

ADENO-ASSOCIATED VIRAL VECTOR-MEDIATED NEUROTROPHIN GENE TRANSFER IN THE INJURED ADULT RAT SPINAL CORD IMPROVES HIND-LIMB FUNCTION

B. BLITS,^d M. OUDEGA,^{a,b,*} G. J. BOER,^d
M. BARTLETT BUNGE^{a,b,c} AND J. VERHAAGEN^d

^aThe Miami Project to Cure Paralysis, University of Miami School of Medicine, Miami, FL 33101, USA

^bDepartment of Neurological Surgery, University of Miami School of Medicine, Miami, FL 33136, USA

^cDepartment of Cell Biology and Anatomy, University of Miami School of Medicine, Miami, FL 33136, USA

^dGraduate School for Neurosciences Amsterdam, Netherlands Institute for Brain Research, Amsterdam, The Netherlands

Abstract—To foster axonal growth from a Schwann cell bridge into the caudal spinal cord, spinal cells caudal to the implant were transduced with adeno-associated viral (AAV) vectors encoding for brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (AAV-NT-3). Control rats received AAV vectors encoding for green fluorescent protein or saline. AAV-BDNF- and AAV-NT-3-transduced 293 human kidney cells produced and secreted BDNF or NT-3, respectively, *in vitro*. The secreted neurotrophins were biologically active; they both promoted outgrowth of sensory neurites *in vitro*. *In vivo*, transgene expression was observed predominantly in neurons for at least 16 weeks after injection. Compared with controls, a modest though significant improvement in hind-limb function was found in rats that received AAV-BDNF and AAV-NT-3. Retrograde tracing demonstrated that twice as many neurons with processes extending toward the Schwann cell graft were present in the second lumbar cord segment of AAV-BDNF- and AAV-NT-3-injected animals compared with controls. We found no evidence, however, for growth of regenerated axons from the Schwann cell implant into the caudal cord.

Our results suggest that AAV vector-mediated overexpression of BDNF and NT-3 in the cord caudal to a Schwann cell bridge modified the local lumbar axonal circuitry, which was beneficial for locomotor function. © 2003 IBRO. Published by Elsevier Science Ltd. All rights reserved.

Key words: AAV vectors, BDNF, NT-3, injury, transplantation, viral vectors.

*Correspondence to: M. Oudega, The Miami Project to Cure Paralysis, University of Miami School of Medicine, P.O. Box 016960, R-48, Miami, FL 33101, USA. Tel: +1-305-243-7161; fax: +1-305-243-3921. E-mail address: moudega@miami.edu (M. Oudega).

Abbreviations: AAV, adeno-associated virus; ANOVA, analysis of variance; BBB, Basso Beattie Bresnahan; BDNF, brain-derived neurotrophic factor; CMV, cytomegalovirus; DMEM, Dulbecco's minimal essential medium; DRG, dorsal root ganglion; ELISA, enzyme-linked immunosorbent assay; FB, Fast Blue; FR, Fluororuby; GFP, green fluorescent protein; m.o.i., multiplicity of infection; NT-3, neurotrophin-3; PAN/PVC, polyacrylonitrile:polyvinylchloride; PB, phosphate buffer; PBS, phosphate-buffered saline; SC, Schwann cell; TU, transducing units.

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doi:10.1016/S0306-4522(02)00970-3

Following an injury to the adult mammalian spinal cord, behavioral recovery is poor. Damaged axons can be enticed to regenerate into and across different types of grafts in the injured cord (Bunge, 2001; Chen et al., 1996; Guest et al., 1997; Oudega et al., 1997; Xu et al., 1995a,b) but fail to exit into the spinal tissue beyond (Bunge, 2001). This failure is at the basis of the lack of behavioral recovery and appears to be due to the axonal growth-inhibitory nature of the graft-cord interface and the spinal tissue beyond (Horner and Gage, 2000; Schwab, 2000).

Manipulating the molecular milieu at or near the injury reduces the hostile nature of adult spinal tissue to axonal growth. For instance, antibody neutralization of growth-inhibitory proteins promoted growth of corticospinal axons around a dorsal hemisection lesion (Schnell and Schwab, 1990; Schnell and Schwab, 1993; von Meyenburg et al., 1998). A similar growth response can be obtained by implanting a bridge with increased levels of growth-promoting factors into the lesion site (Blesch et al., 1999; Blits et al., 2000; Grill et al., 1997; Houweling et al., 1998a). However, these manipulations promote growth around a lesion site but not from a graft into the spinal cord tissue.

Growth of sensory axons across a graft-cord interface was obtained by a continuous infusion of neurotrophins, into the rat dorsal spinal cord, a short distance away from a peripheral nerve graft bridging the transected dorsal columns (Oudega and Hagg, 1996, 1999). Similarly, in a lateral hemisection model, it was shown that a continuous infusion of the neurotrophins, brain-derived neurotrophic factor (BDNF) and/or neurotrophin-3 (NT-3) into the spinal tissue just caudal to a Schwann cell (SC) bridge promoted growth of descending axons from the bridge into the caudal cord (Bamber et al., 2001). The underlying mechanisms are unknown, but it is clear that increased levels of neurotrophic factors promote axonal growth across the otherwise obstructive bridge-cord interface and into the adult spinal tissue.

In the abovementioned studies, neurotrophins were infused into the cord using an osmotic mini-pump attached to a metal infusion device, which was inserted into the spinal tissue. The presence of such a permanent infusion device in the cord could result in additional damage to the spinal tissue. Also, effective use of mini-pumps to promote such growth appears to require infusion of super-physiological concentrations of neurotrophins, which could result in unwanted side effects (Dijkhuizen and Verhaagen, 1999; Eriksdotter et al., 1998; Nordberg, 1996). Moreover, in time, the biological activity of the factors within the pump reservoir may diminish. Clearly, although the principle of

