nervous systems) prevents regenerating sensory afferents from entering the spinal cord and reforming neuronal connections (Carlstedt, 1997; Chong *et al*., 1999). Because of this barrier, dorsal root avulsion injuries are considered central lesions and usually are not surgically repaired. Restoration of sensory function after severe dorsal root injury can only be achieved if both of the aforementioned obstacles are overcome.

Previous studies have demonstrated regeneration of primary sensory axons across a 4-mm gap, but not longer gaps, in transected dorsal roots (McCormack *et al*., 1991; Bloch *et al*., 2001). This regeneration was fruitless, however, as no demonstration of axons re-entering the spinal cord or functional recovery was reported. On the other hand, several experimental approaches have been designed to surmount the regenerative barrier at the DREZ by creating a supportive substrate for axonal growth with intraspinal transplants of embryonic astrocytes (Kliot *et al*., 1990), olfactory ensheathing cells (Ramon-Cueto & Nieto-Sampedro, 1994) or fetal spinal cord tissue (Houle *et al*., 1996), or by introducing appropriate growth factors to increase the intrinsic growth status of dorsal root ganglion (DRG) neurons (Ramer *et al*., 2000; Romero *et al*., 2001). However, in these studies, dorsal roots were injured either by crushing or cutting and coaptation, which represents a less severe injury and only a limited number of clinical cases. A more practical strategy needed to be developed and tested for the treatment of clinically relevant, severe dorsal root injury.

In the present study, we examined whether a combination of tissue engineering techniques and neurotrophin gene therapy could support sensory afferent regeneration across a 6-mm lesion gap in dorsal roots L4–L5 and into the spinal cord to restore sensory function. The findings in this study provide the first possibility for a surgical treatment to induce functional recovery after severe dorsal root injury.

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Materials and methods

Experimental groups

Thirty-five adult (250–350 g) female Sprague–Dawley rats (Harlan Sprague–Dawley, Indianapolis, IN, USA) were divided between two independent experiments. In the first experiment, rats were randomly assigned to four groups: filament-containing tube implantation and NGF/Ad injection ($n = 6$), filament-containing tube implantation and GFP/Ad injection ($n = 4$), empty tube implantation and NGF/Ad injection ($n = 6$), empty tube implantation and GFP/Ad injection ($n = 5$). The second experiment was to determine the effect of a relesion procedure on axonal regrowth and functional recovery. Three experimental groups were tested: filament tube implantation and NGF/Ad injection ($n = 4$), filament tube implantation and GFP/Ad injection ($n = 5$), empty tube implantation and NGF/Ad injection ($n = 5$). All surgical procedures and animal maintenance complied with the NIH guideline regarding the care and use of experimental animals and were approved by the Institutional Animal Care and Research Advisory Committee.

Guidance channel preparation

Filament fabrication

Poly(L-lactide) (PLLA) microfilaments were fabricated by a wet-spinning process (Ngo *et al.*, 2003). Final diameter of filaments was 60–70 μm. The finished fibre was air dried and stored in a desiccator at 4 °C until use.

Manufacture of polymer conduits

Polymer conduits were manufactured by dipping-leaching techniques. Briefly, 0.8 g purified poly(L-lactide-co-DL-lactide, 75 : 25) (PLA, inherent viscosity 1.66 dL/g in $CHCl_3$ at 30 °C) (Birmingham Polymers, Inc., Birmingham, AL, USA) with 1.0 mL triethyl citrate and 0.8 g glucose powder (10–25 μm) were dissolved and homogenized in 10 mL methylene chloride. Polytef sheaths of 1.5 mm outer diameter enclosing stainless steel wires were used as molds for tube dipping. The molds were systematically placed into and removed from the suspension at a constant rate for a total five dips. Every mold incubation time in the suspension was 12 s. Every solvent evaporation time was 40 s. Layers surrounding the mold became the polymer tube, which was further air-dried for 2 days. Then the tube was taken off the mold, vacuumed for 1 day and stored in a desiccator at 4 °C until use. Final thickness of the polymer tubes was ~100 μm.

Final preparation and sterilization of guidance channels

Before being bundled into conduits, PLLA microfilaments were incubated in 70% alcohol for 1 h and dried under a tissue culture hood. Sixteen PLLA microfilaments were bundled through PLA conduits. Conduits with or without filaments were sterilized with 10× penicillin/streptomycin in phosphate buffer (PB) and washed with 0.1 M PB. Finally, the implants were filled with sterile normal saline solution. All the procedures were performed under a tissue culture hood.

Adenoviral vectors

Replication-defective recombinant adenoviruses expressing nerve growth factor (NGF/Ad) or green fluorescent protein (GFP/Ad, control virus) were constructed as described previously (Romero & Smith, 1998; Romero *et al.*, 2000). The functional characterization and spinal cord expression of these adenoviruses have been reported previously (Romero *et al.*, 2000). All plaque-purified adenoviruses were examined for replication-competent adenoviruses (RCA) via PCR. Viruses were then amplified and purified by double caesium chloride gradient ultracentrifugation. The physical number of viral particles was determined by optical absorbency. The number of infectious particles was estimated by crystal violet staining via the agarose overlay method. The quality of these viruses was determined by examining the ratio of infectious to total (live and dead) number of virus, which was always >1 : 50 plaque-forming unit (pfu) to total virus particles.

Surgical procedure

Deeply anaesthetized animals [ketamine (67 mg/kg, i.p.)/xylazine (6.7 mg/kg, i.p.)] underwent a hemilaminectomy at the L1–L2 vertebral segments to expose the lumbar dorsal roots. L4–L5 roots were transected at 5–8 mm from the DREZ and a 4-mm nerve segment was removed. Eight-millimetre long PLLA guidance tubes, with or without inner filament bundles, were then implanted to bridge a 6-mm gap between the proximal and distal nerve stumps. Each side of the nerve was inserted 1 mm into the channel and secured with superglue (Quicktite Loctite). The dorsal roots immediately rostral (L3) and caudal (L6) to the injured site were double-crushed (10 s each) and ligated to prevent collateral sprouting. All lesions were performed unilaterally on the right side.

