Published in final edited form as:

Exp Neurol. 2007 October ; 207(2): 203–217. doi:10.1016/j.expneurol.2007.06.023.

TRANSDUCED SCHWANN CELLS PROMOTE AXON GROWTH AND MYELINATION AFTER SPINAL CORD INJURY

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Abstract

We sought to directly compare growth and myelination of local and supraspinal axons by implanting into the injured spinal cord Schwann cells (SCs) transduced *ex vivo* with adenoviral (AdV) or lentiviral (LV) vectors encoding a bifunctional neurotrophin molecule (D15A). D15A mimics actions of both neurotrophin-3 and brain-derived neurotrophic factor. Transduced SCs were injected into the injury center one week after a moderate thoracic (T8) adult rat spinal cord contusion. D15A expression and bioactivity *in vitro*; D15A levels *in vivo*; and graft volume, SC number, implant axon number and cortico-, reticulo-, raphe-, coerulo-spinal and sensory axon growth were determined for both types of vectors employed to transduce SCs. ELISAs revealed that D15A-secreting SC implants contained significantly higher levels of neurotrophin than non-transduced SC and AdV/GFP and LV/GFP SC controls early after implantation. At 6 wk post-implantation, D15A-secreting SC grafts exhibited 5-fold increases in graft volume, SC number and myelinated axon counts and a 3-fold increase in myelinated to unmyelinated (ensheathed) axon ratios. The total number of axons within grafts of LV/GFP/D15A SCs was estimated to be over 70,000. Also 5-HT, D β H, and CGRP axon length was increased up to 5-fold within D15A grafts. In sum, despite qualitative differences using the two vectors, increased neurotrophin secretion by the implanted D15A SCs led to the presence of a significantly increased number of axons in the contusion site. These results demonstrate the therapeutic potential for utilizing neurotrophin-transduced SCs to repair the injured spinal cord.

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Keywords

spinal cord injury; viral vector transduction; neurotrophin-3; brain derived neurotrophic factor; adenoviral vector; lentiviral vector; supraspinal axons

Introduction

Following spinal cord injury (SCI), regeneration of central nervous system axons is limited, thereby constraining restoration of communication along ascending and descending pathways and resulting in permanent motor and sensory dysfunction. SCI models have been established in rats to mirror human contusion injuries, the most common type of SCI reported clinically. In both rats (Guizar-Sahagun et al., 1994; Hill et al., 2001; Noble and Wrathall, 1985; Stokes and Jakeman, 2002) and humans (Bunge et al., 1993) after contusive injury, cysts form over weeks resulting in an absence of structural support and axonal growth within the lesion.

To provide structural support for axonal growth, Schwann cells (SCs) purified from peripheral nerve or dorsal root ganglia can be grafted into the injured spinal cord (Bunge and Wood, 2006; Oudega and Xu, 2006; Pearse and Barakat, 2006; Pearse and Bunge, 2006) where they elicit axon growth (Martin et al., 1996; Montgomery et al., 1996; Paino et al., 1994; Xu et al., 1995a, 1997). Furthermore, SCs, both *in vitro* (Kleitman et al., 1988; Bahr et al., 1991) and *in vivo* (Blakemore et al., 1977; Duncan et al., 1981; Gilmore and Sims, 1993), are capable of ensheathing and myelinating central axons. In addition to providing physical support for growing axons within the injury site, SCs also generate neurotrophic factors, cell adhesion molecules and extracellular matrix molecules known to promote axonal growth (Guénard et al., 1993; Oudega and Xu, 2006).

In repairing the spinal cord, supraspinal axons originating from motor nuclei in the brain are of particular interest because of their role in locomotion. Transplantation of SCs alone within the contused spinal cord does not elicit supraspinal axon regeneration (Bunge and Wood, 2006). Brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) are known to promote growth of injured supraspinal axons (Coumans et al., 2001; Grider et al., 2005; Grill et al., 1997; Houweling et al., 1998; Jin et al., 2000; Kobayashi et al., 1997; Lu et al., 2005; McTigue et al., 1998; Murray et al., 2002; Schnell et al., 1994; Ye and Houle, 1997), including when they are administered with SCs (Menei et al., 1998; Xu et al., 1995b).

Because different neuronal populations respond to different neurotrophic factors (Nakahara et al., 1996; Ye and Houle, 1997), using multiple growth factors (e.g., both BDNF and NT-3), would be expected to elicit a wider regenerative response. An alternative approach is the use of a bifunctional neurotrophin known as D15A which is capable of mimicking the downstream effects of BDNF and NT-3 through binding to both TrkB and TrkC receptors, respectively (Urfer et al., 1994). D15A is the product of the genetically modified mature human NT-3 gene in which an aspartic acid has been replaced by an alanine at the 15th amino acid position (Urfer et al., 1994).

In the present study, we compared the response of supraspinal descending motor and ascending sensory axons within implants of SCs transduced with adenoviral (AdV) or lentiviral (LV) vectors to secrete D15A in a spinal cord contusion model. *Ex vivo* gene therapy offers the possibility of a continuous supply of neurotrophin by the transplanted cells and largely circumvents the response of the host immune system by reducing exposure to free viral vector particles. It also overcomes the disadvantages that are seen with direct protein infusions: the short half-life of neurotrophins, inability to cross the blood brain

barrier, and trauma from possibly repeated injections or cannulae. In the study described here we combined the provision of a bifunctional neurotrophin with SCs to increase axon number and myelination in the implant. Transduction with D15A is a novel way to combine SC transplantation with a growth factor that activates both TrkB and TrkC receptors to improve repair after SCI.

Materials and Methods

Viral vectors

First generation replication-deficient AdV vectors contained either a green fluorescent protein (GFP) gene or genes coding for GFP and the bifunctional NT-3/BDNF-like molecule, D15A. AdV/D15A and AdV/GFP vectors were kindly supplied by Dr. Scott Whittemore (Abdellatif et al., 2006). The genes were under the transcriptional control of the cytomegalovirus (CMV) promoter. To propagate these vectors, they were first plaque-purified before further amplification in human 293T cells (Blits et al., 2000). Briefly, AdV particles were serially diluted 1:10 in D10 (Dulbecco's modified eagle medium [DMEM], Invitrogen, Carlsbad, CA, supplemented with 10% fetal bovine serum [FBS] and gentamicin [50 µg/ml]). They were then added to 70% confluent 293T cells that contain the E1 gene (Qbiogene, Irvine, CA) in 6-well dishes and overlaid with 1.25% low melting point agarose (SeaPlaque, FMC Corporation, Rockland, ME) in D10. Individual plaques were selected for the presence of GFP, followed by infection at a MOI of 10-20 and amplification in 293T cells in twenty 10-cm dishes. After a cytopathologic effect was observed, viral particles were collected and purified on a CsCl₂ gradient twice. They were dialyzed against TS (137 mM NaCl, 5 mM KCl, 0.7 mM Na₂PO₄, 0.7 mM CaCl₂, 0.5 mM MgCl₂, and 25 mM Tris/HCl, pH 7.4) to desalt and stored at -80°C in TS with 10% glycerol. Viral titers were determined by a plaque assay on confluent 293T cells in a 6-well plate.

