Published in final edited form as:

Exp Neurol. 2012 September ; 237(1): 126–133. doi:10.1016/j.expneurol.2012.06.016.

HSV-mediated Gene Transfer of C3 transferase Inhibits Rho to Promote Axonal Regeneration

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Abstract

Although surgical re-implantation of spinal roots may improve recovery of proximal motor function after cervical root avulsion, recovery of sensory function necessary for fine motor coordination of the hand has been difficult to achieve, in large part because of failure of regeneration of axons into the spinal cord. In order to enhance regeneration, we constructed a non-replicating herpes simplex virus (HSV)-vector carrying the gene coding for bacterial C3 transferase (C3t). Subcutaneous inoculation of the vector into the skin of the forepaw one week after a dorsal C5-T1 rhizotomy resulted in expression of C3t in dorsal root ganglion (DRG) neurons and inhibition of Rho GTPase activity, resulting in extensive axonal regeneration into the spinal cord that correlated with improved sensory-motor coordination of the forepaw.

Keywords

trauma; neural regeneration; gene therapy; herpes simplex virus; Rho GTPase

Introduction

Trauma to the brachial plexus often results in a devastating injuries, with only partial restoration of shoulder function despite best currently available therapies (Giuffre et al., 2010). Peripheral axons are capable of regeneration, but elongation of the central sensory afferents and motor efferents is blocked by the growth inhibitory effects of chondroitin sulfate proteoglycans (CSPG) in the glial scar, and myelin proteins NogoA, myelin associated glycoprotein (MAG) and oligodendrocyte myelin glycoprotein (OMgP) that through their cognate neuronal receptors activate axonal Rho GTPase to inhibit neurite extension (Filbin, 2003; Fournier and Strittmatter, 2001; Huber and Schwab, 2000; Snow et al., 1990; Wang et al., 2002).

The Rho GTPases are a family of monomeric G-proteins that transduce extracellular signals regulating actin cytoskeleton and microtubule polymerization. Activation of Rho GTPase induces a conformational change in the protein that promotes association with scaffolding

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Conflict of Interest Statement

The authors have no competing interests to declare.

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and adaptor molecules resulting in phosphorylation of kinases that act on downstream effectors to regulate the dynamic state of cytoskeletal proteins (Linseman and Loucks, 2008). Clostridial C3 transferase (C3t) is a bacterial protein that inhibits Rho signaling by N-ADP ribosylation of Rho GTPase (Aktories et al., 2004; Jank and Aktories, 2008). Because activation of Rho GTPase serves as a final common path for the several inhibitors of axon growth, and inhibitors of Rho signaling applied extracellularly enhance regeneration after injury (Dergham et al., 2002), we judged that expression of C3t by sensory neurons might also be useful in enhancing axonal regeneration of severed dorsal roots through the dorsal root entry zone (DREZ).

In previous studies we have demonstrated that non-replicating herpes simplex virus (HSV)-based vectors injected into the skin can be used to transduce neurons of the dorsal root ganglion (DRG) *in vivo*, and that expression of neurotrophic peptides from the transduced neurons can be used to protect nerves from degeneration caused by diabetes or chemotherapy (Chattopadhyay et al., 2004; Chattopadhyay et al., 2007; Chattopadhyay et al., 2009). HSV-mediated release of a soluble fragment of the Nogo receptor from transduced DRG neurons to selectively block Nogo signaling enhances regeneration across the DREZ after root injury (Peng et al., 2010). Since C3t has the potential to act downstream of the several inhibitors of axonal regeneration including Nogo, MAG, OMgp and CSPGs, we constructed a non-replicating herpes simplex virus (HSV)-based vector carrying two copies of the bacterial C3t gene (vC3t), and examined the effect of subcutaneous inoculation of vC3t and expression of C3t in dorsal ganglion neurons on axonal regeneration and behavioral parameters of recovery following cervical root injury following cervical root injury.