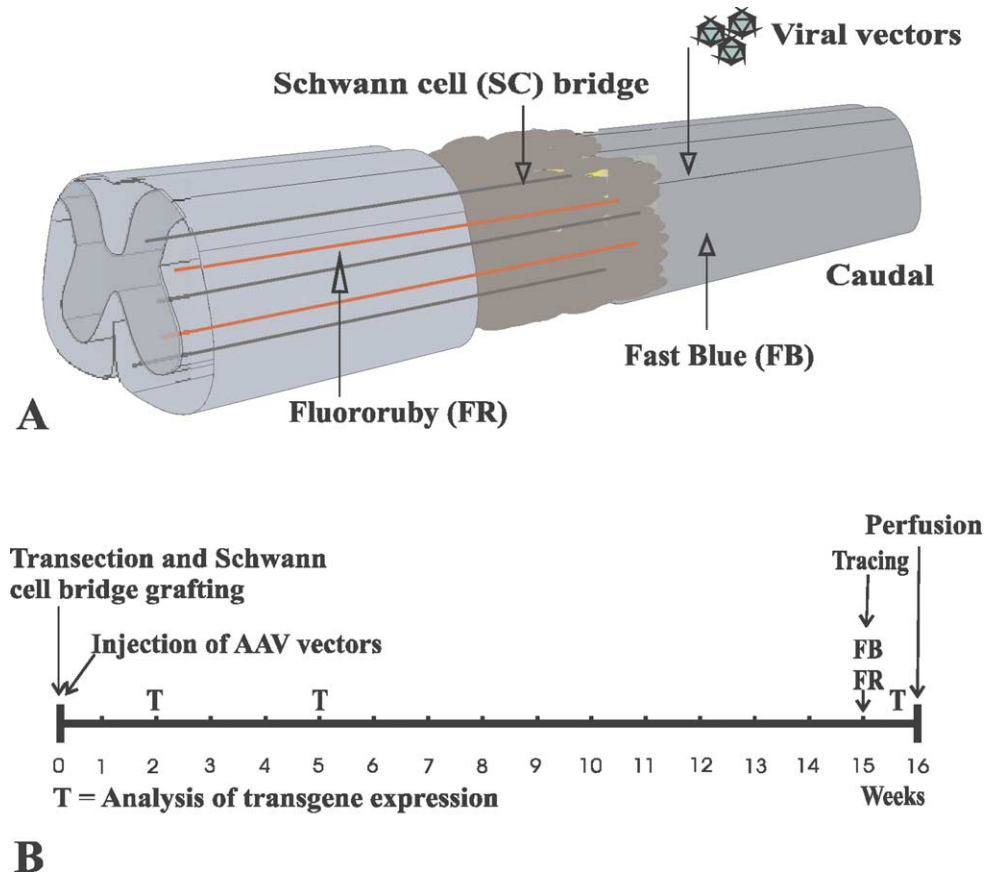


Fig. 1. Schematic representation of the experimental design. (A) Illustration of the completely transected spinal cord and SC bridge that contains ingrowing axons. AAV vectors encoding for BDNF and NT-3 were applied caudally to the SC implant. Neurons that extended axons caudal to the implant (i.e. that had regenerated across the bridge–host cord interface, or had originated in the caudal stump) were retrogradely traced using fast blue (FB). Fluororuby (FR) was injected rostral to the bridge to visualize axons that had traversed the bridge. (B) Time frame of the experiment in weeks. The caudal part of the injured spinal cord was transduced to overexpress neurotrophins with AAV vectors, applied immediately following transection/implantation at 5 mm caudal to the bridge. Analysis of transgene expression (T) was performed at 2, 5 and 16 weeks post-injury. Function of the hind limbs was evaluated weekly using the BBB test until tracing procedures started. Retrograde tracing with FB and anterograde tracing with FR was performed 1 week before termination of the experiment. Finally, the animals were perfusion fixed for histological analysis.

neurotrophin-mediated axonal growth across a graft–cord interface has been proven, other less invasive approaches need to be developed.

Here, we have evaluated the use of gene therapy as an alternative strategy to increase levels of neurotrophic factors in spinal tissue (Hermens and Verhaagen, 1998b). Adeno-associated viral (AAV) vectors encoding for BDNF and NT-3 were generated and evaluated *in vitro*. Using a minimally invasive microinjection, spinal cord cells distal to the SC bridge were transduced with these AAV vectors, and the effects of AAV-mediated neurotrophin expression on behavioral outcome and axonal regeneration were investigated. A schematic representation of the experimental design is presented in Fig. 1.

EXPERIMENTAL PROCEDURES

Viral vector preparation

In order to generate AAV vectors, genes encoding either green fluorescent protein (GFP; AAV–GFP) (Zhang et al., 1996), BDNF (AAV–BDNF) or NT-3 (AAV–NT-3) were subcloned in an AAV

vector plasmid. This plasmid contained the cytomegalo virus (CMV) promoter, the transgene, an SV40 polyadenylation signal, and the Woodchuck post-transcriptional regulatory element flanked by two AAV-2 inverted terminal repeats (Hermens et al., 1999; Loeb et al., 1999; Paterna et al., 2000). In order to isolate the BDNF cDNA, total RNA from adult Wistar rat brain was isolated and first-strand cDNA was transcribed using reverse transcriptase (Gibco BRL, Grand Island, NY, USA) and oligo(dT) primers. BDNF cDNA was amplified from the cDNA synthesis mixture by means of the polymerase chain reaction using an upstream sense primer 5'-CCC GGA ATT CGC CAC CAT GAC CAT CCT TTT CCT TAC T-3' and downstream antisense primer 5'-TTT AGA ATT CCT ATC TTC CCC TTT TAA TTG GT-3' (Amersham Pharmacia Biotech, Little Chalfont, UK). The primers were designed to introduce an *EcoRI* site and a Kozak sequence (GCCACC; Kozak, 1987) in front of the ATG triplet. The amplified DNA fragment was subcloned downstream of the CMV promoter of the expression plasmid pcDNA1/Amp (Invitrogen, Carlsbad, CA, USA). The nucleic acid sequence was verified by DNA sequencing using a USB sequencing kit. The NT-3 gene was isolated from pc5-NT-3 (Dijkhuizen et al., 1998, 1997). The production of rAAV vector was described previously (Hermens et al., 1999). The amount of transducing units (TU) of the vector stocks was determined by infecting 293 cells. The number of cells that were trans-

duced with AAV–GFP was determined under UV radiation using a Zeiss microscope. To detect neurotrophin expression, transduced cells were subjected to standard *in situ* hybridization procedures using digoxigenin(dig)-labeled probes. Within five randomly chosen microscopic fields, positive cells were counted, multiplied by the dilution factor and 3769, the number of microscopic fields needed to cover the 6-cm dish when using a 20× objective. Titers were all in the range of 10^9 TU/ml.

Quantification and bioactivity of recombinant protein *in vitro*

To determine the amount of neurotrophins produced by AAV vector-transduced cells, 293 human kidney cells were seeded into a 6-well plate (4.5×10^5 cells/well). The next day, the cells were infected with recombinant AAV vectors (AAV–GFP, AAV–BDNF or AAV–NT-3) at a multiplicity of infection (m.o.i.) of 1. To allow second-strand synthesis in the AAV vector-treated cultures, cells were grown for 48 h before the medium was refreshed with 1.5 ml of Dulbecco's minimal essential medium (DMEM)/10% fetal calf serum and cells were allowed to grow for another 24 h. The acquired conditioned medium was divided into three portions and quickly frozen in a dry ice/ethanol bath and kept at -20°C . One portion was used to determine the amount of neurotrophin protein by using an enzyme-linked immunosorbent assay (ELISA) (Promega, Madison, WI, USA) according to the manufacturer's protocol. The other two portions were used in a bioassay to test the biological activity of the secreted transgene product.