The LV/GFP and LV/D15A SCs were generated by the Miami Project Viral Vector Core, by methods previously described (Follenzi and Naldini, 2002; Blits et al., 2005). The genes encoding either GFP or D15A were subcloned into a LV vector plasmid, p156RRLsinPPT_hCMVMCSpre (generously provided by Dr. L. Naldini, University of Torino). Besides the LTR and necessary packaging signals, this plasmid contained the CMV promoter and the Woodchuck post-transcriptional regulatory element (WPRE). Co-transduction of SCs with LV/GFP and LV/D15A was performed in the absence of polybrene.

Schwann cell preparation

Purified populations of SCs were collected from dissociated sciatic nerves of adult female Fischer rats, according to Morrissey et al. (1991). Briefly, sciatic nerves were dissected and the perineurium was removed. Nerves were cut into 1 mm explants and placed into D10 medium. Contaminating fibroblasts were removed by transplanting only the explants to new dishes weekly. Two weeks later, explants were incubated overnight in 0.25% dispase/0.05% collagenase in DMEM and then dissociated by trituration. From this point on, SC medium was changed to D10 with three mitogens [D10+3M: 2 µM forskolin, 20 µg/ml pituitary extract, 20 µg/ml heregulin]. SCs were further purified by subsequent incubation for 30 minutes in conditioned medium from mouse hybridoma cells containing the Thy1.1 antibody, and adding rabbit complement to destroy remaining fibroblasts. Next, SCs were replated and maintained in culture with mitogens until needed when they were trypsinized and resuspended; their purity was 95-98%.

The *ex vivo* transduction efficiency of the two viral systems was assayed using GFP expression of SCs. SCs (5×10^5 /well) at 70% confluency were seeded in 12-well plates with

D10+3M and transduced overnight with varying MOI (20, 50, 100, 200, 500, and 1000) of AdV vectors. Polybrene (1 μ g/ml; Sigma, St. Louis, MO), previously shown to increase AdV and retroviral transduction efficiency of other cell types in culture (Arcasoy et al., 1997), was added to the medium to enhance AdV vector transduction. The following day, medium was refreshed and the cultures were incubated for 3 more days. The cell cultures were fixed with 4% paraformaldehyde and stained with Hoechst to allow a comparison of Hoechst labeled nuclei and GFP positive cells. The highest percentage of GFP positive cells with no obvious signs of cytotoxicity following incubation was designated the optimal MOI and was used for all subsequent *in vitro* and *in vivo* studies. LV vector-transduced SCs were transduced at a MOI of 50 as earlier optimized by the Viral Vector Core. Cells were harvested and stored in liquid nitrogen, where they could be thawed and expanded as needed.

Gene expression quantification

In order to quantify neurotrophin secretion, 1×10^5 SCs in 3 ml of D10+3M and polybrene (1 μ g/ml) were infected overnight in a 6-well dish with either AdV/GFP or AdV/GFP/D15A vectors at a MOI of 200. LV vector-transduced SCs (MOI=50) were thawed and incubated in D10+3M. From AdV- and LV-transduced SCs, conditioned media were collected daily for three days before freezing the cells in a dry ice/ethanol bath for storage at -80°C. The amount of neurotrophin in the conditioned media was determined using an ELISA recognizing NT-3, according to the manufacturer's protocol (Promega, Madison, WI).

D15A bioactivity

A neurite outgrowth assay using E15 lumbar dorsal root ganglia (DRG) was employed to demonstrate neurotrophin bioactivity. Twelve-well dishes were coated with polyornithine (500 mg/ml) overnight. Lumbar DRG were removed on embryonic day 15, pooled in L15, placed in wells containing 600 μ l of conditioned medium collected from transduced SC cultures at day 3, and incubated for three days at 37°C in 5% CO₂. After rinsing with PBS three times, 600 μ l of conditioned medium, conditioned medium containing anti-human NT-3 antibodies (83 μ g/ml), D10 alone, or D10 supplemented with BDNF (730 ng/ml) and NT-3 (730 ng/ml) was added to the wells. Each culture group was prepared in triplicate; the experiment was conducted twice.

The explants were fixed in 4% paraformaldehyde for 15 minutes, permeabilized in 100% methanol at -20°C for 10 minutes and rinsed with TBS containing 1% normal goat serum (TBS-NGS). The explants were then incubated with a mouse monoclonal anti-neurofilament antibody (RT97 1:5 in TBS-NGS; Developmental Studies Hybridoma Bank, Iowa City, IA) for 1 hour at room temperature. They were washed three times with TBS-NGS and incubated with a biotin conjugated goat anti-mouse IgG antibody (1:200 in TBS-NGS, Santa Cruz Biotechnology, Santa Cruz, CA) for 30 minutes at room temperature. Explants were rinsed again three times with TBS-NGS and incubated for 1 hour in peroxidase-conjugated streptavidin (DAKO Corporation, Carpinteria, CA). The explants were washed a final three times in TBS and stained with 3, 3' diaminobenzidine tetrahydrochloride (DAB, 0.05%) in Tris-immidazole buffer (pH 7.5) with 0.04% (NH₄)₂SO₄NiSO₄ and 0.01% H₂O₂.

Animal injury

Adult female Fischer rats (180-200g) (n=219 total usable animals; Table1) were housed two per cage and provided food and water *ad libitum*. All animal procedures and care were approved by the University of Miami Animal Care and Use Committee, in accordance with NIH and the *Guide for the Care and Use of Animals*. The animals' backs were shaved and cleaned with betadine. For injury and implantation procedures, animals were anaesthetized

with a humidified mixture of 1-2% vaporized halothane, 60% nitrous oxide and 40% oxygen.

Following anesthesia, T7-T9 vertebrae were exposed and a dorsal laminectomy was performed at the T8 level to expose the dura. The T6 and T12 vertebrae were held with stabilization clamps, and the T8 level of the spinal cord was placed under the shaft of the MASCIS weight drop device. The moderate contusion was created by dropping a 10-gram rod from a height of 12.5 mm onto the exposed cord. Compression distances between 1.25 and 1.75 mm, and impact height and velocity errors below 6%, were acceptable values to ensure consistency. Muscles were subsequently sutured together and the skin was closed using Michel clips. The rats then received intramuscular gentamicin (5 mg/kg) for seven days and 3 ml of Ringer's solution subcutaneously. The analgesic, Buprenex (Reckitt Benckiser, Richmond, VA; 0.01 mg/kg) was administered subcutaneously for 24 hours. Animals, returned to their cages, were allowed access to a heating pad. Temperature and hydration status were carefully monitored for twenty-four hours after injury. Bladders were expressed twice a day for 3-7 days until spontaneous voiding began.