Materials and Methods

Vector construction

Two non-replicating HSV vector constructs (111 and 121) were constructed, each containing the full coding sequence of C3 exoenzyme (GenBank: CAA35828.1) with an HA tag at the 5' terminal under the control of the human cytomegalovirus immediate-early promoter (HCMV IEp). Full length C3t was amplified from a plasmid (kind gift of Professor R. Aktories, University of Freiburg, Germany) and cloned into BamHI-EcoRI cut plasmid HCMV-polyA/SASB3-16. The HCMV-HA-C3-polyA/SASB3-16 plasmid was co-transfected with the non-replicating HSV recombinant UL41E1G6 (kindly provided by Dr. Joseph Glorioso, University of Pittsburgh) into complementing 7b cells. UL41E1G6 is deleted for the essential immediate early genes *ICP4* and *ICP27*. The deletion of the *ICP4* loci also removes the promoter for the *ICP22* and *ICP47* genes, rendering the recombinant (and the resulting vector) defective in expression of 4 HSV IE genes. The C3t construct lacks a leader sequence, so that transgene-mediated C3t is not released from transfected cells. Three runs of extraction were performed to select clear plaques and the identity of the insert confirmed by PCR followed by DNA sequencing. Control vector vGFP was constructed to be identical to vC3t, but contained the green fluorescent protein (GFP) gene in place of C3t (Zhou et al., 2009). Control vector vZ has been described previously (Chattopadhyay et al., 2009).

Tissue culture and vector infection

HEK 293 cells (ATCC, Manassas, VA) were grown in ATCC-formulated Eagle's Minimum Essential Medium supplemented with 10% fetal bovine serum. Dissociated DRG neurons were grown in culture as previously described (Peng et al., 2010). Both HEK 293 cells and DRG neurons were infected with either vC3t or vGFP at multiplicity of infection (MOI) 1 for 2 hrs and replaced with fresh medium. The cell lysates from HEK 293 cells and DRG

were collected at 24 and 48 hrs for determination of C3t expression by RT-PCR or by Western blot. The experiments were repeated three times.

Experimental animals and surgical procedures

Female Sprague–Dawley rats weighing 200 to 250g were used in all experiments. Housing conditions and experimental procedures were approved by the University of Michigan Committee on Use and Care of Animals. For studies of transgene expression and activity *in vivo*, two sets of 10 rats were inoculated in the plantar surface of left forepaw with 30 μ l of vector containing 1.0×10^9 plaque-forming units (pfu) of vC3t or vGFP. The third group of 10 rats was inoculated with 30 μ l of saline and used as control. Seven days later, the left C6–C8 DRGs were collected from each animal and samples were used for either determination of C3t expression by RT-PCR or for determination of RhoA activity. For *in vivo* regeneration studies and behavior analysis twenty four female Sprague–Dawley rats weighing 200 to 250 g were anesthetized with isoflurane and the cervical spinal cord and approximately 2 mm of the dorsal roots were exposed via a partial dorsal laminectomy. The C5-T1 spinal cord was identified using the T2 spinal process as a landmark, the dura was pierced and C5-T1 dorsal roots crushed 3 times, for 5 seconds per crush, unilaterally (left) between the dorsal root ganglia and DREZ using fine forceps. Muscle and fascia were sutured closed, and the skin was closed with autoclips. Following surgery, all animals were maintained under preoperative conditions and were eating and drinking within 3 hr after surgery. Approximately one hour after injury, animals were inoculated in the plantar surface of left forepaw with 30 μ l of vector containing 1.0×10^9 pfu of vC3t or vZ. Ten additional rats underwent similar surgery but no laminectomy or dorsal root crush injury was performed (sham group). After completion of the behavioral testing, the rats were reanesthetized with isoflurane, and the left median nerve was exposed and 5 μ l of 5% cholera toxin subunit B (CTB, List Biological Laboratories, Campbell, CA) injected into the left median nerve. Seven days later tissue was collected for analysis.