The biological activity of the AAV vector-derived recombinant protein was determined using a fetal rat dorsal root ganglion (DRG) neurite outgrowth assay (Blits et al., 2000; Dijkhuizen et al., 1998). Briefly, E17 rat embryos were dissected and the DRG aseptically removed from the spinal cord. DRG were pooled in ice-cold L-15 Leibovitz medium with L-glutamine (Gibco BRL) and transferred in 50- μl DMEM/10% fetal calf serum onto glass coverslips coated with poly-L-ornithine (500 mg/ml in 0.1-M boric acid pH 8.3) and laminin (10 mg/ml; Roche, Basel, Switzerland). The glass coverslips were then placed in a 6-well plate ($n=$ three per well) and kept overnight in an incubator at 37°C , 5% CO_2 . Next, the DRG were immersed for 24 h in 100- μl DMEM/10% fetal calf serum containing exogenous 25 ng/ml BDNF, NT-3 or in conditioned medium. This medium was replaced and the cultures were grown for 48 h at 37°C , 5% CO_2 followed by fixation in 4% paraformaldehyde for 2 h. Neurite outgrowth in these cultures was evaluated after staining with the anti-neurofilament antibody, RT97 (1:200; Roche; Dijkhuizen et al., 1997).

Purification of rat SCs

Purified SC cultures were obtained from sciatic nerves of adult female Fischer rats (Charles River Laboratories, Wilmington, MA, USA) as described previously (Morrissey et al., 1991; Xu et al., 1995). To determine the purity of the SCs, a sample of the harvested cells was plated in a culture dish, cultured for 3 h, stained for S100, and then coverslipped with Citifluor (UKC Chemical Laboratory, Canterbury, England) containing 100- μM Hoechst nuclear dye (Sigma, St. Louis, MO, USA) to label all cells. The percentage of S100-positive SCs in the Hoechst-labeled cultures was 95–98%.

Preparation of an SC bridge

SCs were harvested and rinsed twice in DMEM/F12 and then mixed gently in 1% fibrinogen (Sigma) with 2% CaCl_2 , 2% gentamicin (Gemini Bioproducts, Inc., Calabasas, CA, USA), and 2% aprotinin (7.7 IU/ml; Sigma) to a final concentration of 1.4×10^8 cells/ml. Using an Eppendorf pipette, this mixture was gently inserted into a polyacrylonitrile:polyvinylchloride (60%:40%) copolymer (PAN/PVC) tube (inner diameter approximately 2.7 mm;

gift from Tyrone Hazlett, CytoTherapeutics, Providence, RI) containing 2 μl of 25 U/ml thrombin (Sigma). The mixture was allowed to clot and then kept overnight in L-15 (Gibco BRL) at 37°C , 7% CO_2 . The next day, the SC and fibrin clot was carefully removed from the PAN/PVC tube and kept in L-15 (Gibco BRL) with 0.1% gentamicin in a tabletop laminar flow hood until implantation within a few hours.

Surgery

Rats were housed according to NIH and USDA guidelines. The Institutional Animal Care and Use Committee of the University of Miami approved all animal procedures. The number of animals used in these experiments as well as their suffering was minimized. Forty-five female adult Fischer rats (160–180-g body weight; Charles River Laboratories) were anesthetized with an i.m. injection of rat anesthesia cocktail (2.57 mg ketamine, 0.51 mg xylazine and 0.09 mg acepromazine per 100-g body weight). The back was shaved and aseptically prepared with Betadine. Lacrilube ophthalmic ointment (Allergen Pharmaceuticals, Irvine, CA, USA) was applied to the eyes to prevent drying, and Bicillin was administered intramuscularly (0.02 ml/100-mg body weight, 300 U/ml; J. Buck, Inc., Owings Mills, MO, UAS). During surgery, animals were kept on a heating pad to maintain body temperature at $37 \pm 0.5^\circ\text{C}$.

Following laminectomy at the T8–T9 vertebral level, the spinal cord was completely transected and a 3-mm cord segment was removed at the T9–T10 spinal cord level. All spinal roots visible in the transection gap were removed. After hemostasis was achieved, a 4-mm-long SC bridge was implanted between the rostral and caudal spinal cord stumps. Subsequently, rats were placed in a spinal cord fixator to prevent damage from injection due to respiratory movements. Next, 2 μl of a AAV–BDNF and AAV–NT-3 mixture or AAV–GFP (2×10^6 TU total) or saline ($n=15$ for all three groups) were stereotactically injected into the spinal gray matter 5 mm caudal to the caudal bridge–host interface using a glass needle (diameter approximately 80 μm) attached to a 10- μl Hamilton syringe. A piece of silicone sheathing (0.13 mm thick; Specialty Manufacturing, Inc., Saginaw, MI, USA) was placed over the implanted area, and the muscles and skin were sutured separately. Immediately after surgery, rats received an s.c. injection of 10 ml lactated Ringers solution. Animals received dexamethasone-1-phosphate for 3 days (5 mg/100-g body weight i.m.; Sigma) to reduce swelling of the spinal cord and possible inflammatory responses (Hermens and Verhaagen, 1998a). The rats were allowed to recover in warmed cages with food and water readily available. To prevent urinary tract infection, Bicillin was administered intramuscularly at 3, 6 and 9 days postsurgery. Bladders were emptied manually twice a day until bladder function returned. In case urinary tract infection occurred later, Bicillin was administered every other day for a week.

Assessment of locomotor performance

Hind-limb function of the rats was assessed weekly, starting 2 weeks before surgery, using the Basso, Beattie and Bresnahan (BBB) open-field locomotor test (Basso et al., 1995). Two independent investigators oblivious of the different experimental treatments determined BBB scores. Behavioral analysis was performed until the animals (AAV–BDNF/NT-3 group, $n=7$; GFP group, $n=7$; saline group, $n=11$) were subjected to axonal tracing. Statistical analysis was performed using a two-way analysis of variance (ANOVA) with repeated measures, followed by the posthoc Newman-Keuls test. Values were considered statistically different when $P \leq 0.05$.