Schwann cell implantation

Seven days after injury, the contusion site was re-exposed. Meanwhile, primary SCs at 80-90% confluence were trypsinized from 100 mm dishes, twice pelleted at 1,500 rpm for 5 min and washed with D10, and resuspended in DMEM. SCs were collected the morning of scheduled surgery, kept on ice, and implanted within three hours. Animals were divided into five implant groups: non-transduced SCs, GFP-only AdV vector-transduced SCs (AdV/GFP SCs), D15A/GFP-containing AdV vector-transduced SCs (AdV/GFP/D15A SCs), GFP-only LV vector-transduced SCs (LV/GFP SCs), and D15A/GFP-containing LV vector-transduced SCs (LV/GFP/D15A SCs) (Table 1). Animals survived for up to seven weeks after the initial injury. One injection of 6 μ l SCs in DMEM ($\sim 2 \times 10^6$ cells) was performed over two minutes into the injury epicenter at the midline of the spinal cord at a 75° angle to the cord using a 10 μ l Hamilton syringe with a fitted glass capillary tube (150 μ m inner diameter). The syringe was pulled out slowly over a period of one minute, and a small piece of gel foam was used to patch the hole created in the spinal cord to prevent leakage of SCs out of the injection site. Post-operative procedures were performed as described above.

Tissue ELISA

To determine neurotrophin levels in the injury/graft site, homogenized tissue was subjected to an ELISA recognizing NT-3 (Ying et al., 2003). Two days after initial injury, two days after implantation, or one, three or six weeks after implantation (Table 1), animals were anesthetized with rat cocktail (43 mg/ml ketamine, 8.6 mg/ml xylazine, 1.4 mg/ml acepromazine, 0.2 ml/100 g body weight). Spinal cord segments including the injury/graft sites (~ 8 mm) were removed, quickly frozen (dry ice/ethanol temperature) and stored at -80°C. Later, tissue was weighed and homogenized by placing in 10X weight per volume lysis buffer (137 mM NaCl, 20 mM Tris, 1% NP-40 [Calbiochem, San Diego, CA], 10% glycerol, 1 mM PMSF, 0.5 mM sodium vanadate, 1 μ g/ml leupeptin, 10 μ g/ml aprotinin, pH 8.0) and gently homogenized with pestle and mortar followed by sonication for 10 seconds. Samples were centrifuged at 12,000 rpm for five minutes and the supernatants harvested for storage at -80°C. Measurements were normalized to total protein, as determined by a standardized Bradford protein assay. The amount of secreted NT-3 was expressed as the total amount of NT-3 in the sample per milligram of total protein.

Anterograde tracing

Animals were randomly divided into two groups for corticospinal tract and reticulospinal tract tracing (Table 1). Three weeks after implantation, animals were anesthetized with rat

cocktail and anterograde tracing was performed. For group 1, biotinylated dextran amine (BDA, 10,000 MW, 10% in PBS, Molecular Probes, Eugene, OR) was stereotactically injected at 8 locations over 1.5 min/injection site into the hindlimb motor cortex (Bregma -1.5, -2, -2.5, -3 mm, 2.5 mm lateral to the midline, depth 1 mm; 0.5 μ L/site, bilateral). Animals in group 2 received two stereotaxic injections over 1 min/injection site of dextran coupled to rhodamine (D-Rh, 10% in PBS, Molecular Probes) into the reticular formation (Bregma -10.8 mm, 0.6 mm lateral to the midline, depth 8.5 mm; 0.3/ μ L site, bilateral). Injections were performed using a 1 μ L Hamilton syringe with a fitted glass capillary tube (100 μ m inner diameter). The needle was slowly removed to prevent leakage and the wound was covered with Gelfoam. The skin was sutured closed and animals received 3 ml of Ringer's solution subcutaneously. Temperature and hydration status were carefully monitored for 24 hours.

Histology and immunohistochemistry

Seven weeks after injury (six weeks post-implantation), animals (Table 1) were euthanized with rat cocktail and, after an injection of 300 units of heparin into the heart, were transcardially perfused with 300 ml of cold (approximately 4°C) 0.9% NaCl solution, followed by 500 ml of phosphate-buffered 4% paraformaldehyde. Brains and spinal cords were dissected immediately after perfusion and placed in 4% paraformaldehyde overnight. The next day, the fixative was replaced with a solution of 30% w/v sucrose and 0.1% sodium azide. The center of the injury/implant area was identified, and 7 mm rostral and caudal (14 mm total) were removed and embedded in gelatin (Oudega et al., 1994) overnight at 37°C, after which the tissue in the gelatin block was immersed in 4% paraformaldehyde overnight, followed by 30% w/v sucrose until analysis. A freezing microtome was used to section the injury/implant area (T6-T10) sagittally at 30 μ m. The sections were stored at 4°C in 0.1 M phosphate buffer containing 0.1% sodium azide (pH 7.4).

We used specific antibodies against 5-hydroxytryptamine (5-HT), dopamine- β -hydroxylase (D β H) and calcitonin gene-related peptide (CGRP) to characterize the axonal regeneration response of populations of supraspinal motor and ascending sensory neurons to grafts of D15A-secreting SCs. Briefly, sections were rinsed once with PBS and incubated in a blocking solution of 5% NGS for one hour at room temperature. Non-transduced and AdV vector-transduced SC animal groups were then incubated overnight at room temperature with a mouse monoclonal p75 (IgG-192,1:2, from hydridoma cells)/5%NGS/0.3% Triton X-100 and a rabbit polyclonal primary antibody against a selected fiber type, 5-HT (1:8000, Immunostar, Hudson, WI), D β H (1:2800, Immunostar) or CGRP (1:1000, Peninsula, San Carlos, CA). Sections were then rinsed three times with PBS and incubated with secondary antibodies, goat anti-mouse IgG AlexaFluor 488 (1:200, Molecular Probes) and goat anti-rabbit AlexaFluor 594 (1:200, Molecular Probes) with 1% NGS for 1 hour at room temperature. The IgG-192 p75 antibody was omitted from the LV vector-transduced SC groups because of their robust GFP expression at the time of sacrifice. Sections were rinsed three times with PBS and subsequently mounted on gelatin-coated glass slides, air dried, and coverslipped with Vectashield (Vector Labs, Burlingame, CA).