RT-PCR

Total RNA was isolated from HEK 293 cells, *in vitro* DRG neurons or rat DRG using TRIzol isolation reagent (Invitrogen). cDNA prepared from mRNA was amplified using following primer sets: β -actin-forward: 5'-CAG TTC GCC ATG GAT GAC GAT ATC-3' and β -actin-reverse: 5'-CAC GCT CGG TCA GGA TCT TCA TG-3' for β -actin, C3t-forward: 5'-GGA TCC GCC ACC ATG TAC CCC TAC GAC TTG TCC AAC TAC ACC GCT TAT TCA AAT ACT TAC CAG-3' and C3t-reverse: 5'-GAA TTC TTT AGG ATT GAT AGC TGT GCC CAT CAT TGT TGC TG-3' for C3t. All reactions involved initial denaturation at 94°C for 5 min, followed by 26 cycles for β -actin, 26 cycles for C3t in 293 cells, 28 cycles for C3t in P10 DRG and 36 cycles for C3 in rat DRG (94°C for 30 sec, 68°C for 2 min and 1 cycle 68°C for 8 min) using a GeneAmp PCR 2700 (Applied Biosystems, Carlsbad, CA).

Western blot

Cultured cells were homogenized in lysis buffer containing 50 mM Tris, 10 mM NaCl, 1% NP40, 0.02% sodium azide, and protease inhibitor cocktail (Sigma Aldrich, St. Louis, MO) pH 7.4. Aliquots containing 20 μ g of protein were analyzed as previously described (Peng et al., 2010). Primary antibodies used were a rabbit antibody against the hemagglutinin tag (anti-HA, 1:400; Sigma-Aldrich) and a mouse antibody to β III tubulin (1:2000; Sigma-Aldrich). Peroxidase-coupled secondary antibodies (Calbiochem, San Diego, CA) were used for amplification and protein bands visualized using X-OMAT AR film (Kodak, Rochester, NY) after chemiluminescence (DuPont NEN, Boston, MA). The membranes were stripped and reprobed with mouse anti- β -actin (1: 2000; Sigma-Aldrich) as a loading control. The

intensity of each band was determined by quantitative chemiluminescence using a PC-based image analysis system (ChemiDoc XRS System, Bio-Rad).

Rho activity assay

P10 DRG were plated on 24-well plates at 2×10^5 cells per well, and vGFP or vC3t (MOI 1) added 30 minutes after plating. The medium was changed after 2 hrs and cell lysates collected 24 hrs after plating. DRG were homogenized in lysis buffer containing 50 mM Tris, 10 mM NaCl, 1% NP40, 0.02% sodium azide, and protease inhibitor cocktail (Sigma Aldrich) pH 7.4. RhoA activity was measured as previously described (Peng et al., 2010) using a G-LISA kit (Cytoskeleton, Denver, CO). The RhoA G-LISA kit contains a Rho-GTP-binding protein linked to the wells of a 96 well plate. Active, GTP-bound Rho in cell/tissue lysates will bind to the wells while inactive GDP-bound Rho is removed during washing steps, and the bound active RhoA is detected with a RhoA specific antibody. Four independent samples with three conditions were used for quantitation.