Anterograde and retrograde tracing procedures

For anterograde and retrograde tracing, animals (AAV–BDNF/NT-3 group, $n=7$; GFP group, $n=7$; saline group, $n=11$) were

anesthetized using rat cocktail. During tracer injections, animals were kept on a heating pad to maintain body temperature at 37 ± 0.5 °C. One week before termination of the experiment, axons that were traversing the SC cable from rostral to caudal were labeled using 2×0.2 - μ l Fluororuby (FR, 10,000 MW; Molecular Probes, Eugene, OR, USA) injected 5 mm rostral to the bridge, 0.6 mm from the dorsal spinal artery at a depth of 1 mm bilaterally. In the same animals, the retrograde Fast Blue (FB) tracing technique was used to determine the origin of fibers that traversed the SC graft and entered the caudal spinal cord, and also to label neurons in the L2 region that extended their processes toward the implantation site. A volume of 0.4 μ l of 5% FB (Sigma) was administered (0.1 μ l at 0.6 mm from the dorsal sulcus, 1 and 2 mm deep, bilaterally) approximately 6 mm caudal to the caudal bridge–spinal cord interface. In all cases, the tracer was applied using a 1-ml Hamilton syringe with a glass needle attached. The injection area was covered with gelfoam, the muscles and skin were sutured separately, and the animals were returned to their cages.

Histological procedures

Rats were anesthetized with rat cocktail and transcardially perfused with chilled 0.1-M phosphate-buffered saline pH 7.4 (PBS) containing heparin, followed by ice-cold 4% paraformaldehyde. The spinal cord was removed, postfixed in the same fixative for 3 h, and then cryoprotected in 30% sucrose in 0.1-M phosphate buffer (PB) pH 7.4. In some animals, the AAV vector-mediated transgene expression *in vivo* was evaluated at 2, 5, and 16 weeks postinjection (n =two per time point). The reporter gene GFP was detected using GFP immunocytochemistry, as described previously (Peel et al., 1997), and *in situ* hybridization was used to detect overexpression of BDNF and NT-3. From all other animals, the rostral and caudal cord stumps (including 1–2 mm of the bridge) were embedded in 10% gelatin (Difco, Becton Dickinson, Sparks, MD, USA) in PBS and sagittally sectioned (40 μ m) on a freezing microtome. Transverse sections (40 μ m) were cut from the second lumbar cord segment (L2). All microtome sections were collected in PB with 0.1% sodium azide and stored at 4 °C until further processing. To analyze the presence of FR-labeled axons and FB-labeled neurons, every sixth section of the rostral and caudal cord segments was mounted on gelatin-coated glass slides, coverslipped with Citifluor (UKC Chemical Laboratory) and examined under fluorescence microscopy.

Quantitative analysis of regenerating fibers

All FB-labeled neurons in the rostral cord were counted. In addition, in five randomly chosen transverse sections of the L2 segment, the total number of FB-labeled neurons and motor neurons, which were recognized by their location in the ventral horn and their typical morphology, was determined. The number of animals used for these quantifications was $n=7$ in the AAV–BDNF/NT-3 and AAV–GFP groups and $n=11$ in the saline group. Statistical analysis was performed using a two-way ANOVA followed by the posthoc Newman-Keuls test. Values were considered statistically different when $P \leq 0.05$.

RESULTS

AAV vector-infected 293 cells secrete the transgene product *in vitro*

The amount of BDNF and NT-3 that was present in the medium following transduction of 293 cells, with a m.o.i. of 1, was determined using an ELISA assay. Following infection with AAV–GFP, –BDNF or –NT-3 could not be detected in

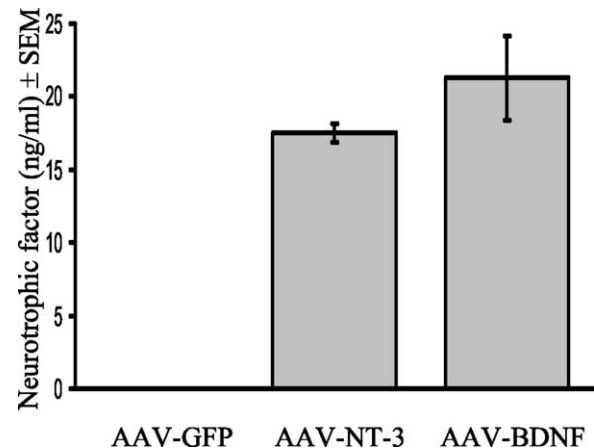


Fig. 2. AAV vector-transduced 293 cells produce and secrete the transgene product. An enzyme-linked immunosorbent assay was performed on conditioned medium derived from 293 cells that were infected with AAV vectors at a multiplicity of infection of 1. Vectors that were used encoded either the reporter gene (GFP) or neurotrophins, BDNF and NT-3. The amount of neurotrophins present in the medium is expressed as nanograms/milliliter. Infection with a vector encoding a reporter gene did not result in neurotrophin expression. Following infection with a vector encoding a neurotrophin, approximately 25 ng of neurotrophin per ml was measured in the medium after 24 h in all cases.

the medium (Fig. 2). Infection with AAV–NT-3 resulted in 17.5 ± 0.6 ng/ml NT-3 (mean \pm S.E.M.). AAV–BDNF-infected cells secreted 21.3 ± 2.9 ng/ml BDNF (Fig. 2).

BDNF and NT-3 secreted from AAV vector-infected cells are biologically active *in vitro*

BDNF and NT-3 secreted by 293 cells infected with the newly generated AAV vectors induced a robust outgrowth of DRG neurites in a previously described assay (Blits et al., 2000; Dijkhuizen et al., 1998). Non-neuronal cells migrated from DRG explants after receiving the usual medium or medium from AAV–GFP-infected 293 cells; neurite outgrowth was poor (Fig. 3A, B). Non-neuronal cells also migrated from the DRG explants after receiving medium from AAV–BDNF- or AAV–NT-3-infected 293 cells. In contrast to the controls, neurite outgrowth in these cultures was robust (Fig. 3C, D). This outgrowth was comparable to that observed when recombinant BDNF or NT-3 was added to the medium of DRG cultures (not shown). AAV vector-mediated NT-3 induced outgrowth of predominantly thick-appearing neurites, whereas AAV vector-mediated BDNF was observed to induce outgrowth of mainly thin-appearing neurites.