Estimation of graft volume was obtained by 3-D reconstruction of serial sections using Neurolucida software. The graft area in each 30 μ m sagittal section (at intervals of 180 μ m) was delineated using GFP fluorescence or p75 immunolabeling. Total fiber lengths of 5-HT, D β H and CGRP immunostained fibers were obtained using computer assisted microscopy and stereological analysis, via an isotropic virtual plane program (MicroBrightField, Inc., Williston, VA). Random, blinded analysis at 63X was performed using an unbiased counting frame superimposed over the graft area (counting frame area, 3600 μ m²; sampling box height, 18 μ m; top and bottom guard zones, 1 μ m each; distance between isotropic planes, 15 μ m; 3 mutually orthogonal orientations; sampling intervals, $x = 175 \mu$ m, $y = 350 \mu$ m).

Schwann cell number estimation

Implants were outlined at 10X and then examined under a 63X oil immersion objective. Using Stereoinvestigator software and the optical fractionator sampling design (counting frame area, 625 μm^2 ; sampling intervals, $x = 250 \mu\text{m}$, $y = 250 \mu\text{m}$), 30 μm sagittal sections (at intervals of 180 μm) containing implants were systematically sampled for an estimation of LV/GFP-positive SC number at six weeks post-implantation.

Myelinated axon counts and total axon estimation

To count the number of axons myelinated by SCs in the graft/injury center, a 1 mm transverse slice from the center was removed after perfusion and prepared for electron microscopy (Xu et al., 1995a). In 1 μm , toluidine blue-stained plastic sections at 63X (oil), outlines of graft areas could be discerned by the distinct morphologic characteristics of SC myelin sheaths ("signet ring" appearance; Bunge and Wood, 2006). Using the optical fractionator sampling design (counting frame area, 625 μm^2 ; sampling intervals, $x = 250 \mu\text{m}$, $y = 250 \mu\text{m}$), the graft epicenter was systematically sampled to obtain the total number of SC-myelinated axons.

Thin sections of the same regions were viewed in a Philips CM10 electron microscope. Electron micrographs were taken at 6,600X, at the four corners and center of 20 random grid squares, when myelinated or unmyelinated axons were present. Both unmyelinated axons and SC myelinated axons were counted for each field. The myelinated axon counts and the ratios of myelinated to unmyelinated axons were used to estimate the total number of axons crossing the graft center (Table 1).

Two 1 μm , toluidine blue-stained plastic sections were selected from the center of the injury/graft from each animal (Table 1). The spared peripheral white matter in each section was first outlined using software (MicroBrightField) and computer-assisted microscopy. The contoured region of each section was divided into grids and sampling of numbers of preserved central myelinated axons within 20 μm^2 regions of each of these grids was performed at 63X (oil) using the optical fractionator method. Counts from each of the two sections per animal were first averaged and then these counts were averaged within a group for statistical comparison among groups.

Traced fiber analysis

For quantifying supraspinal labeled axons, analysis was performed according to Hill et al. (2001). Briefly, a zero point was designated as the rostral-most part of the lesion cavity at the level of the central canal. Nine lines, perpendicular to the rostral-caudal axis, were drawn 500 μm apart from 2 mm rostral to 2 mm caudal from the zero point; axons that crossed these lines were counted at 40X. In order to normalize the number of traced fibers among animal groups, the total number of fibers was quantified 6 mm rostral to the lesion site and the percentage of total axons crossing designated lines caudal to this point was determined. Sections (30 μm) were examined at 120 μm intervals.

Behavioral assessment

All animals were subjected to behavioral testing once a week from injury until sacrifice. Two non-biased observers analyzed hindlimb performance using a Basso, Beattie and Bresnahan (BBB) scoring system (Basso et al., 1995). A BBB sub-score also was obtained because some animals did not exhibit the same progression of functional improvements as delineated by the BBB score (Pearse et al., 2004).

Statistics

Data are presented as means and standard errors of the means for each group. All statistical analyses were performed using the software program, Instat Version 3.06 (Graphpad Software, Inc.). A two-way analysis of variance (ANOVA) and Tukey's post-hoc analysis were performed on *in vivo* ELISA data and one-way repeated measures ANOVA was performed on BBB data, with a Bonferroni post-test to establish significance. All other data sets were subjected to one-way ANOVAs, followed by Student's *t*-test post-test comparisons of the individual means. A statistically significant difference was accepted at $P < 0.05$.

Results

Schwann cells are transduced with LV and AdV vectors

The generation of replication-defective AdV vectors from human 293T cells yielded titers of 5×10^{10} pfu/ml for both D15A and GFP vectors. AdV vector-transduced SCs did not begin to express GFP until approximately 24 hours after infection; at three days after transduction AdV/GFP/D15A and AdV/GFP transduced SCs exhibited GFP expression in about 30% of cultured cells.

LV/GFP/D15A and LV/GFP SCs exhibited robust transduction efficiency with GFP expression in greater than 95% of the cells after thawing from a frozen cell stock that had been infected earlier at an MOI of 50. The titer for both LVs was 1×10^9 TU/ml. LV/GFP transduced SCs continued to express GFP after several passages in culture, and expression was similar in both LV/GFP/D15A and LV/GFP SC cultures.

Transduced SCs secrete bioactive D15A in culture

D15A levels in conditioned supernatant from transduced SCs were determined using an NT-3 ELISA capable of recognizing the NT-3 backbone of D15A. AdV/GFP/D15A and LV/GFP/D15A SCs expressed significantly higher levels (105 ± 9.7 , 179 ± 19.9 SEM ng/ml/24 hours) than non-transduced SC and GFP controls (which did not contain detectable levels) three days after transduction (two-way ANOVA, $P < 0.001$).

The bioactivity of the secreted D15A molecule was confirmed using an embryonic rat DRG neurite outgrowth assay after incubation in conditioned media for three days (Fig.1). Neurofilament staining revealed increased neurite outgrowth in DRG cultures incubated with conditioned media from AdV/GFP/D15A and LV/GFP/D15A SC cultures (Fig. 1B,D) compared to conditioned media from AdV/GFP and LV/GFP SC cultures (Fig. 1A,C). D10 media supplemented with BDNF and NT-3, in the same concentrations as secreted D15A, increased DRG neurite outgrowth compared to unconditioned and conditioned media from non-transduced SCs (Fig. 1F). The neurite outgrowth response to the D15A molecule was attenuated when neutralizing antibodies against NT-3 were added (Fig. 1E).