Immunohistochemistry

HEK 293 cells and P10 DRG neurons grown *in vitro* were infected with vC3t or vGFP at MOI of 1 for 2 hours. After 24 hours *in vitro* the cells were fixed, blocked and probed with mouse antibody against β III tubulin (anti-Tuj1 1:1000, Covance, Princeton, NJ) antibody overnight. The second antibodies utilized were fluorescent anti-rabbit IgG Alexa Fluor 594 or anti-mouse IgG Alexa Fluor 488 (1:2000, Invitrogen). For *in vivo* regeneration experiments, rats were perfused transcardially with 0.9% NaCl followed by freshly made 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The spinal cords were removed and blocked in the following segments: C5-T1 with the respective roots attached, C4-C5 and C3-C4. The tissues were post-fixed in the same solution for 8 h, and cryoprotected with 30% sucrose in 0.1 M phosphate buffer for 2 days at 4 °C. Cryostat sections of C5-T1 spinal cord with attached roots (25 μ m) were thaw-mounted onto cold Superfrost microscope slides (Fisher, Pittsburgh, PA), blocked with 5% normal goat serum in PBS-T, incubated with anti-Tuj1 (1:5000, Covance), anti-calcitonin gene related peptide (anti-CGRP 1:500 Peninsula Laboratories, San Carlos, CA), and anti-cholera toxin subunit B (anti-CTB 1:500, Sigma) overnight at 4 °C followed by fluorescent anti-rabbit IgG Alexa Fluor 594 or anti-mouse IgG-Alexa Fluor 488 (1:2000; Invitrogen) for 1 h at room temperature. Cryostat sections (25 μ m) of C4-C5 and C2-C4 of spinal cord were mounted and blocked as above for immunodetection of CTB (anti-CTB 1:500 Sigma) using DAB-HRP detection (ABC Kit, Vector Labs, Burlingame, CA) or anti-rabbit IgG-Alexa Fluor 488. All sections were mounted in water-based Fluoromount-G (Electron Microscopy Sciences, Hatfield, PA). The fluorescent images were captured using a Nikon Eclipse E1000 microscope and analyzed using Metamorph software (Molecular Devices, Sunnyvale, CA). The area occupied by positive immunostained fibers within the selected area of DREZ (dotted area) situated 400 μ m distal from the transition zone was analyzed as previously described (Peng et al., 2010) in sections of spinal cord with the root attached corresponding to three cervical roots from each of four animals in each group (sham, vZ and vC3t).

Neurite outgrowth assay

Neurite outgrowth was performed as previously described with minor modifications (Peng et al., 2010; Zhou et al., 2009). P10 DRGs were plated on poly-D-lysine (100 μ g/ml) and laminin (4 μ g/ml) coated 8 well chamber slides at 2×10^4 cells per well. vC3t or vZ at MOI 1 was added 30 min after plating for 2 hours. After 24 hrs *in vitro* DRG neurons were fixed and immunostained with an antibody against neuron-specific β III tubulin (anti-Tuj1, Covance) followed by anti-mouse IgG Alexa Fluor 488 (1:2000, Invitrogen). In the myelin study, the chamber slides were precoated with 3 μ g of purified CNS myelin (Norton and Poduslo, 1973) in each well. The total process outgrowth for β III tubulin positive neuron

was determined using Metamorph imaging software (Molecular Devices). More than 200 randomly selected individual neurons were analyzed per condition in each experiment and the experiment was repeated 3 times.

Behavioral Analysis

Two behavioral analyses were performed: the grid walk and the cylinder test. For the grid walk the animals were placed on a 1-meter long horizontal runway of metal grid bars elevated 30 cm from the ground with regular gaps (4 cm). The number of errors of foot placing was counted in each crossing. Three crossings were counted per animal at each time point. All animals were pretrained in the task 1 week prior to surgery. Behavioral measures were determined weekly for the 7 week duration of the study by an observer blinded to treatment group. For the cylinder test, the rats were placed in a transparent cylinder (16 cm diameter, 21 cm high) and videotaped. Spontaneous wall touches of the impaired (left) and contralateral forelimb (right) were counted. A total of 20 touches were counted and the percentage of impaired forelimb touch over total 20 touches was used for quantification. The rats were tested once before injury. Quantitation was performed in behaviors from ten animals per group and presented as percent of sham control group value.

Data analysis

Statistical significance of the difference between vector-treated and control was determined by one-way ANOVA with post-hoc comparisons where appropriate. Parametric statistics, using the general linear model for repeated measures, were used to identify significant effects of treatment condition. The results were examined using the software package SPSS 12.0 for Windows (SPSS Inc). Data are expressed as mean $\pm$ standard error of mean (SEM), with $p < 0.05$ considered significant.