Four weeks after injury and tube implantation, animals received a second hemilaminectomy (under anaesthesia, as above) at the T13-L1 vertebral segments to expose the lumbar spinal cord. All spinal cord microinjections were performed as described previously (Romero & Smith, 1998; Romero *et al.*, 2000). Before adenoviral administration the animals received 100 μg intraperitoneally of a 1 : 1 combined solution of rat CD-4 (W3/25) and CD-45 (MRC OX-22) antisera to suppress the immune system transiently. Each animal then received eight injections (0.3 μL; 0.5 mm apart and 0.6 mm deep) of adenoviral vectors (2.5×10^6 pfu/μL) along the L4–L5 DREZ. The injections were made through a beveled glass micropipette connected to a Nanoliter injector (World Precision Instruments, Inc., Sarasota, FL, USA) at 20 nL/ s. This injection profile resulted in abundant NGF expression in the injected cord (≈10-fold greater than that in control spinal cord), concentrated in the dorsal spinal cord extending up to the DREZ (Romero *et al.*, 2000; Tang *et al.*, 2004). After injection, dorsal musculature was sutured, the skin incision was closed with autoclips, and the rats were allowed to recover for another 4 weeks. Behavioural testing was performed before the surgery and once a week afterwards until the animals were killed.

For relesion experiments, dorsal roots L4 and L5 were re-exposed 4 weeks after adenovirus injection. The guidance tube was carefully isolated and removed; the regenerated nerve cable was recut and ligated to prevent any axonal regeneration. The wound was closed, and the rats were allowed to recover for another 2 weeks.

Histological preparation

At the end of experiments, animals were anaesthetized using sodium pentobarbital (Nembutal, 0.1 mL/100 g), perfused transcardially with 0.9% saline, followed by 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.5. The nerve implant and the injected spinal cord were extracted and incubated in the same fixative for ~24 h.

For implant retrieval, PLA tubes were removed by longitudinal dissection. Filament bundles were dissolved by placing the implant in the series of dehydration ethanol (50%, 70%, 90%, 100% ethanol),

three changes of methylene chloride, and rehydration ethanol (above ethanol solution, reverse order). Once rehydrated, the tissue was cryoprotected using 30% sucrose in 0.1 M PB at 4 °C for another 2 days. Transverse sections (15 μm) were cryocut at the distal end of the regenerated tissue (1 mm proximal to the nerve stump), mounted onto slides and stored at −20 °C. Spinal cord samples were cryoprotected in a 30% sucrose solution. Thirty-micrometre-thick tissue sections were cut on a cryostat and divided into five alternate sets. The tissue sections were stored in cryoprotectant solution at −20 °C until processed.

Immunocytochemistry

Slide (dorsal root specimen) or floating (spinal cord specimen) sections were incubated with polyclonal antiserum against rat calcitonin gene-related peptide (CGRP; 1 : 20 000; Sigma). Tissue sections of both control and experimental groups were developed simultaneously by using identical incubating solutions. Visualization was achieved by tissue incubation in biotinylated secondary antibodies and then the Vectastain Elite ABC reagents (Vector Laboratories, Burlingame, CA, USA), followed by developing using a peroxidase substrate, 3,3 diaminobenzidine (DAB), generating a brown colour.

Image analysis

All stained tissue sections were viewed with a Nikon E800 light microscope at 100× to 400× magnification. Images were captured with Metamorph Imaging Software 5.0 (Universal Imaging Corp., West Chester, PA, USA). To quantify the CGRP fibre-occupying area, a standard optical density threshold was applied to each image and staining densities equal to or greater than the threshold measured. For regenerated dorsal roots, two distal sections per animal were quantified; and density calculations were of the entire nerve cross-section, including filaments. For spinal cord samples, two sections within L4 and L5 segments were randomly selected and for each section the measurements were taken from the right dorsal horn lateral to midline and above the plane of the spinal canal.

Behavioural test

The latency of paw withdrawal from a radiant heat source was used to measure the rats' response to noxious thermal stimuli, as previously described (Hargreaves *et al*., 1988). All animals were tested before the surgery to establish baseline latencies, after which testing was performed once every week. To describe the testing briefly, we placed the rats beneath an inverted clear plastic chamber on a glass floor. After a 5 min habituation period, the plantar surface of the hindpaw was exposed to a beam of radiant heat applied through the glass floor. Paw withdrawal latency (PWL) was detected automatically by a photocell and was taken as a behavioural index of the nociceptive threshold. Therefore, a score that was increased significantly over baseline represented analgesia (antinociception). A 22 s maximum was used to assure that no tissue damage occurred to the paw. Individuals conducting these experiments were always blinded as to the treatment. PWL readings were always taken in duplicate at ≈10 min intervals.

Statistical analysis

Raw data from image analyses were evaluated using two-sample Student's *t*-test or one-way ANOVA followed by Tukey's *posthoc* tests. Raw data from behavioural tests were analysed using two-way ANOVA with repeated measures to determine the effect of treatment over time. Data represent the mean ± SEM. *P*-values below 0.05 were considered as statistically significant. All statistical analyses were performed with SAS® version 9.0.

Results

The effectiveness of a combined therapy for severe dorsal root injury was investigated using the paradigm shown in Fig. 1. For this study, regrowth and functional recovery of unmyelinated, peptidergic sensory afferents were analysed. These axons are a class of small-diameter, polymodal nociceptive sensory axons containing a specific neuropeptide marker, calcitonin gene-related peptide (CGRP) (Chung *et al*., 1988; Snider & Wright, 1996), and they have been shown to regenerate in the presence of nerve growth factor (Ramer *et al*., 2000; Romero *et al*., 2001). In each animal, dorsal roots L4 and L5 on the right side were transected, leaving a 6-mm long lesion gap. A PLA guidance channel, either empty or filled with PLLA microfilament bundles, was implanted to bridge the two nerve stumps. To reduce sprouting from adjacent segments, L3 and L6 dorsal roots were crushed and tied to prevent regeneration.