AAV vector expression patterns *in vivo*

AAV vectors transduced spinal cord cells when injected immediately after injury and implantation of the SC bridge (Fig. 4, A–D). Transgene expression (NT-3 not shown) was found in numerous cells, predominantly neurons, in approximately a 6-mm length of the spinal cord. The expression of the transgene persisted for 16 weeks, the duration of the experiment (Fig. 4D). Moreover, many

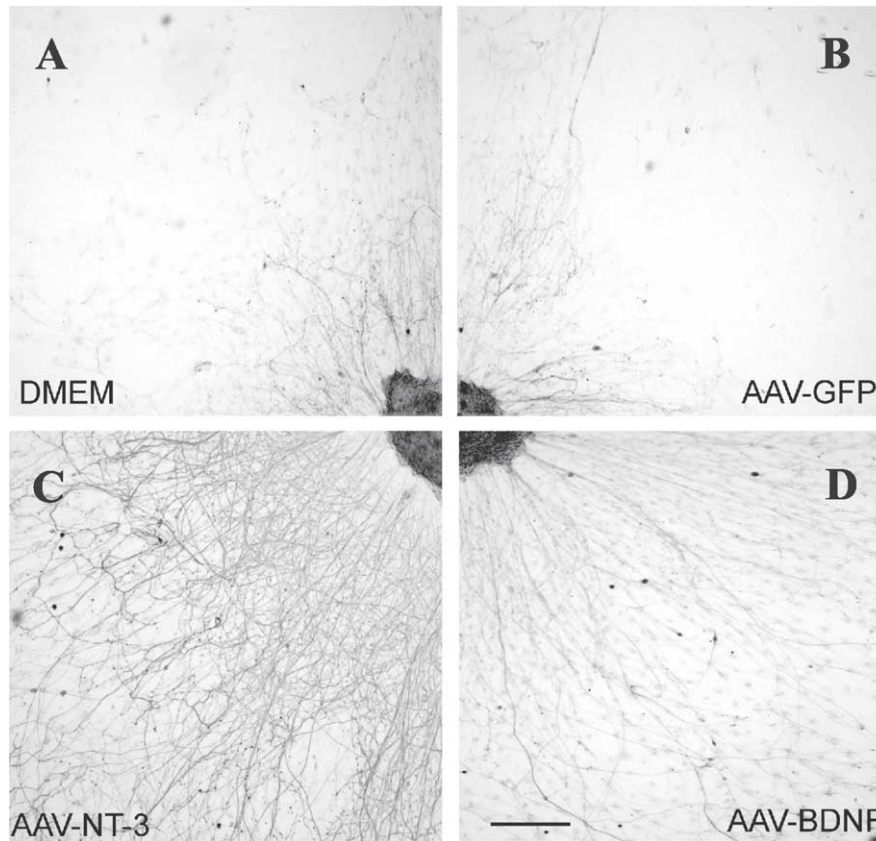


Fig. 3. BDNF and NT-3 from AAV vector-infected 293 cells promote neurite outgrowth from dorsal root ganglia *in vitro*. Dorsal root ganglion explants were cultured with conditioned media from AAV vector-infected 293 cells and then stained for neurofilaments using the RT97 antibody. Conditioned medium was removed from (A) mock-infected 293 cells, (B) AAV-GFP-infected cells, (C) AAV-NT-3-infected cells or (D) AAV-BDNF-infected cells. In the control situations (A and B) non-neuronal cells migrated out of the explant and some outgrowth of neurites was observed. With AAV-NT-3- and AAV-BDNF-conditioned medium, however, a robust neurite outgrowth was induced. Scale bar=1 mm.

transduced neurons were found to extend neurites into the SC bridge (Fig. 4E).

Administration of AAV-BDNF and AAV-NT-3 results in improved hind-limb performance

Hind-limb performance was evaluated using the BBB locomotor test (Basso et al., 1995). One week after SC implantation and injection of vectors (2×10^6 TU total), the hind limbs of the treated animals were completely paralyzed. Hind-limb performance gradually improved in all experimental groups. At 3 weeks post-implantation, the mean scores of animals treated with AAV-BDNF and AAV-NT-3 were higher than those for the other groups, and they remained higher thereafter (Fig. 5). The ANOVA test revealed that the AAV-BDNF/AAV-NT-3 group was significantly different from both control groups. A statistically significant difference ($P < 0.05$) between the animals treated with AAV-BDNF and AAV-NT-3 and the control groups was present at 7 weeks after implantation and injection of vectors. At 12 weeks, animals treated with AAV-BDNF and AAV-NT-3 exhibited an average score of 6.9 ± 0.1 (mean \pm S.E.M., $n=7$), which was higher than the final score of the group that received AAV-GFP (5.8 ± 0.6 ; $n=7$) or saline (5.6 ± 0.3 ; $n=11$). A BBB score of 6.9 indi-

cates that the animals were able to move all three joints of the hind limbs extensively. In none of the animals was weight-supported stepping observed (Fig. 5).

Injection of AAV-BDNF and AAV-NT-3 caudal to an SC bridge does not result in axonal growth into the cord

Following injection of FR rostral to the SC bridge, many anterogradely labeled axons were found to have grown into and across the implant but not through the caudal bridge–host cord interface and into spinal tissue beyond (Fig. 6). Apparently, treatment with AAV vectors encoding for BDNF and NT-3 (2×10^6 TU total) did not promote re-entry of regenerating axons into the caudal spinal cord. Also, application of FB approximately 6 mm from the graft–cord interface in the caudal spinal cord did not result in detection of labeled neurons rostral to the bridge, indicating again that axons did not exit the bridge into the caudal spinal cord.

Treatment with AAV-BDNF and AAV-NT-3 results in more traced neurons in the L2 cord

We determined the total number of FB-labeled neurons and the number of FB-labeled motor neurons in five ran-