Results

HSV vector vC3t produces C3t in vitro

In order to enhance neurite growth in vitro and axonal regeneration following injury, we constructed a non-replicating HSV vector containing two copies of the bacterial C3 transferase (C3t) gene with an HA tag under the control of the HCMV IEp (Figure 1). Control vectors contained either GFP (vGFP) or lacZ (vZ) transgenes instead of C3t (Chattopadhyay et al., 2009; Zhou et al., 2009). Two different C3t-expressing vector constructs were analyzed (111 and 121) for expression of the C3t transgene. Transfection of HEK 293 cells with the C3t-containing vector constructs resulted in expression of C3t mRNA detected by RT-PCR directed in part at the HA tag (Figure 2A) and protein (Figure 2B) 24 hrs post infection. The expression of C3t by vector in transduced HEK 293 cells induced the expression of the neuronal isoform β III tubulin (Figure 2B). For subsequent experiments we chose to use construct 121, which we designated as vC3t, because of higher expression levels. Infection of HEK 293 cells with vector construct vC3t resulted in process formation by those cells visualized by fluorescent immunocytochemistry with anti- β III tubulin antibody (Figure 2C).

Vector-mediated expression of C3t in DRG neurons blocks RhoA activity and increase expression of β III tubulin

Primary DRG neurons in culture were transfected with vC3t at MOI 1. Infection with vC3t resulted in the expression of C3t mRNA and protein in DRG neurons (Figure 3A, B) and caused a concomitant reduction in basal Rho activity measured by G-LISA as compared to control or DRG transduced with vector control (Figure 3C). Similar to the effect in HEK 293 cells, transfection of postnatal DRG neurons with vC3t resulted in an increase in β III-

tubulin protein that was not observed in DRG neurons treated with control vector (Figure 3D).

Expression of C3t in DRG neurons allows the cells to overcome myelin inhibition of neurite extension

Neurite growth was assessed in P10 DRG *in vitro* in the absence or presence of purified CNS myelin (Peng et al., 2010). In the absence of myelin (Figure 4A), transfection with vC3t caused a significant increase in the number and length of neurites measured as total neurite outgrowth when compared to control DRG or DRG infected with control vector (vZ) measured by β III tubulin immunostaining 24 hrs after plating and infection. In addition vC3t blocked the inhibitory effect of myelin on neurite extension from P10 DRG neurons cultured on a substrate containing 3 μ g of purified CNS myelin protein per well (Figure 4B). Taken together the results show that expression of C3t in DRG neurons by vC3t is able to enhance neurite outgrowth early in development and overcome inhibition of neurite growth by myelin by inhibiting Rho-GTPase and enhancing β III-tubulin expression. Based on these *in vitro* data we performed a number of experiments to determine if vC3t administered *in vivo* from skin inoculation may enhanced axonal regeneration following injury.

vC3t expresses C3t and blocks Rho activity in DRG *in vivo*

To examine the potential use of vC3t in animal models we delivered the vector by subcutaneous inoculation in the left forepaw of adult rats (schematic representation Figure 5A, B). C3t mRNA expression was observed in the left C6–C8 DRG in animals inoculated with vC3t but not in control animals or animals receiving vGFP (Figure 5C). The expression of C3t in DRG *in vivo* correlated with inhibition of Rho activity in these cervical DRG homogenates measured by G-LISA at 1 week after injection (Figure 5D).

vC3t enhances sensory regeneration into DH of spinal cord

To determine the capacity of vC3t to enhance regeneration of sensory fibers after cervical dorsal root injury, adult rats were injured with unilateral dorsal root crush (C5 to T1), and approximately 1 hour later injected subcutaneously in the left forepaw with 30 μ l of 1×10^9 pfu/ml of vC3t or vZ to transduce the DRG. At 8 weeks post injury and vector delivery immunohistochemical analysis was performed on the DREZs and dorsal horn at the levels of the injury (A) and in the dorsal columns at C4 (B) and above C4 (C) (schematic of tissue analysis Figure 6). Subcutaneous injection of vC3t in the left forepaw enhanced unmyelinated axon growth across the ipsilateral dorsal root entry zone and dorsal horn as compared with control vector as demonstrated by CGRP immunoreactive axon terminals in the gray matter of lamina I–II suggestive of regeneration of unmyelinated and small myelinated axons (Figure 6A). We also found increased β III tubulin immunostained fibers in the white matter crossing the dorsal root entry zone (Figure 6A) in the animals treated with subcutaneous injection of vC3t in the forepaw suggestive of growth of additional axons. To determine whether the increase in labeled fibers represented regeneration of injured axons or sprouting of uninjured fibers, we injected CTB into the median nerve to label axons in transit. CTB is retrogradely transported predominantly in myelinated fibers. At 8 weeks post-injury and vector delivery there was an increase in CTB staining in the dorsal root entry zone of animals treated with vC3t as compared with control vector, consistent with regenerating axons (Figure 7A). These results suggest that C3t inhibition of Rho GTPase promotes CNS regeneration after injury and enhances growth of unmyelinated and myelinated fibers.