Initial studies showed that 4 weeks after injury and implantation, a nerve-like tissue cable formed in most of the guidance tubes, and CGRP-immunoreactive afferents regenerated across the lesion gap (Fig. 1B and C) through either filament-containing (Fig. 1B, D and F) or empty (Fig. 1C, E and G) tube implants up to the DREZ (Fig. 1D–G). Although axons regenerated through both types of implant, they stopped at the DREZ and failed to regenerate into the dorsal spinal cord. Based on these results, we hypothesized that expression of growth-promoting molecules on the CNS side of the DREZ might entice axons to regenerate past the CNS/PNS border and into the spinal cord to re-establish neuronal connections.

For those experiments, animals received eight injections of adenovirus expressing NGF (NGF/Ad) or GFP (GFP/Ad) into the spinal cord along the L4, L5 DREZ (Fig. 1A) 4 weeks after dorsal rhizotomies and tube implantation. Animals were killed 4 weeks after virus injection and 8 weeks after receiving dorsal root implants. The 4-week delay in virus injection was used because initial experiments showed that axons had regenerated through the tubes and reached the DREZ within this time period. At completion of the experiment, dorsal root implants and spinal cords were extracted to analyse regrowth of CGRP(+) afferents across the lesion gap and into the spinal cord. All treated animals were behaviourally tested for nociceptive responses before injury and then once a week afterwards to investigate the effect of injury and treatment on nociceptive sensation.

Filament bundling promotes long-distance regeneration in rat dorsal roots

Eight weeks after dorsal root lesions and implantation (4 weeks after virus injection), nerve regeneration across a 6-mm lesion gap was examined. Thin fibroblast capsules often surrounded the polymer implants. This fibrous capsule helped hold together the guidance channels, which showed definitive signs of degradation at this time point. Examination of the dorsal roots showed the formation of nerve-like tissue cable in 10 out of 11 empty PLA implants (Fig. 2D) and in all 10 filament-containing implants (Fig. 2C). Figure 2A and B shows representative photos of CGRP-positive afferents that have regenerated toward the distal end of the lesion gap. In filament-containing

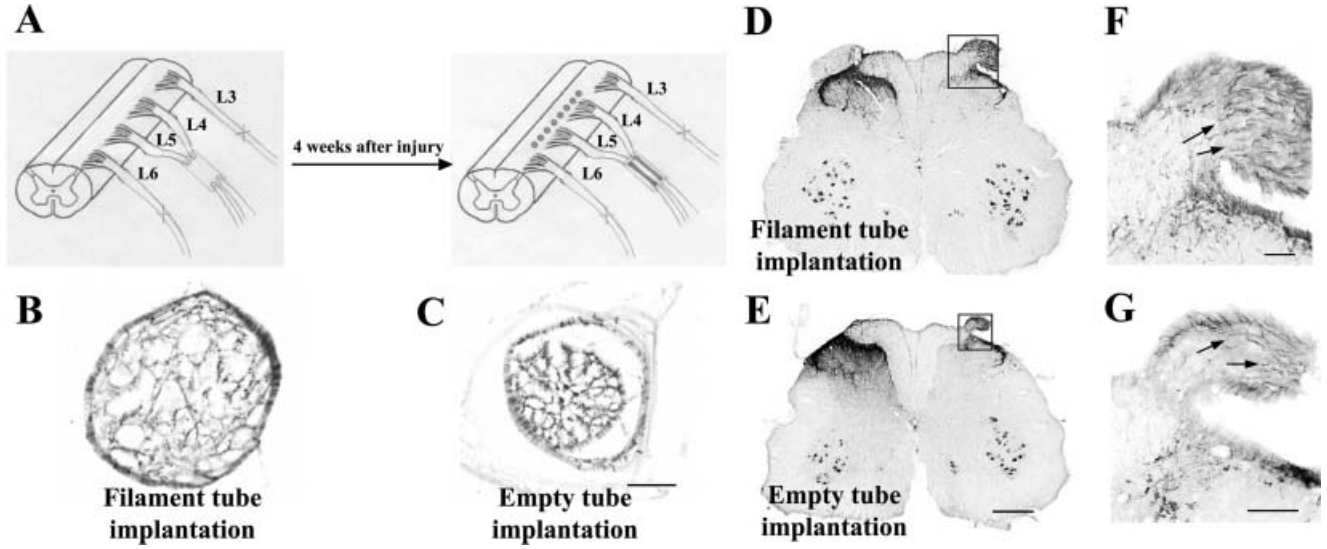

FIG. 1. Schematic illustration of experimental design. Right L4, L5 dorsal roots were cut and a PLA tube, with or without filament bundling, was implanted to bridge a nerve gap of 6 mm in the root. L3, L6 dorsal roots were crushed and ligated to reduce collateral sprouting (A). A pilot study showed that 4 weeks after the initial surgery, CGRP(+) nociceptive afferents were able to regenerate to the distal end of the implant (B and C) and up to the DREZ (D and E). Arrows in high magnification images (F and G) demonstrate that regenerating afferents stop at DREZ. At this time, eight injections of NGF/Ad or GFP/Ad were made along L4, L5 DREZ into the ipsilateral spinal cord (circles in A). The animals were allowed to survive another 4 weeks, after which they were killed for histological analysis. In addition, behavioural test for thermal nociception was performed before the surgery and then once a week until killing. Scale bar, 100 μm (B and C); 500 μm (D and E); 100 μm (F and G).