Neurotrophin secretion is elevated in vivo

Analysis of neurotrophin secretion by implanted SCs (and tissue surrounding the injury/implant site) using an NT-3 ELISA revealed differences in neurotrophin levels among the various implant cell groups. AdV/GFP/D15A ($P < 0.001$) and LV/GFP/D15A ($p < 0.004$) SC grafts secreted significantly higher levels of neurotrophin at 2 days post-implantation (8237 ± 1549 and 4359 ± 1201 pg NT-3/mg total protein, respectively; Fig. 2) compared to non-transduced SC control grafts (106 ± 23 pg) (Fig.2); the levels found in the control AdV/GFP and LV/GFP SC grafts (194 ± 53 and 113 ± 11 pg, respectively) were similar to the non-transduced SC control grafts. Levels of neurotrophin secreted by AdV/GFP/D15A SC grafts were significantly higher than those in LV/GFP/D15A grafts at two days post-implantation

($P < 0.001$). By one week after implantation, AdV/GFP/D15A SC grafts (6462 ± 1002 pg) continued to secrete significantly higher levels of neurotrophin compared to other implant groups ($P < 0.001$), whereas in LV/GFP/D15A SC implants (1212 ± 311 pg), neurotrophin content was diminished to levels that were not significantly different from the non-transduced SC control (Fig. 2) and GFP control groups. Neurotrophin levels in AdV/GFP/D15A and LV/GFP/D15A implants at three and six weeks post-implantation (6 wk, 816 ± 318 and 806 ± 123 pg, respectively) were not significantly different from those in AdV/GFP, LV/GFP and non-transduced control groups (90 ± 7 , 238 ± 94 , 178 ± 22 pg, respectively), possibly due to the small number of samples. Neurotrophin levels in thoracic spinal cord two days after injury (before implantation) did not differ from levels in control SC grafts at any time point after implantation. Thus, D15A-secreting AdV or LV vector-transduced SC grafts exhibited significantly enhanced neurotrophin secretion early after implantation.

Schwann cell implants increase in size when expressing D15A

The graft borders in each spinal cord section at 6 weeks post-implantation were defined visually by GFP expression (for LV vector-transduced cells) or p75 receptor immunoreactivity (for AdV vector-transduced cells). In contrast to the decrease in secretion of D15A (Fig. 2), LV vector-transduced SCs (LV/GFP; LV/GFP/D15A) continued expressing GFP *in vivo* for 6 weeks after implantation whereas GFP expression in AdV vector-transduced SCs (AdV/GFP; AdV/GFP/D15A) diminished in intensity over 6 weeks (data not shown). Measurements of GFP fluorescent or p75 receptor immunoreactive areas revealed differences in implant sizes among groups (Fig. 3). AdV/GFP/D15A and LV/GFP/D15A SC grafts possessed significantly increased graft volumes (2.55 ± 0.18 mm³ and 2.41 ± 0.32 mm³, respectively; one-way ANOVA, $P < 0.001$) when compared to AdV/GFP and LV/GFP (1.22 ± 0.09 mm³ and 0.83 ± 0.15 mm³, respectively) and non-transduced control (0.716 ± 0.12 mm³) implants (Fig. 3). Under microscopic examination, the majority of the grafts were widest at or near the injury center, where the injection site was located, and tapered off along the rostrocaudal axis. There was occasional implanted SC migration along the central canal.

To begin to explore possible reasons for changes in implant volumes between GFP-only and neurotrophin secreting grafts, counts of GFP positive cells were performed on the LV vector-transduced SC implants. This quantification was possible because LV vector-transduced cells expressed GFP for at least six weeks after implantation. From the original implanted population of an estimated 2×10^6 SCs, numbers of GFP-labeled SCs present within the injured spinal cord at 6 weeks were significantly higher in the LV/GFP/D15A group (88740 ± 8093 SEM) compared to LV/GFP control animals ($518,400 \pm 77387$ SEM) (26% vs. 5%, respectively; unpaired t-test, $P < 0.001$). Examination of the GFP cell number and volume data demonstrated a positive correlation ($R^2 = 0.91$) between total implant cell number and implant volume.

When the rim of the spinal cord appeared thinner around the enlarged D15A SC implants (Fig. 3B), we questioned whether the enlargement resulted in damage to spared white matter in the periphery of the spinal cord. Central myelinated axon number in this region was determined in injury only, LV/GFP and LV/GFP/D15A implant groups (Table 2). Possibly because the *n* was small, significance was not found. There were no significant differences among groups (one way ANOVA, Bonferroni post-test), suggesting that the enlarged implant did not cause additional loss of intact spinal cord around the implant.

Schwann cell myelinated axons are increased in D15A-secreting graft centers

To assess the effect of increased neurotrophin on axon growth and myelination by SCs, we examined injury/graft centers for peripheral myelin (Fig. 4A-E). SC myelinated axon counts at 6 weeks post-implantation demonstrated a 3.5 to 5 fold increase in D15A-secreting grafts compared to both GFP-only and non-transduced SC control grafts (Fig. 4F). Both AdV/GFP/D15A and LV/GFP/D15A SC grafts contained significantly increased numbers (18232 ± 1577 and 26224 ± 1879 , respectively; one-way ANOVA, $P < 0.001$) of axons myelinated by SCs at the injury center. The counts in LV/GFP/D15A SC implants were considerably higher than in the AdV/GFP/D15A graphs (one-way ANOVA, $P < 0.01$) (Table 3). Non-transduced SC implants contained 5158 ± 786 myelinated axons at the injury epicenter and AdV/GFP and LV/GFP SC implants contained similar numbers (4718 ± 1158 and 4640 ± 971 , respectively).

Total axon number is increased in D15A-secreting SC grafts

Ultrastructural analysis revealed statistically significant differences in myelinated to unmyelinated (ensheathed) axon ratios between LV/GFP/D15A SC grafts and control grafts (Table 3). D15A/SC implants contained more myelinated axons for every unmyelinated axon ($x = 1:1.65$) compared to the three control SC implants ($x = 1:6.9$). By combining the information on number of myelinated axons with these ratios, estimates of total axon number were obtained. In LV/GFP/D15A implants, over 70,000 axons were estimated to be present, compared to the mean of 41,000 for the control groups.