vC3t enhances regeneration of large myelinated fibers into dorsal columns

CTB containing large axons were seen at 8 weeks post-injury and vector delivery in the cervical (C4–C5) dorsal column using HRP-DAB immunolabeling. The labeled axons were limited to the left cuneate fasciculus, the location of ascending myelinated fibers from the left forelimb, and were seen in animals treated with vC3t (arrows) but not in control vector treated animals (Figure 7B). The CTB labeled fibers appear to occupy the anatomical distribution as Wallerian degeneration induced by the injury. The results were confirmed in longitudinal sections, where CTB labeled regenerating axons were found ascending in the ipsilateral cuneatus fasciculus of the dorsal spinal cord (C3–C4) in the vC3t treated group but not in the vZ treated group using immunofluorescence detection (Figure 7C). No CTB labeled fibers were seen contralateral to the injury. Taken together, these results demonstrate that large myelinated fibers extending directly from primary afferent into the dorsal columns regenerate rostrally through several segments of spinal cord. No CTB labeling was seen in the medulla suggesting that axons had not reached the nucleus cuneatus at the time of study 8 weeks.

vC3t improves forelimb function

Two behavioral test of forepaw function were performed at weekly intervals for 7 weeks as described in Methods. Animals injected with vC3t subcutaneously in the forepaw showed significant improvement in behavior as measured by forepaw slips in gridwalk testing (Figure 8A) and forepaw reaching in the cylinder test (Figure 8B) beginning 2 weeks after injury and persisting for 7 weeks.

Discussion

Rho-ROCK signaling pathway is a common downstream mediator of inhibitory cues, blocking neurite extension, exerted by endogenous myelin proteins and the astrocytic glial scar. The mechanism of activation used by each ligand may vary but for Nogo66 binding to the neuronal receptor causes activation and translocation of Rho kinase (ROCK) II to a membrane compartment (Alabed et al., 2006). Commercially available inhibitors of Rho GTPase (C3 transferase derivative) or ROCK (Y-27632) prevent myelin inhibition of neurite growth in vitro and their local application promotes CNS axonal regeneration after thoracic spinal cord injury in rodents (Dergham et al., 2002; Fournier et al., 2003). Similarly, neurite growth inhibitory activity associated with chondroitin sulfate proteoglycans of the glial scar can be reversed with inhibitors of Rho-ROCK (Dergham et al., 2002; Monnier et al., 2003). The importance of this signaling pathway in regeneration is reflected in the observation that knockout mice deficient for ROCK II show significant improvement in behavior after brachial dorsal root crush injury or thoracic spinal cord injury compared to control mice (Duffy et al., 2009).

A novel C3 transferase peptide fragment (aa. 154–182) lacking the domain responsible for enzymatic inhibitory activity but causing a decrease in the amount of active RhoA has been applied directly to the injury site to promote functional recovery and regeneration of corticospinal tract axons following thoracic spinal cord injury (Boato et al., 2010). This peptide had no effect in DRG neurite outgrowth assay *in vitro*. Adenoviral vector delivery of C3 transferase has been reported in a model of glaucoma in vitro where C3t transgene expression increased outflow facility in an organ cultured of monkey eye anterior segments (Liu et al., 2005).