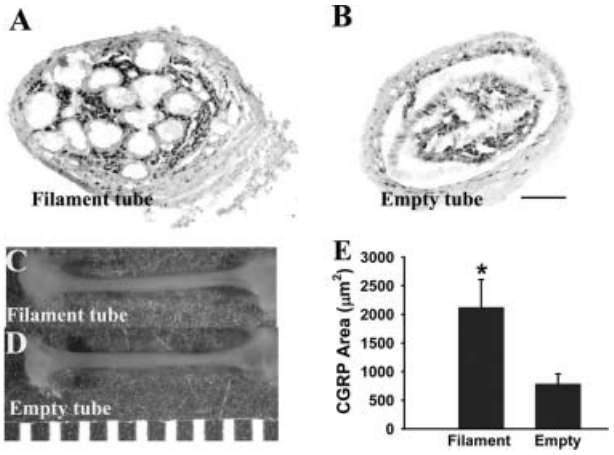

FIG. 2. Filament-loaded guidance tubes improved nerve regeneration across a 6-mm long gap after dorsal root injury. Eight weeks after injury and tube implantation, a regenerated nerve cable was present in 10 of 11 empty tube-implanted rats (D), compared to all 10 of filament tube-implanted rats (C). Representative pictures show transverse sections of the distal end of the regenerated cable within filament tube (A) and empty tube (B). Quantification of CGRP(+) axons at the distal end revealed a significant increase in filament-bundled group (E). Ruler with 1 mm division scale is shown in the background for C and D. Values are expressed as mean ± SEM, $n = 10$–11. *$P < 0.05$, analysed by a Student's t-test. Scale bar, 100 μm.

implants, filaments appeared well integrated within the tissue. Individual filaments were surrounded by several fibroblast-like cell layers; and axons were distributed evenly along the filaments. Previously we showed that filament-containing implants enhanced sciatic nerve regeneration over long nerve lesion gaps. To determine if these implants enhance regeneration across dorsal root lesion gaps, the area of CGRP-positive staining was measured. Quantification of the area of CGRP-positive staining was used to give a relative estimate of the extent of axonal regeneration. Filament-containing guidance tubes showed a more than 2.5-fold increase (Fig. 2E, 2123 ± 482 vs. 786 ± 173 μm^2, $n = 10$ and 11, $F_{1,19} = 6.15$, $P = 0.02$) in the amount of CGRP axon labelling at the distal end of implants when compared to empty tubes. These data indicate that longitudinally aligned filaments effectively enhanced regeneration of sensory afferents over a long distance in the dorsal root.

Neurotrophin gene therapy induced extensive regeneration of sensory axons into the spinal cord after severe dorsal root injury and nerve conduit implantation

Four weeks after adenoviral injections (and 8 weeks after injury and guidance channel implantation), CGRP fibre distribution within the spinal cord was compared between GFP/Ad-injected (Fig. 3B and D) and NGF/Ad-injected (Fig. 3A and C) rats in both filament-containing (Fig. 3A and B) and empty tube implant groups (Fig. 3C and D). Interestingly, regeneration of nociceptive axons into the spinal cord from both types of implants showed similar growth patterns. With either implant type, GFP/Ad-injected cord showed a nearly complete loss of CGRP(+) fibers on ipsilateral (right) side, except for a small contingent remaining in Lissauer's tract. This verified that injury-induced degeneration fails to initiate spontaneous regeneration of CGRP-positive sensory axons across the DREZ even after implantation of guidance channels within the nerve. NGF expression, in contrast, resulted in robust regeneration of CGRP fibers throughout the dorsal spinal cord, including the cuneate fasciculus, laminae I–VI of the dorsal horn, the lateral funiculus, and even into the ventral spinal cord. Quantification of CGRP(+) fibre growth showed that injury caused more than 80% decrease in the area occupied by CGRP fibers in the ipsilateral spinal cord. NGF/Ad injections resulted in robust regeneration of sensory afferents into the spinal cord of both filament-containing (651400 ± 82730 vs. 30250 ± 8845 μm^2) and empty (596200 ± 131600 vs. 32170 ± 3281 μm^2) implant groups

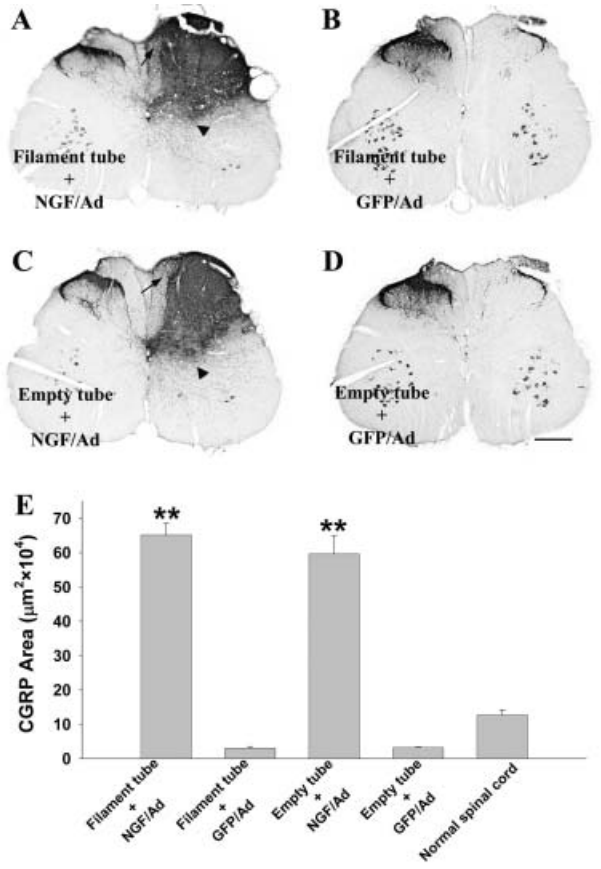

FIG. 3. NGF expression 4 weeks after dorsal root injury and guidance tube implantation induced robust regrowth of CGRP(+) afferents into spinal cord by 8 weeks postinjury. NGF/Ad injection into filament (A) or empty tube (C) implanted rats caused reinnervation of the ipsilateral (right) spinal cord, with robust regrowth of CGRP(+) afferents within the cuneate fasciculus (arrow), throughout dorsal cord and extending to ventral cord (arrowhead), when compared to GFP/Ad injected animals (B and D). Quantification of CGRP fibre-occupying area showed a dramatic increase of fibre growth in NGF/Ad-injected cord, which is higher than that in normal spinal cord. Values are expressed as mean ± SEM, $n = 4$–6. $^{**}P < 0.01$, compared with GFP/Ad group, analysed by one-way ANOVA, followed by a Tukey *posthoc* test. Scale bar, 500 μm.