5-HT, D β H and CGRP fiber growth is increased within D15A-secreting implants

Immunolabeling for 5-HT, D β H and CGRP fibers revealed significantly increased axon lengths within the neurotrophin-secreting grafts. The stereologic method we used enabled quantification of total axon length (i.e., growth) within the graft rather than revealing the total number of axons. AdV/GFP/D15A SC implants contained significantly increased (1773 ± 226 mm; one-way ANOVA, $P < 0.01$) serotonergic axon growth compared to non-transduced (573 ± 14 mm), LV/GFP/D15A (1098 ± 204 mm), AdV/GFP (929 ± 50 mm) and LV/GFP (573 ± 130 mm) SC implants, although serotonergic axons penetrated all SC implants (Fig. 5). All D15A containing implants harbored significantly increased (adenoviral, 1501 ± 137 ; lentiviral, 1387 ± 376 mm; one-way ANOVA, $P < 0.01$) growth of D β H positive axons, compared to controls (SCs, 311 ± 48 ; AdV/GFP, 791 ± 67 ; LV/GFP, 612 ± 130 mm). Sensory CGRP fibers were increased significantly (one-way ANOVA, $P < 0.01$) in length in the AdV/GFP/D15A SC implants (2181 ± 324 mm) compared to non-transduced SC (352 ± 46 mm), AdV/GFP control (857 ± 135 ; LV/GFP, 245 ± 46 mm) and LV/GFP/D15A (773 ± 190 mm) SC implants.

CST and RST fibers do not grow into SC implants

Anterograde tracing was performed to identify CST and RST fibers above and in the implant. Examination of injection sites and the rostral cord showed that neuronal uptake and anterograde transport of the tracers was successful in $>95\%$ of animals injected. The CST fibers within dorsal columns clearly were disrupted by the contusion injury. The number of anterogradely labeled CST fibers was significantly increased 500 to 2000 μ m rostral to the rostral border of the LV/GFP/D15A SC implants, compared to controls (two-way ANOVA, $P < 0.01$; Fig. 6A). Elevated labeled fiber counts rostral to the rostral border of AdV/GFP/D15A SC implants also were found, but they were not significantly different from controls. We observed that many labeled CST fibers terminated in end bulbs (not shown) and did not extend into the implant.

Some degree of RST fiber sparing in the lateral and ventral regions of the thoracic spinal cord was observed. No significant differences in RST fiber length were found among animal groups (two-way ANOVA; Fig. 6B). Whereas the number of labeled fibers approaching the lesion steadily decreased, approximately 20% of labeled RST fibers were observed 2 mm caudal to the zero point, in lateral and ventral white matter. Similar to CST fibers, numerous RST fibers terminated in end bulbs (not shown). Many RST labeled fibers terminated at the graft periphery, but no labeled RST axons extended into the graft. GFAP staining showed that labeled fibers stopped at the site of the astrocytic scar (data not shown).

In animals traced for the CST and RST, the results found after AdV/GFP and LV/GFP SC implantation were not significantly different from those in non-transduced SC-implanted spinal cords.

Behavioral recovery is not improved

Recovery of hindlimb function was assessed for all groups. Open field locomotion was evaluated weekly using the BBB and additional sub-scoring tests. No significant differences in BBB scores or sub-scores between D15A and control implant groups were observed (data not shown).

Discussion

The effects of neurotrophins combined with SCs on axonal regeneration have been studied in several models of SCI (Bamber et al., 2001; Menei et al., 1998; Tuszynski et al., 1998; Weidner et al., 1999; Xu et al., 1995b). This combination provides a permissive substratum and stimulatory molecules for axonal growth. The study presented herein examined for the first time the combined effects of SCs and a molecule with BDNF and NT-3 activity (D15A) on axonal regeneration in a clinically relevant moderate contusive SCI model. We found that AdV and LV vector-transduced SCs secreted D15A for at least three days after transduction *in vitro* and also after implantation into the contused spinal cord. D15A-secreting SC implants exhibited significantly greater volume than non-transduced SC and GFP controls, at least in part due to a five-fold greater number of SCs and myelinated axons. There was a significant increase in growth of 5-HT-, β -H-, and CGRP-positive fibers in AdV/GFP/D15A SC implants compared to GFP and non-transduced SC controls. Thus, These findings demonstrate a positive growth response of injured axons to the combination of SCs and the bifunctional neurotrophin.

Whereas both types of vectors carrying D15A led to a significantly improved response, qualitative differences were observed between them. D15A was expressed in 30% of the cells with AdV vectors, in contrast to expression of LV/GFP/D15A in >95% of the cultured cells. And yet, the amount of neurotrophin was higher in implants of AdV- than LV-transduced cells. GFP was not expressed in SCs transduced with AdV vectors for as long a period as it was in LV vector-transduced SCs. More myelinated axons were counted and total axons estimated in LV/GFP/D15A than in AdV/GFP/D15A implants. Finally, increased growth of dopaminergic fibers was detected in LV/GFP/D15A but not AdV/GFP/D15A grafts. These results point to the need to better understand the consequences *in vivo* of introducing each type of viral vector and possible differences in regulation of the genes that they carry. Some potential explanations for the differences that we observed are included in paragraphs below.

Examination of the injury/implant site for neurotrophin expression showed that significantly higher levels were detected in LV/GFP/D15A SC implants at two days and, in AdV/GFP/D15A SC implants, from two days to one week, compared to non-transduced SC and GFP/SC implants. The D15A levels diminished thereafter in both LV vector- and AdV vector-

transduced SC implants. This was anticipated for implants of AdV vector-transduced SCs (Boer et al., 1997; Ruitenberg et al., 2002) because of the possibility of episomal gene loss through cell division. Neurotrophin decreases in implants of LV vector-transduced SCs, however, were unexpected. Earlier studies had demonstrated that LV vector-transduced olfactory ensheathing cells expressed the transgene for up to four months (Abdellatif et al., 2006; Ruitenberg et al., 2002). The transgene in both the AdV and LV vectors is under control of the CMV promoter. The difference between the two is that the LV vector also contains a WPRE sequence that enhances transgene expression. With the AdV vectors, there could be some residual expression of native adenoviral genes, since only the E1 and part of the E3 gene are removed. The E4 gene is known to enhance the expression of the CMV promoter (Yew et al., 1999).

In the implants containing LV/GFP/D15A SCs, neurotrophin levels decreased dramatically over time while GFP expression remained high. Truncated forms of the TrkB receptor may be over expressed, thereby binding the neurotrophin and restricting its availability (Biffo et al., 1995). By 42 days, truncated TrkB is substantially increased around the contusion cavity (Liebl et al., 2001). Also, in larger implants that contain more SCs, more SC p75 NGFR will be present. Because the neurotrophin will bind to this receptor, it will be internalized and thus removed from the environment. There is also the possibility that GFP and D15A protein levels are subject to different modes of regulation at varying times after implantation, e.g., D15A may be degraded rapidly following synthesis at later times; it is known that the stability of GFP is relatively high (Li et al., 1998). Moreover, the method of visualization of GFP expression, cell staining, differs from the evaluation of NT-3 by ELISA. Because the sensitivities of these two methods may differ, the expression of GFP and NT-3 may be assessed as different. Using a marker protein such as GFP to gauge the temporal expression of the relevant protein is problematic (Abdellatif et al., 2006).