Using a HSV-vector (vC3t) for delivery and expression of C3t in neurons is a novel therapeutic tool that targets small Rho-GTPases downstream of many receptors signaling axon growth inhibitory cues from myelin proteins and astroglial proteoglycans following

CNS injury. HSV has the added advantage of being highly neurotropic and reaching the sensory neurons from skin inoculation by retrograde transport where establishes long term latent state (Glorioso and Fink, 2004). A substantial proportion of the relevant DRG neurons are transduced using this delivery system in vivo (Chattopadhyay et al., 2012). One of the limitations in the use of recombinant C3t to enhance regeneration after injury is the possibility of off-target effects resulting from inhibition of RhoA activity in uninjured neurons. Because we have not added a leader sequence to the C3t transgene, vector-mediated expression of C3t is restricted to cells transfected by the vector and thus the possibility of off target effects is reduced. vC3t represents a novel means to enhance recovery after trauma to the nervous system. In the dorsal root injury model, subcutaneous injection of vC3t in the forepaw to inhibit Rho activity in dorsal root ganglion neurons promotes regeneration of small and large myelinated sensory axons across the DREZ and into the spinal cord, and improves behavioral outcomes. The results presented with a single gene expressing HSV vector (vC3t) are encouraging for future therapies to improve axonal regeneration as delivery of a similar HSV vector backbone has shown no toxicity in phase I clinical trial (Fink et al., 2011).

Neurotrophic factors delivered either intrathecally or subcutaneously or by a transduced nerve graft have been used successfully to enhance sensory regeneration after dorsal root injury (Liu et al., 2009; Ramer et al., 2000; Ramer et al., 2002; Wang et al., 2008); and following spinal cord injury (Hollis and Tuszynski, 2011). Taken together the data presented suggest that targeted delivery of the vC3t in combination with a trophic factor gene could potentially offer additional advantages of enhancing regeneration not only by blocking RhoA inhibition of axon growth but by increasing cAMP-PKA (Gao et al.; Zhou et al., 2009) and ras-MAPK (Arevalo and Wu, 2006) signaling to support regeneration. Developments in the regulation of expression cassettes from HSV vectors may provide additional advantages towards the treatment of these chronic neurologic conditions (Wu et al., 2011; Wu et al., 2011).

Acknowledgments

We thank Professor R. Aktories, University of Freiburg, Germany from providing molecular reagents without which the work would not have been possible.

List of Abbreviations

C3t	C3 transferase
CSPG	chondroitin sulfate proteoglycans
DREZ	dorsal root entry zone
DRG	dorsal root ganglion
GFP	green fluorescent protein
HCMV IEp	human cytomegalovirus immediate-early promoter
HSV	herpes simplex virus
MAG	myelin associated glycoprotein
MOI	multiplicity of infection
OMgP	oligodendrocyte myelin glycoprotein
pfu	plaque-forming units
ROCK	Rho kinase

vC3t	C3t gene
vGFP	control vector expressing GFP
vZ	control vectors expressing lacZ