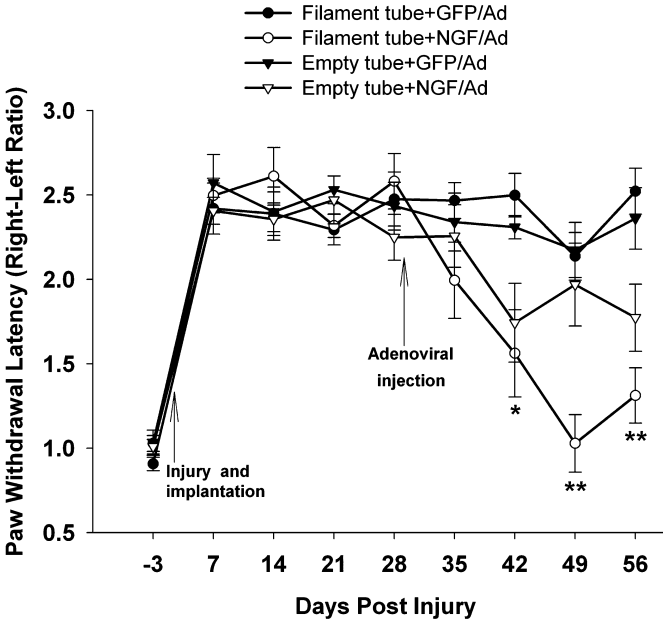

FIG. 4. NGF/Ad injection in filament-tube implantation rats significantly improved sensory function after severe dorsal root injury. Paw withdrawal latency (PWL) was measured on both hindlimbs before and 1 week, 2 weeks, 3 weeks through to 8 weeks after the injury. The experimental (right) to control (left) side ratio of PWL were used to assess nociceptive function change on the ipsilateral hindlimb. Dorsal root injury caused a complete loss of nociceptive function in all four experimental groups, which remained unchanged for 4 weeks before the virus injection. NGF/Ad injection into filament tube-implanted rats significantly reduced the PWL from 2 weeks postinjection, to a near-normal level by 3 and 4 weeks postinjection. NGF/Ad injection into empty tube implantation group also decreased the PWL, but failed to reach statistical difference compared with control group. Values are expressed as mean ± SEM, $n = 4$–6. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with GFP/Ad group, analysed by two-way ANOVA.

(Fig. 3E). The high density of axons within the dorsal horn is most likely reflective of both regenerating axons through L4 and L5 as well as sprouting of axons from L2 regions or higher (Romero *et al.*, 2001). Interestingly, even though filament-containing guidance tubes showed more robust nerve repair than empty tubes, there was no significant difference in spinal cord CGRP fibre density between these two implant groups.

Filament-containing guidance tubes combined with neurotrophin gene therapy led to significant sensory recovery after severe dorsal root injury

To investigate functional recovery, both hindlimbs were examined for changes in nociceptive response to thermal stimuli before the injury and once every week afterwards for 8 weeks. The temporal latency for hindlimb withdrawal from the noxious stimulus was measured for the experimental (right) and control (left) sides and plotted as right : left ratios for paw withdrawal latencies (PWL, Fig. 4). Prior to injury, PWL for either hindlimb ranged between 8 and 10 s, resulting in a ratio of 1 : 1. After injury and implantation, all four experimental groups displayed an absence in nociceptive response for the right hindlimb, with PWL reaching the cutoff limit of 22 s. Sensory response remained absent for the entire 4-week period prior to virus injections. GFP/Ad injections failed to induce functional recovery within a 4-week postinjection period, whereas NGF/Ad injections led to differential recovery depending on the type of guidance tube implanted into the dorsal root. NGF treatment combined with filament-containing tubes led to a decrease in PWL, starting 1 week after injections and becoming significant by 2 weeks ($F_{3,38} = 6.12$, $P = 0.02$, two-way ANOVA). Functional recovery reached a value indistinguishable from preinjury levels 3 and 4 weeks after injection ($P = 0.72$ at 4 weeks, when compared with preinjury value, two-way ANOVA). Therefore, filament-containing tube implantation combined with NGF/Ad injections effectively restored neuronal connectivity and nociceptive sensation within the previously denervated area. Unexpectedly, NGF treatment combined with empty guidance tubes only slightly reduced the ipsilateral PWL compared to GFP/Ad-injected animals, and the difference was not statistically significant at the observed time points ($P = 0.09$, two-way ANOVA).

Relesioning the dorsal root reduces NGF-induced regrowth and eliminates functional recovery

To further verify that NGF/Ad injections induced regeneration of CGRP(+) afferents into the spinal cord resulting in sensory recovery of the ipsilateral hindlimb, we recut and ligated the regenerated nerve