Volumes of D15A-containing SC implants were up to five times greater than in AdV/GFP, LV/GFP and non-transduced SC (identified by p75 immunostaining) control implants at six weeks post-implantation. The larger volumes were due, at least in part, to the 5-fold greater number of SCs that we observed in the implants of LV/GFP/D15A-transduced SCs. The larger number of GFP-labeled SCs could be due to increased proliferation from heightened neurotrophin levels (Girard et al., 2005) or to the mitogenic signal (Wood and Bunge, 1975) on the increased numbers of axons within the implant due to elevated neurotrophin levels (McTigue et al., 1998). Alternatively, D15A may have enhanced implanted SC survival (Girard et al., 2005). Neurotrophins have been shown previously to be important for SC survival *in vitro* as antibodies to NT-3 cause significant (>60%) cell death (Meier et al., 1999). A small contribution to the larger volume of D15A-containing implants may have been made by increased migration of host SCs into the implant. Migration of SCs into contusion/graft sites is commonly seen after SCI (Bunge and Wood, 2006) and NT-3 is known to enhance SC migration via TrkC binding and modulation of the *c-Jun* N-terminal kinase cascade (Yamauchi et al., 2003).

Descending 5-HT-positive axons that originate in the raphe nuclei do not respond to grafts of non-transduced SCs placed into the injured thoracic spinal cord (Martin et al., 1996; Xu et al., 1995a, 1997). In the present study, however, growth of 5-HT axons, quantified by estimating total axon length, was observed within AdV/GFP/D15A SC grafts but not within LV/GFP/D15A SC implants. This differential response is possibly explained by higher and more prolonged secretion of neurotrophin by AdV/GFP/D15A SCs compared to LV/GFP/D15A SCs. An improved response of serotonergic neurons has been observed in previous studies that employed exogenous delivery or enhanced cellular secretion of BDNF and/or NT-3 (Coumans et al., 2001; Grider et al., 2005; Jin et al., 2000; Lu et al., 2005; Menei et al., 1998; Murray et al., 2002; Xu et al., 1995b; Ye and Houle, 1997).

An important advantage of SCs over other cell types tested for spinal cord repair is their ability to myelinate axons within the transplant. In this study we counted an average of 5158 ± 786 myelinated axons within the injury/graft epicenter of GFP-only or non-transduced SCs grafts. This number is similar to the mean determined by Takami et al. (2002) (5212 ± 1783) who used the same implantation paradigm. In both types of D15A implants, the number of SC myelinated axons was significantly increased. This may suggest either direct effects of D15A on the myelination process or indirect effects due to not only an increase in axon number but also, in the presence of heightened neurotrophin levels, increased girth of implant axons beyond the threshold required for myelination (Windebank et al., 1985).

BDNF acts as a positive modulator for myelination in culture (Chan et al., 2001). On the other hand, NT-3 prevents myelination by embryonic SCs *in vitro* (Chan et al., 2001), most likely through activation of TrkC receptors (Cosgaya et al., 2002). D15A binds to TrkB and TrkC receptors with similar affinity as BDNF and NT-3 (Urfer et al., 1994). In our *in vivo* study, the SCs possibly expressed lower levels of TrkC than TrkB. In an earlier study with other neurotrophic factors, Oudega et al. (1997) observed a similar increase in myelinated to unmyelinated axon ratios in insulin-like growth factor-1/platelet-derived growth factor-containing SC implants compared to control SC implants (1:3 vs. 1:7, respectively). Glial cell line-derived neurotrophic factor also enhances myelination, leading to a change in the ratio from 1.8 to 1.25 in SC implants (Iannotti et al., 2003).

We found that the total number of fibers, as estimated from the counts of SC-myelinated axons and the myelinated to unmyelinated axon ratios, reached more than 70,000 in the LV/GFP/D15A SC implants. This is the highest number of axons in a contusion site that we have observed in any combination therapy so far. The strategy tested here was designed primarily to promote axonal growth into the implant; achieving more axonal growth into the implant would increase the probability of more fibers exiting the implant to grow into the spinal cord. During the six weeks of testing, the average BBB score was similar in all groups at all times. One possible explanation is that fibers did not exit the implant to enter the caudal cord where they could have influenced hindlimb function. In the Takami et al. (2002) study, significant differences in BBB scores were not observed until at least 8 weeks after SC implantation. Thus, a longer time point after implantation may be necessary to observe improvement in functional recovery of animals with neurotrophin-secreting SC implants.

Another possible explanation is that the accumulation of chondroitin sulfate proteoglycan around the implant precluded axons from growing into the spinal cord (Plant et al., 2001; Takami et al., 2002). In contrast to D15A-transduced SCs, D15A-transduced glial restricted precursor cells (GRPCs) introduced into rat contusion lesions led to significant improvements in transcranial magnetic motor-evoked potential responses and hindlimb locomotor function (Cao et al., 2005). Thus, conduction through demyelinated regions was restored. Although the occurrence of axonal regeneration is certainly possible, the focus was on remyelination of axons that had remained intact but were demyelinated by the contusion injury. The functional improvements were found only in the D15A-treated animals, most likely because the D15A enhanced the differentiation of GRPCs into oligodendrocytes and increased their myelination capacity. Axonal growth from the implant, therefore, was not an issue although it is known that these precursor cells reduce expression of the inhibitory chondroitin sulfate proteoglycans (Hill et al., 2004) in contrast to SCs (Plant et al., 2001; Takami et al., 2002).

In sum, neurotrophins and SCs can provide chemotropic, trophic and structural support to create a more supportive environment for survival of neurons and regrowth of axons after injury. It was the goal of our experiment to combine these two therapies to promote more fiber growth into the implant than occurs in an unmodified SC implant. This goal was

accomplished in that there was up to a 5-fold increase in the number of myelinated axons and increased total axon length of 5-HT-, D β H- and CGRP-positive fibers in the implant. Myelinated and non-myelinated axons together were estimated to number over 70,000. The next step is to add to this paradigm modification of the graft host cord interface and beyond to enable fibers to re-enter the spinal cord and potentially improve functional performance of the hindlimbs. This study is one of a series to investigate which combination strategy will best repair the spinal cord to adequately improve functional outcome.

Acknowledgments

We gratefully acknowledge Drs. S. Whittemore (University of Louisville School of Medicine) for providing the adenoviral vectors, and Dr. L. Naldini (University of Torino) for the lentiviral vector plasmid; the Miami Project Viral Vector Core for lentiviral vector-transduced SCs; the Miami Project core animal facility for contusions and post-operative animal care; Yelena Pressman for SCs; and Margaret Bates for myelinated axon counts and electron microscopy analysis; and Dr. P. Tsoulfas (Miami Project) for comments on the manuscript. We also thank Dr. Helen Bramlett (Miami Project) for help with statistical analysis. This research was funded by NIH grants P01 NS38665 and R01 NS09923, the Buoniconti Fund and the Miami Project to Cure Paralysis.