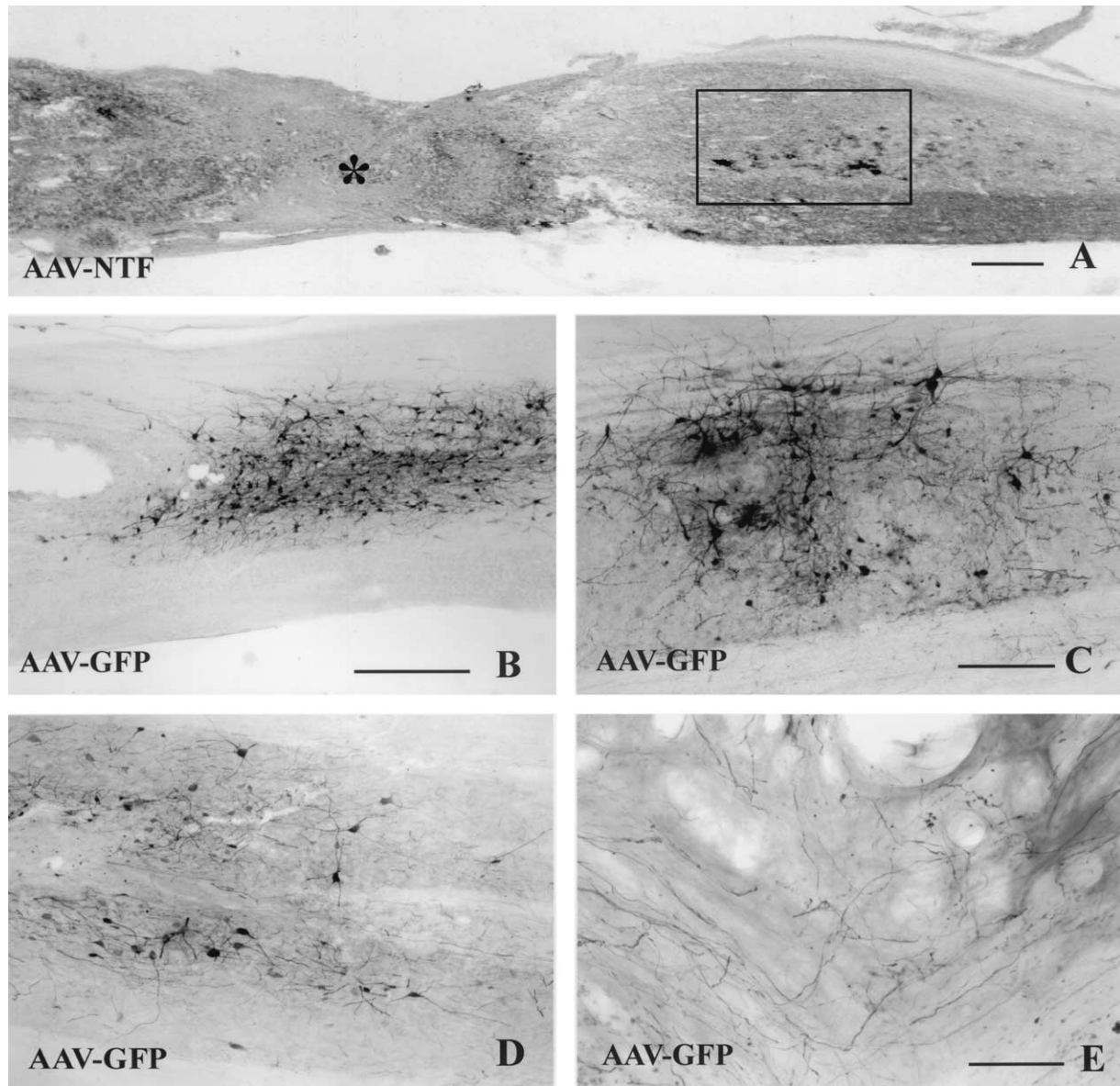


Fig. 4. AAV vectors transduce spinal cord cells *in vivo*. Analysis of transgene expression using AAV vectors (2×10^6 TU total). (A) Overview of a transected, SC-implanted and transduced spinal cord. This AAV-BDNF NT-3-injected spinal cord was subjected to *in situ* hybridization at 14 days post-injury with a probe directed against BDNF. BDNF-overexpressing cells are visible caudal to the SC implant in the rectangle. The rectangle also indicates a comparable area depicted in panel B. The asterisk indicates the SC bridge. Rostral cord is at the left. (B) Spinal cord subjected to GFP immunocytochemistry at 14 days post-injection of AAV-GFP caudal to the bridge. (C) GFP immunocytochemistry at 35 days post-injection of AAV-GFP. Many cells expressed the transgene. (D) Same as C but at 16 weeks post-injection, illustrating transgene expression persisted throughout the experiment. (E) Micrograph of the bridge, subjected to GFP immunocytochemistry at 14 days post-injection of AAV-GFP. Some GFP-positive fibers have grown from the caudal injection site into the SC bridge. Scale bars=1 mm (A and B), 250 μ m (C and D) and 100 μ m (E).

domly chosen transverse sections of L2 segments (Fig. 7). The number of FB-labeled neurons was 466 ± 106 (mean \pm S.E.M., $n=5$) in saline-injected animals and 476 ± 54 ($n=3$) in AAV-GFP-injected animals. In contrast, in animals that received AAV-BDNF and AAV-NT-3 (10^6 TU total), the total number of FB-labeled neurons was 919 ± 191 ($n=4$), significantly higher than the number in the control groups ($P=0.022$; $F=2,34$). The number of labeled motor neurons was 33 ± 10 in rats with a saline injection, 35 ± 4 in rats with an AAV-GFP inject-

ion and 58 ± 16 in rats with an AAV-BDNF and AAV-NT-3 injection. These values were not significantly different.

DISCUSSION

Successful transduction of cells in the injured spinal cord was achieved *in vivo* with AAV-BDNF and AAV-NT-3 that were administered immediately after transection and implantation of an SC bridge into the adult rat thoracic spinal

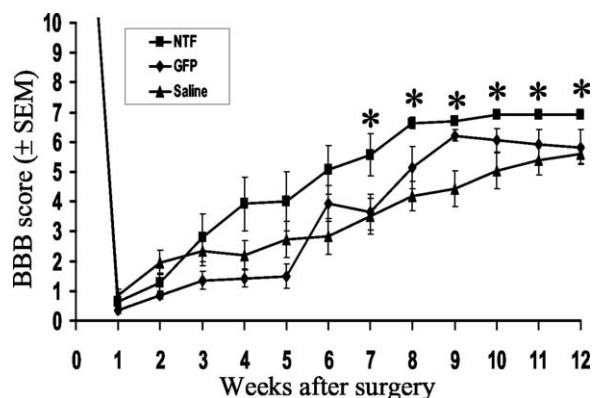


Fig. 5. AAV-mediated overexpression of BDNF and NT-3 leads to improved hind-limb performance. Following transection of the spinal cord, implantation of the SC bridge and subsequent injection of viral vectors, all animals exhibited the same degree of loss of function. In time, improvement in hind-limb locomotion was observed in all animals. The AAV-NT-3/BDNF (AAV-NTs)-treated animals ($n=7$), however, showed better functional recovery when compared with the AAV-green fluorescent protein-treated ($n=7$) and saline-treated ($n=11$) animals. The analysis of variance test revealed that the AAV-BDNF/AAV-NT-3 group was significantly different from both control groups. The asterisks indicate statistical significance ($P<0.05$).

cord. Predominantly neurons were transduced with the AAV vectors for at least 16 weeks after injection into the cord. A significant, albeit modest, improvement in hind-limb performance was observed in animals that were treated with AAV-BDNF and AAV-NT-3 compared with control animals. In the treated animals, significantly more neurons in the L2 cord segment extended processes toward the SC implant. Regenerating axons were found in the SC bridge but not beyond.