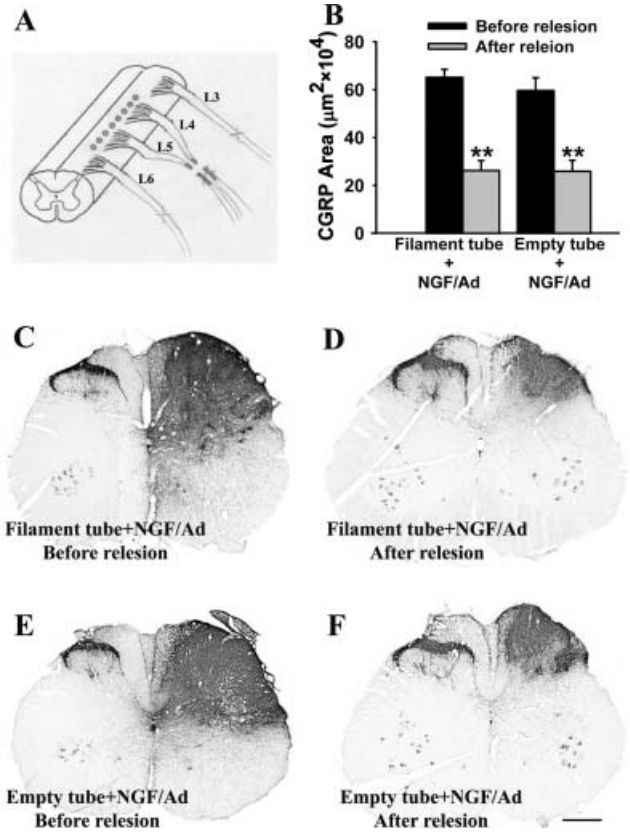

FIG. 5. Relesioning the regenerated nerve cable removed most NGF-induced sensory fibre regrowth. To determine the origin of fibre regrowth and functional recovery, we recut and ligated the regenerated nerve 4 weeks after NGF/Ad injection (8 weeks after injury and tube implantation). Behavioural analysis was performed for 2 weeks postrelesion, after which the animal was killed and spinal cord stained for CGRP(+) afferents (A). In both the empty (E and F) and filament tube (C and D) implantation group, relesion substantially reduced the amount of NGF-induced fibre growth, as confirmed by quantification of CGRP fibre-occupying area (B). There are also sprouting fibers induced by NGF/Ad, which are unaffected by the relesion (D and F). Values are expressed as mean ± SEM, $n = 4$–6. $^{**}P < 0.01$, compared with the cord without relesion, analysed by one-way ANOVA, followed by a Tukey *posthoc* test. Scale bar, 500 μm.

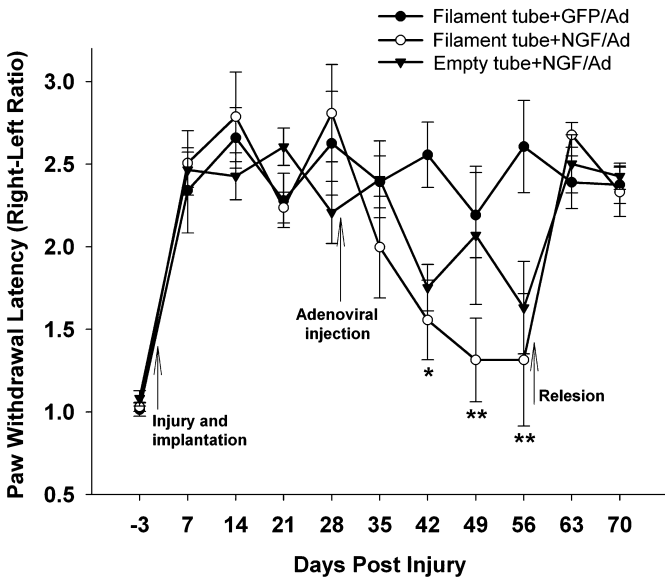

FIG. 6. Relesioning the regenerated dorsal root abolished NGF-induced functional recovery. In filament tube implantation and NGF/Ad treatment group, PWL was reduced by NGF/Ad injection, but returned to the preinjection level after relesion, indicating that NGF induced nociception recovery through regeneration of sensory axons. In GFP/Ad group, PWL went up after the injury and then remained at similar level throughout the experimental time. Values are expressed as mean ± SEM, $n = 4$–5. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with preinjection level, analysed by two-way ANOVA.

cable within the guidance tube 4 weeks after virus injections. Behavioural tests were performed for 2 weeks after the relesion, followed by processing and histological analysis (Fig. 5A). In both filament-containing (Fig. 5C and D) and empty (Fig. 5E and F) tube implantation groups, relesion eliminated most of the NGF-induced fibre regrowth (≈60%, Fig. 5B, $F_{3,17} = 21.3$, $P < 0.01$), indicating that regeneration of axons through the guidance channels accounted for the majority of labelling within the spinal cord. The remaining CGRP-positive fibers most likely represent NGF-induced axonal sprouting from adjacent spinal cord regions containing intact sensory axons. This observation is consistent with our previous results (Romero *et al*., 2001). Behaviour analysis revealed a total abolishment of functional recovery upon relesion in animals receiving filament-containing tubes and NGF/Ad-treatment, indicating functional recovery resulted from regeneration of nociceptive axons through the dorsal roots and into the spinal cord (Fig. 6).

Discussion

The dorsal root injury model described here is significant from both a basic science and a clinical viewpoint. Our model simultaneously addresses two regenerative barriers and highlights several problems associated with regeneration of the nervous system. First, centrally projecting DRG axons have an inherently poor capacity to regenerate after injury when compared to peripheral axons (McCormack *et al*., 1991; Chong *et al*., 1996). While injury to peripherally projecting axons stimulates robust up-regulation of regeneration-associated molecules required for axonal sprouting and elongation, dorsal root injury results in minimal increase in these proteins. One consequence is a reduced rate of growth for dorsal root axons to approximately half the rate of peripherally lesioned DRG axons (Wujek & Lasek, 1983; Jenkins *et al*., 1993; Broude *et al*., 1997). This growth reduction for the central process is more apparent and problematic after excision of nerve pieces four or more millimetres in length. Even when these axons regenerate they encounter a second barrier at the PNS/CNS transitional zone. This barrier is most likely established due to the nonpermissive environment at the dorsal root entry zone, formed by reactive astrocytes, isomorphic gliotic tissues, and associated neurite-inhibitory factors (Siegal *et al*., 1990; Pindzola *et al*., 1993; Aldskogius & Kozlova, 2002). Dorsal root lesions during development or in neonates result in spontaneous regeneration because the DREZ barrier has not fully formed (Carlstedt *et al*., 1988). These characteristics provide an excellent model to study growth-promoting strategies between the peripheral and central nervous systems. In addition, dorsal root injury itself is an important clinical entity that lacks effective treatment. Brachial plexus avulsions often occur during difficult childbirth or traffic accidents, causing sensory loss and some motor control impairments, often accompanied by intractable pain (Berman *et al*., 1998; Terzis *et al*., 2001).