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Figure 1. Medium conditioned by SCs transduced with AdV/GFP/D15A or LV/GFP/D15A contains biologically active neurotrophin that supports neurite outgrowth in DRG cultures Embryonic DRG, incubated for three days in conditioned medium from (B) AdV/GFP/D15A or (D) LV/GFP/D15A SCs, exhibited robust neurite outgrowth when compared to (A) AdV/GFP and (C) LV/GFP SCs. These cultures may be compared to examples of similar cultures provided medium containing neutralizing NT-3 antibodies (E) and medium containing appropriate concentrations of added BDNF and NT-3 (F). Neurofilament staining. Scale bar = 1 mm.

Figure 2. Spinal cord tissue containing AdV/GFP/D15A or LV/GFP/D15A SC implants contains significantly higher levels of neurotrophin than non-transduced SC or GFP only controls Spinal cord tissue was removed, homogenized and subjected to an NT-3 ELISA. Total protein was determined using a Bradford protein assay. At 2 days, a statistically significant difference was achieved in D15A-transduced SC implants compared to control non-transduced SC grafts; AdV/GFP/D15A implants generated significantly higher levels of neurotrophin than did LV/GFP/D15A SC grafts. By 1 wk, only the levels in AdV/GFP/D15A reached significance. The levels of neurotrophin secreted by control AdV/GFP and LV/GFP implanted tissue were similar to spinal cord implanted with non-transduced SCs. Error bars, SEM. *** $P < 0.001$; ** $P < 0.01$.

Figure 3. D15A-secreting SC implant volumes are significantly increased at six weeks post-implantation

LV/GFP SCs are depicted in (A). In comparison, larger volumes are observed in LV/GFP/D15A SC grafts (B). (C) Volume quantification of implants, identified by GFP expression of LV vector-transduced SCs or p75 immunoreactivity of AdV vector and non-transduced SCs. A statistically significant difference was accepted at a P value of *** $P < 0.001$ as compared to controls (one-way ANOVA). r, rostral. Scale bar = 1 mm. Error bars, SEM.

Figure 4. Myelinated axon counts in injury/graft centers are increased within D15A-secreting SC implants, at six weeks post-implantation

SC myelinated axons, evident as dark rings, were present in all implanted groups. Many more SC myelinated axons were present in transverse sections of the (B) AdV/GFP/D15A and (D) LV/GFP/D15A SC groups, when compared to (A) AdV/GFP, (C) LV/GFP and (E) non-transduced SC groups. (F) Quantification reveals significantly more axons myelinated by SCs in the epicenters of D15A-transduced SC grafts. A statistically significant difference was accepted at a P value of $***P < 0.001$ compared to controls and $**P < 0.01$ comparing AdV/GFP/D15A with LV/GFP/D15A. Scale bar = 10 μ m. Error bars, SEM.

Figure 5. D15A-secreting SC implants contain significantly increased lengths of 5-HT, DβH and CGRP-positive fibers at 6 weeks post-implantation

Confocal images at 400X magnification show AdV/GFP/D15A SC implants containing (A) 5-HT fibers and (C) CGRP fibers, compared to AdV/GFP SC implants (B,D). Significantly increased lengths of (E) 5-HT, (F) DβH and (G) CGRP immunostained fibers were observed within AdV/GFP/D15A SC grafts, when compared to AdV/GFP, LV/GFP/D15A, LV/GFP, and non-transduced SCs. (F) LV/GFP/D15A SC grafts also contained increased DβH fiber length. A statistically significant difference was accepted at a P value of $**P < 0.01$ as compared to controls (one-way ANOVA). Scale bar = 50 μ m. Error bars, SEM.

Figure 6. D15A-secreting SC implants did not improve regrowth of anterogradely-traced corticospinal or reticulospinal fibers into the implant

The rostral-most end of the lesion/implant site at the level of the central canal was designated as the zero point. Lines perpendicular to the longitudinal axis were drawn 500 μm apart, and labeled axons that crossed these lines were counted. **(A)** The presence of LV/GFP/D15A SC implants led to significantly increased numbers of BDA-traced CST fibers rostral to the zero point compared to non-transduced SC implants. AdV/GFP/D15A SC implants were elevated compared to controls, but not significantly. **(B)** No differences were observed in dextran-rhodamine-traced RST fibers among animal groups. A statistically significant difference was accepted at a P value of $**P < 0.01$ as compared to controls (two-way ANOVA). Error bars, SEM.

Table 1

Number of animals used for each analysis

Group	Implanted tissue ELISA (all time points) [*]	Anterograde tracing		Immunohistochemistry, graft volume, SC # ^{**}	Myelinated axons (total axons, wm sparing [•])
		CST	RST		
Non-transduced SCs	12	9	8	5	5 (3)
Adenoviral groups					
AdV/GFP	12	9	8	5	5 (3)
AdV/GFP/D15A	24	9	8	5	5 (3)
Lentiviral groups					
LV/GFP	12	9	8	5	5 (3)
LV/GFP/D15A	24	9	8	5	5 (3)

^{*} Non-transplanted animals, n=3^{**} SC number was assessed only in LV vector-transduced implants.[•] White matter sparing was evaluated by counting preserved central myelinated axons. BBB testing was performed on animals in the immunohistochemistry and myelinated axon groups, total n=50.

Table 2

Counts of central myelinated axons in the rim of the spinal cord around the implant

	SCI only	LV/GFP SCs	LV/GFP/D15A SCs
RAT 1	16225		
2	12585		
3	20214		
4		9088	
5		5184	
6		18496	
7			12928
8			14336
9			8848
AVG	16341	10923	12037
SEM	2935	3422	2193

Table 3Estimation of total axon numbers in graft centers ^a

Implant group	Myel. Axons ^b	Ratio	Estimated total number of axons
Non-transduced SCs	5158 ± 786	1:8.3	45908
AdV/GFP/D15A SCs	18232 ± 1577 ***	1:1.5	49618
AdV/GFP SCs	4718 ± 1158	1:7.5	43673
LV/GFP/D15A SCs	26624 ± 1879 ***	1:1.8	72108 *
LV/GFP SCs	4640 ± 971	1:4.9	32641

^a 1 µm toluidine blue-stained transverse sections, 6 weeks post-implantation^b Mean ± SEM

* P<0.05 compared to control groups (one-way ANOVA)

** P<0.01, AdV/GFP/D15A compared to LV/GFP/D15A5 (see Fig.4)

*** P<0.001 compared to control groups (one-way ANOVA)