In this study, we used newly generated AAV vectors that were first evaluated for their ability to transduce cells *in vitro* and *in vivo*. All three vectors, AAV-GFP, AAV-BDNF, and AAV-NT-3, infected and transduced 293 human kidney cells, which then expressed the respective transgenes. Medium from the cells transduced with AAV-BDNF and AAV-NT-3 induced robust outgrowth of DRG axons, indicating that the cells secreted biologically active transgene products. Cells transduced with AAV-NT-3 displayed

outgrowth of mainly thick-appearing DRG axons, whereas that from AAV-BDNF transduced cells induced growth of mainly thin-appearing DRG axons. This difference in axon morphology may reflect the specificity of the two neurotrophins; NT-3 mainly exerts its effect through the trkC receptor, which is present on thicker DRG axons, and BDNF through the trkB receptor, which is present on thinner DRG axons (Davies, 2000). There is also the possibility, of course, that the observed difference in axon thickness was related to a difference in axon fasciculation caused by the neurotrophins or by the non-neuronal cells that migrated from the DRG explants.

In vivo, a persistent AAV vector-mediated transduction of cells was achieved without detectable signs of toxicity. In fact, at 16 weeks after injection into the cord, AAV vector-mediated transgene expression in neurons was still present. Moreover, transduced neurons had extended processes into the SC bridge. These results indicate that transduction by the new AAV vectors is long-term and non-toxic.

Following treatment with AAV vectors encoding BDNF and NT-3, a modest yet significant improvement in hind-limb performance was observed compared with control animals. The average BBB score in the group that was treated with AAV-BDNF and AAV-NT-3 at 12 weeks after injury/implantation and vector application was approximately 7, which reflects extensive movements of all three hind-limb joints. Using retrograde labeling techniques, it was found that administration of AAV-BDNF and AAV-NT-3 increased the number of labeled interneurons and relay neurons in the L2 cord segment. BDNF and NT-3 secreted by the AAV vector-transduced cells may have promoted either survival and/or sprouting of these neurons. Possibly, the interneurons and relay neurons, which are located in the intermediate gray and the dorsal horn of the spinal cord, respectively, may have become integrated in local locomotor circuitries that participated in hind-limb locomotor function.

The injection of AAV-BDNF and AAV-NT-3 caudal to the bridge did not promote axonal regeneration from the implant into the spinal tissue beyond, as is typical for transplantation of a SC bridge only. Both vectors were

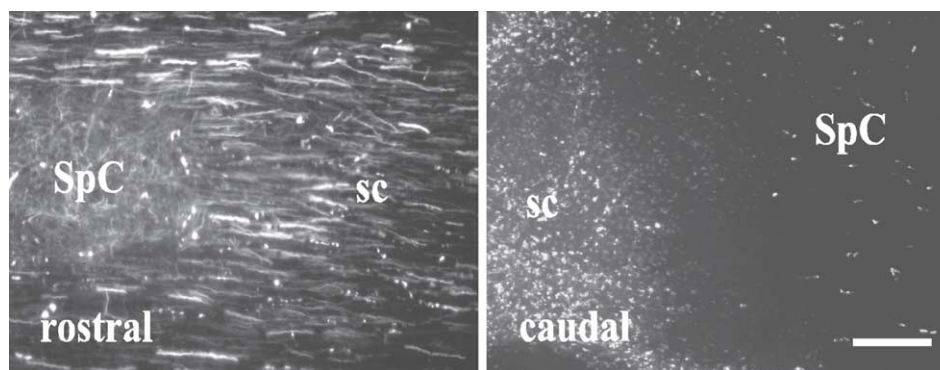


Fig. 6. Descending axons traverse the SC implant but do not re-enter the caudal cord. Photographs show the rostral and caudal bridge–host interfaces. FR-traced axons grew into the SC bridge, but did not grow beyond the caudal bridge–host interface into the caudal spinal cord. SpC=spinal cord, SC=Schwann cell bridge. Scale bar=200 μ m.

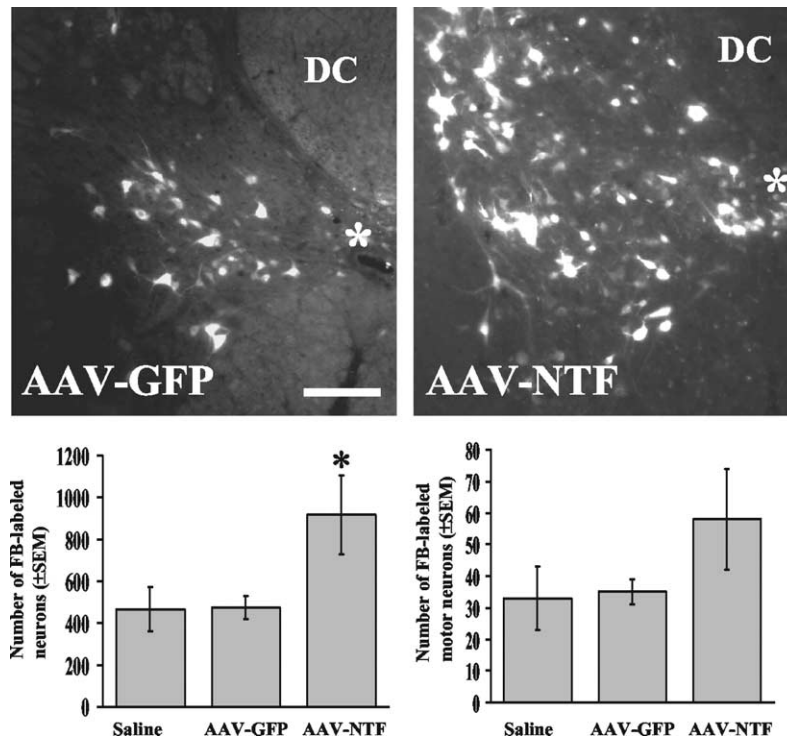


Fig. 7. AAV-mediated overexpression of BDNF and NT-3 results in more neurons at L2 extending neurites toward the SC implant. Photographs show FB-labeled neurons in a transverse section of the L2 segment following AAV-GFP and AAV-NTF application. A significant increase in FB-labeled neurons was present in the L2 segment in the AAV-NT3/BDNF-treated animals ($P=0.022$). The bars in the graphs represent means \pm S.E.M. Although there was a trend, the number of FB-labeled motor neurons in the L2 segment did not differ significantly from the other groups ($P=0.08$). DC=dorsal column. Scale bar=200 μ m.