For functional recovery to occur in this model, regenerating axons first needed to be directed across the dorsal root lesion gap and, secondly, into the spinal cord to restore synaptic connections with second order sensory neurons. To enhance regeneration of DRG

sensory axons across the first barrier (a 6-mm nerve gap) two guidance tubes were analysed. Filament-containing PLA guidance tubes significantly enhanced regeneration of sensory axons when compared to empty guidance tubes. The longitudinally aligned filaments within these tubes are thought to serve as a migratory substrate for non-neuronal cells to efficiently organize tissue cable formation and reconstruct the damaged nerve (Rangappa *et al*., 2000; Ngo *et al*., 2003; Cai *et al*., 2004). Guidance tubes containing filament bundles markedly improved Schwann cell migration and axonal regeneration as well as axonal maturation and fasciculation, especially across long gap injuries (Ngo *et al*., 2003; Cai *et al*., 2004). The guidance tubes themselves were fabricated to be biodegradable and permeable to metabolites and most proteins. These PLA guidance channels showed less nerve compression and improved axonal growth and maturation when compared with nondegradable silicone tubes (our unpublished data). In the present experiments, the degradation of the polymer is slow and mostly intact after 10 weeks; however, modification of the polymer can be made without jeopardizing filament performance (Ngo *et al*., 2003; Cai *et al*., 2004).

After NGF/Ad injections, the recovery of sensory function in the filament-containing guidance tube group was statistically significant when compared to all other groups. Despite the fact that the empty guidance tube group showed comparable density of CGRP(+) nociceptive fibre within the spinal cord, only a modest return of function was observed. This difference in behavioural performance might be due to differences in the number of axons growing across the dorsal root gap. The density of axons regenerating through the filament-containing implants was > 2.5-fold higher than that observed through the empty guidance tubes. This higher density of axons strongly indicates that there is a direct relationship between the number of regenerating axons and the extent of functional recovery. Larger numbers of regenerating axons would increase the number of receptive fields in the periphery communicating to second order neurons within the spinal cord. Reinnervation by more receptive fields would result in better perception of physiologically relevant stimuli and the return to near baseline of thermal nociception. The high density of CGRP-positive axons within the spinal cords of either group is reflective of the potent sprouting behaviour of these axons in the presence of NGF (Romero *et al*., 2000, 2001; Tang *et al*., 2004). Sprouting axons could potentially be from two sources, the first being branching of regenerating axons from appropriate dermatomes and the other being spouting of axons through Lissauer's tract from spinal cord regions above or below the lesioned roots. We have previously observed robust sprouting of axons from lumbar regions above or below the lesioned nerve segments in the absence of regeneration (Romero *et al*., 2001). This form of sprouting does not mediate functional recovery of the denervated region, but may result in mechanical allodynia (Tang *et al*., 2004). Sprouting of c-fibers, however, can be controlled by coexpression of semaphorin 3A, a potent chemorepulsive factor for these axons (Tanelian *et al*., 1997; Tang *et al*., 2004).

The dorsal root cable contains different subpopulations of sensory axons, which express different high-affinity tyrosine kinase receptors and respond to different neurotrophic factors (Snider & Wright, 1996; Snider & McMahon, 1998). The CGRP-positive axons studied here represent only one subpopulation, which is TrkA-positive and NGF-responsive. Other populations include medium-sized myelinated mechanoreceptor axons expressing TrkB and interacting with brain-derived neurotrophic factor (BDNF), and large myelinated proprioceptive axons expressing TrkC and responding to neurotrophin-3 (NT-3). Ramer *et al*. (2000) showed on a dorsal root crush injury model that intrathecal infusion of different neurotrophic factors resulted in selective ingrowth of different populations of sensory afferents, which corresponds to the known effects of neurotrophins on distinct classes of sensory axons. Future studies need to determine whether expression of other neurotrophic factors in the spinal cord could induce regeneration and functional recovery of other classes of sensory afferents, in order to get a more complete reconstruction and recovery of sensory function after severe dorsal root injury. Functional regeneration of nociceptive sensory afferents was effectively induced by use of a filament-containing PLA guidance channel bridging the 6-mm dorsal root lesion, followed by neurotrophin gene therapy within spinal cord. The findings in this study may help to develop treatments for severe dorsal root injury, and may also be applied for regenerating other nervous system structures.

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Abbreviations

Ad, adenovirus; CGRP, calcitonin gene-related peptide; DREZ, dorsal root entry zone; DRG, dorsal root ganglion; GFP, green fluorescent protein; NGF, nerve growth factor; PLA, poly(L-lactide-co-DL-lactide, 75 : 25); PLLA, poly(L-lactide); PWL, paw withdrawal latency.

References

Aldskogius, H. & Kozlova, E.N. (2002) Strategies for repair of the deafferented spinal cord. *Brain Res. Brain Res. Rev.*, **40**, 301–308.

Berman, J.S., Birch, R. & Anand, P. (1998) Pain following human brachial plexus injury with spinal cord root avulsion and the effect of surgery. *Pain*, **75**, 199–207.

Bertelli, J.A., Orsal, D. & Mira, J.C. (1994) Median nerve neurotization by peripheral nerve grafts directly implanted into the spinal cord: anatomical, behavioural and electrophysiological evidences of sensorimotor recovery. *Brain Res.*, **644**, 150–159.

Bloch, J., Fine, E.G., Bouche, N., Zurn, A.D. & Aebischer, P. (2001) Nerve growth factor- and neurotrophin-3-releasing guidance channels promote regeneration of the transected rat dorsal root. *Exp. Neurol.*, **172**, 425–432.

Broude, E., McAtee, M., Kelley, M.S. & Bregman, B.S. (1997) c-Jun expression in adult rat dorsal root ganglion neurons: differential response after central or peripheral axotomy. *Exp. Neurol.*, **148**, 367–377.

Cai, J., Peng, X., Nelson, K.D., Eberhart, R. & Smith, G.M. (2004) Synergistic improvements in cell and axonal migration across sciatic nerve lesion gaps using bioresorbable filaments and heregulin-beta1. *J. Biomed. Mater. Res.*, **69**, 247–258.

Carlstedt, T. (1997) Nerve fibre regeneration across the peripheral-central transitional zone. *J. Anat.*, **190**, 51–56.

Carlstedt, T., Anand, P., Hallin, R., Misra, P.V., Noren, G. & Seferlis, T. (2000) Spinal nerve root repair and reimplantation of avulsed ventral roots into the spinal cord after brachial plexus injury. *J. Neurosurg.*, **93**, 237–247.

Carlstedt, T., Cullheim, S., Risling, M. & Ulfhake, B. (1988) Mammalian root-spinal cord regeneration. *Prog. Brain Res.*, **78**, 225–229.

Chong, M.S., Woolf, C.J., Haque, N.S. & Anderson, P.N. (1999) Axonal regeneration from injured dorsal roots into the spinal cord of adult rats. *J. Comp. Neurol.*, **410**, 42–54.

Chong, M.S., Woolf, C.J., Turmaine, M., Emson, P.C. & Anderson, P.N. (1996) Intrinsic versus extrinsic factors in determining the regeneration of the central processes of rat dorsal root ganglion neurons: the influence of a peripheral nerve graft. *J. Comp. Neurol.*, **370**, 97–104.

Chung, K., Lee, W.T. & Carlton, S.M. (1988) The effects of dorsal rhizotomy and spinal cord isolation on calcitonin gene-related peptide-labeled terminals in the rat lumbar dorsal horn. *Neurosci. Lett.*, **90**, 27–32.

Hargreaves, K., Dubner, R., Brown, F., Flores, C. & Joris, J. (1988) A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. *Pain*, **32**, 77–88.

Houle, J.D., Skinner, R.D., Garcia-Rill, E. & Turner, K.L. (1996) Synaptic evoked potentials from regenerating dorsal root axons within fetal spinal cord tissue transplants. *Exp. Neurol.*, **139**, 278–290.

Jenkins, R., McMahon, S.B., Bond, A.B. & Hunt, S.P. (1993) Expression of c-Jun as a response to dorsal root and peripheral nerve section in damaged and adjacent intact primary sensory neurons in the rat. *Eur. J. Neurosci.*, **5**, 751–759.

Kliot, M., Smith, G.M., Siegal, J.D. & Silver, J. (1990) Astrocyte-polymer implants promote regeneration of dorsal root fibers into the adult mammalian spinal cord. *Exp. Neurol.*, **109**, 57–69.

Liu, S., Bodjarian, N., Langlois, O., Bonnard, A.S., Boisset, N., Peulve, P., Said, G. & Tadie, M. (1998) Axonal regrowth through a collagen guidance channel bridging spinal cord to the avulsed C6 roots: functional recovery in primates with brachial plexus injury. *J. Neurosci. Res.*, **51**, 723–734.

McCormack, M.L., Goddard, M., Guenard, V. & Aebischer, P. (1991) Comparison of dorsal and ventral spinal root regeneration through semipermeable guidance channels. *J. Comp. Neurol.*, **313**, 449–456.

Ngo, T.T., Waggoner, P.J., Romero, A.A., Nelson, K.D., Eberhart, R.C. & Smith, G.M. (2003) Poly (L-lactide) microfilaments enhance peripheral nerve regeneration across extended nerve lesions. *J. Neurosci. Res.*, **72**, 227–238.

Pindzola, R.R., Doller, C. & Silver, J. (1993) Putative inhibitory extracellular matrix molecules at the dorsal root entry zone of the spinal cord during development and after root and sciatic nerve lesions. *Dev. Biol.*, **156**, 34–48.

Ramer, M.S., Priestley, J.V. & McMahon, S.B. (2000) Functional regeneration of sensory axons into the adult spinal cord. *Nature*, **403**, 312–316.

Ramon-Cueto, A. & Nieto-Sampedro, M. (1994) Regeneration into the spinal cord of transected dorsal root axons is promoted by ensheathing glia transplants. *Exp. Neurol.*, **127**, 232–244.

Rangappa, N., Romero, A., Nelson, K.D., Eberhart, R.C. & Smith, G.M. (2000) Laminin-coated poly (L-lactide) filaments induce robust neurite growth while providing directional orientation. *J. Biomed. Mater. Res.*, **51**, 625–634.

Romero, M.I., Rangappa, N., Garry, M.G. & Smith, G.M. (2001) Functional regeneration of chronically injured sensory afferents into adult spinal cord after neurotrophin gene therapy. *J. Neurosci.*, **21**, 8408–8416.

Romero, M.I., Rangappa, N., Li, L., Lightfoot, E., Garry, M.G. & Smith, G.M. (2000) Extensive sprouting of sensory afferents and hyperalgesia induced by conditional expression of nerve growth factor in the adult spinal cord. *J. Neurosci.*, **20**, 4435–4445.

Romero, M.I. & Smith, G.M. (1998) Adenoviral gene transfer into the normal and injured spinal cord: enhanced transgene stability by combined administration of temperature-sensitive virus and transient immune blockade. *Gene Ther.*, **5**, 1612–1621.

Siegal, J.D., Kliot, M., Smith, G.M. & Silver, J. (1990) A comparison of the regeneration potential of dorsal root fibers into gray or white matter of the adult rat spinal cord. *Exp. Neurol.*, **109**, 90–97.

Snider, W.D. & McMahon, S.B. (1998) Tackling pain at the source: new ideas about nociceptors. *Neuron*, **20**, 629–632.

Snider, W.D. & Wright, D.E. (1996) Neurotrophins cause a new sensation. *Neuron*, **16**, 229–232.

Tanelian, D.L., Barry, M.A., Johnston, S.A., Le, T. & Smith, G.M. (1997) Semaphorin III can repulse and inhibit adult sensory afferents *in vivo*. *Nature Med.*, **3**, 1398–1401.

Tang, X.Q., Tanelian, D.L. & Smith, G.M. (2004) Semaphorin3A inhibits nerve growth factor-induced sprouting of nociceptive afferents in adult rat spinal cord. *J. Neurosci.*, **24**, 819–827.

Terzis, J.K., Vekris, M.D. & Soucacos, P.N. (2001) Brachial plexus root avulsions. *World J. Surg.*, **25**, 1049–1061.

Wujek, J.R. & Lasek, R.J. (1983) Correlation of axonal regeneration and slow component B in two branches of a single axon. *J. Neurosci.*, **3**, 243–251.