introduced because different brainstem neuronal populations express *trkB* and *trkC*, the receptors for BDNF and NT-3, respectively (Xu et al., 1997). This lack of axonal growth into the caudal spinal cord may explain why none of the treated animals reached a BBB score of 9, i.e. weight support in stance, which implies the involvement of higher brain centers. Why did our treatment with AAV-BDNF and AAV-NT-3 fail to promote axonal growth from the graft into the caudal spinal cord? Previously, it was shown that a continuous infusion of BDNF or NT-3 into the spinal cord, a short distance away from a peripheral nerve or SC implant, promotes re-entry of sensory axons (Oudega and Hagg, 1999) and descending spinal axons (Bamber et al., 2001), respectively, into the spinal tissue. However, in these studies the injury was confined to the dorsal columns (Oudega and Hagg, 1999) or the lateral half of the cord (Bamber et al., 2001) and relatively high levels (1–10 μ g/day) of neurotrophins were infused. The transection/implantation model used in the present study undoubtedly caused more damage to the spinal tissue, which resulted in a graft–host cord interface highly inhibitory for axonal growth. Possibly the transduced cells did not secrete high enough amounts of neurotrophins to engender growth of axons across such an obstructive interface. We do not know how much neurotrophin was present at different time points after transduction of the cord cells with the AAV vectors. It is also possible that the secreted neurotrophins were inactivated by binding to truncated neurotrophin re-

ceptors present in spinal cord and neural scar tissue (Armanini et al., 1995; Frisen et al., 1993).

Another explanation for the lack of axonal growth into the distal cord is that the secreted neurotrophins did not diffuse close enough to the bridge–cord interface. In the study of Bamber et al. (2001), the infusion device was inserted into the spinal cord at 2.5 mm from the caudal graft–cord interface, whereas in the present study the injection with AAV vectors was 5 mm away from the distal interface. In fact, the most rostral transduced cells (caudal to the bridge) were approximately 2 mm away from the caudal bridge–host cord interface. It is well known that diffusion of NT-3 and BDNF is limited; for BDNF, for example, it is 1 mm or less in adult CNS tissue (Anderson et al., 1995). Clearly, in the present study the injection did not cause transduction of cells at the graft–cord interface. Previous studies revealed that it is difficult to transduce both peripheral and central neural scar tissue with adenoviral vectors (Dijkhuizen et al., 1998; Blits et al., 1999). This is surprising because fibroblasts and astrocytes, cell types that can be transduced by adenoviral vectors, are a major part of neural scar tissue.

Axonal growth from a graft into spinal cord mediated by increased levels of neurotrophins is based on the principle that the hostile nature of spinal tissue to axonal growth (due to an unfavorable balance between growth-inhibitory and growth-promoting molecules; Horner and Gage, 2000; Schwab, 2000) is overcome. Cai and colleagues (Cai et

al., 1999) demonstrated that exposure of, for instance, sensory axons to neurotrophins blocks the inhibition of growth by myelin-associated glycoprotein and myelin *in vitro*. It was suggested that neurotrophin-induced elevation of cAMP levels blocks the inhibition of axonal growth (Cai et al., 1999). More recently, it was shown that elevation of cAMP *in vivo* promotes sensory regeneration in the spinal cord (Qiu et al., 2002). A neurotrophin-mediated shift in the unfavorable molecular balance may result in axonal growth across the graft–host cord interface and into the spinal tissue. The present and previous (Oudega et al., 2000) results suggest that the result of manipulations that cause such a molecular shift in spinal tissue depend on the injury/implantation model, which determines the severity of the damage to the spinal cord. With a more severe injury, a more aggressive approach may be necessary to promote axonal growth from an implant into the spinal cord. The design of a more powerful repair strategy that is based on the use of AAV vectors may have to include multiple injections of the vectors. Also, the application of other non-toxic vector systems, such as AAV type 5 or lentiviral vectors, may more effectively increase the level of neurotrophins. Because the need for high levels of neurotrophins may result in side effects, such as gait disturbance, weight loss, or seizures (Ankeny et al., 2001; Ditttrich et al., 1996; Klein et al., 1999), an alternative approach could be the delivery of relatively low levels of neurotrophic factors combined with, for example, strategies to neutralize or reduce axonal growth-inhibitory molecules such as Nogo (Schnell and Schwab, 1990, 1993; von Meyenburg et al., 1998) or chondroitin sulfate proteoglycan (Moon et al., 2001; Yick et al., 2000). Combination strategies may be key to achieve adequate regrowth of different populations of axons (Bunge, 2001; Oudega et al., 2000).

The improved hind-limb function, which was found despite the lack of axons growing into the caudal spinal cord, may also have been caused by neurotrophin-mediated modifications at the neuronal level in the denervated lumbar spinal cord segments. The AAV-transduced cells seen at the lumbar levels may have secreted enough neurotrophins to, for instance, enhance neuronal excitability or stimulate neurotransmitter synthesis and turnover in the neurons that are involved in the local locomotor circuitry (Altar et al., 1994; Siuciak et al., 1996). Neurotrophic factors are also known to potentiate synaptic transmission (Arvanov et al., 2000; Kang and Schuman, 1995; Thoenen, 1995), depress postsynaptic potentials (Frerking et al., 1998), activate cation channels (Kafitz et al., 1999) or induce changes in myelination (McTigue et al., 1998). All these events could contribute to an improved functional performance. In fact, following application of BDNF in spinal cord lesion models, these mechanisms, as well as decreased free radical formation (Ankeny et al., 2001; Houweling et al., 1998b), have been hypothesized previously to result in improved functional outcome. It has been demonstrated that trophic factors can activate the central pattern generator, which results in spontaneous airstepping and accelerated locomotor recovery in the open field (Jakeman et al., 1998). Further investigation of the induc-

tion of locomotor patterns by neurotrophic factors will provide more insight into their role in functional behavior in normal and spinal cord-injured animals. This may lead to novel therapeutic strategies aimed at stimulating or directing spinal plasticity to enhance the function of spared neural circuits (Edgerton et al., 2001; Mendell et al., 2001; Muir and Steeves, 1997).

Acknowledgements—The authors are grateful to Andrew Weber, Pratik Desai, Annemarie Ali, and Jill Claasens for histology; Fred de Winter and Gerben van der Meulen for assistance with the figures; and Yelena Pressman for cell culture. Lyudmila Ruskova, Rosemary Abril and Kim Looor are thanked for their help with animal care and behavioral testing. Tyrone Hazlett (CytoTherapeutics, Providence, RI, USA) is thanked for providing the PANI/PVC tubes. Generation of AAV vectors would not have been possible without the help and knowledge of Wim Hermens, Paul Dijkhuizen and Olivier ter Brake. Martin Oudega is a Werner Heumann Memorial Scholar. This work was funded by N.W.O. GB/MW Pioneer grant 030-94-142 (J.V.), Internationaliseringsfonds Graduate School Neurosciences (B.B.), NIH grants NS09923 (M.B.B.), PO1NS38665 (M.O. and M.B.B.) and the Miami Project to Cure Paralysis.

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(Accepted 30 October 2002)