References

- Aktories K, Wilde C, Vogelsang M. Rho-modifying C3-like ADP-ribosyltransferases. *Rev Physiol Biochem Pharmacol.* 2004; 152:1–22. [PubMed: 15372308]
- Alabed YZ, Grados-Munro E, Ferraro GB, Hsieh SH, Fournier AE. Neuronal responses to myelin are mediated by rho kinase. *J Neurochem.* 2006; 96:1616–1625. [PubMed: 16441511]
- Arevalo JC, Wu SH. Neurotrophin signaling: many exciting surprises! *Cell Mol Life Sci.* 2006; 63:1523–1537. [PubMed: 16699811]
- Boato F, Hendrix S, Huelsenbeck SC, Hofmann F, Grosse G, Djalali S, Klimaschewski L, Auer M, Just I, Ahnert-Hilger G, Holtje M. C3 peptide enhances recovery from spinal cord injury by improved regenerative growth of descending fiber tracts. *J Cell Sci.* 2010; 123:1652–1662. [PubMed: 20406886]
- Chattopadhyay M, Goss J, Wolfe D, Goins WC, Huang S, Glorioso JC, Mata M, Fink DJ. Protective effect of herpes simplex virus-mediated neurotrophin gene transfer in cisplatin neuropathy. *Brain.* 2004; 127:929–939. [PubMed: 14749293]
- Chattopadhyay M, Mata M, Goss J, Wolfe D, Huang S, Glorioso JC, Fink DJ. Prolonged preservation of nerve function in diabetic neuropathy in mice by herpes simplex virus-mediated gene transfer. *Diabetologia.* 2007; 50:1550–1558. [PubMed: 17508196]
- Chattopadhyay M, Walter C, Mata M, Fink DJ. Neuroprotective effect of herpes simplex virus-mediated gene transfer of erythropoietin in hyperglycemic dorsal root ganglion neurons. *Brain.* 2009; 132:879–888. [PubMed: 19244253]
- Chattopadhyay M, Zhou Z, Hao S, Mata M, Fink DJ. Reduction of voltage gated sodium channel protein in DRG by vector mediated miRNA reduces pain in rats with painful diabetic neuropathy. *Molecular Pain.* 2012;8. [PubMed: 22296727]
- Dergham P, Ellezam B, Essagian C, Avedissian H, Lubell WD, McKerracher L. Rho signaling pathway targeted to promote spinal cord repair. *J Neurosci.* 2002; 22:6570–6577. [PubMed: 12151536]
- Duffy P, Schmandke A, Sigworth J, Narumiya S, Cafferty WB, Strittmatter SM. Rho-associated kinase II (ROCKII) limits axonal growth after trauma within the adult mouse spinal cord. *J Neurosci.* 2009; 29:15266–15276. [PubMed: 19955379]
- Filbin MT. Myelin-associated inhibitors of axonal regeneration in the adult mammalian CNS. *Nat Rev Neurosci.* 2003; 4:703–713. [PubMed: 12951563]
- Fink DJ, Wechuck J, Mata M, Glorioso J, Goss J, Krisky D, Wolfe D. Gene Therapy for Pain: Results of a Phase I Clinical Trial. *Ann Neurol.* 2011; 70:207–212. [PubMed: 21796661]
- Fournier AE, Strittmatter SM. Repulsive factors and axon regeneration in the CNS. *Curr Opin Neurobiol.* 2001; 11:89–94. [PubMed: 11179877]
- Fournier AE, Takizawa BT, Strittmatter SM. Rho kinase inhibition enhances axonal regeneration in the injured CNS. *J Neurosci.* 2003; 23:1416–1423. [PubMed: 12598630]
- Gao Y, Nikulina E, Mellado W, Filbin MT. Neurotrophins elevate cAMP to reach a threshold required to overcome inhibition by MAG through extracellular signal-regulated kinase-dependent inhibition of phosphodiesterase. *J Neurosci.* 2003; 23:11770–11777. [PubMed: 14684879]
- Giuffre JL, Kakar S, Bishop AT, Spinner RJ, Shin AY. Current concepts of the treatment of adult brachial plexus injuries. *J Hand Surg Am.* 2010; 35:678–688. [PubMed: 20353866]
- Glorioso JC, Fink DJ. Herpes vector-mediated gene transfer in treatment of diseases of the nervous system. *Annu Rev Microbiol.* 2004; 58:253–271. [PubMed: 15487938]
- Hollis ER 2nd, Tuszynski MH. Neurotrophins: Potential Therapeutic Tools for the Treatment of Spinal Cord Injury. *Neurotherapeutics.* 2011

- Huber AB, Schwab ME. Nogo-A, a potent inhibitor of neurite outgrowth and regeneration. *Biol Chem.* 2000; 381:407–419. [PubMed: 10937871]
- Jank T, Aktories K. Structure and mode of action of clostridial glucosylating toxins: the ABCD model. *Trends Microbiol.* 2008; 16:222–229. [PubMed: 18394902]
- Linseman DA, Loucks FA. Diverse roles of Rho family GTPases in neuronal development, survival, and death. *Front Biosci.* 2008; 13:657–676. [PubMed: 17981578]
- Liu S, Bohl D, Blanchard S, Bacci J, Said G, Heard JM. Combination of microsurgery and gene therapy for spinal dorsal root injury repair. *Mol Ther.* 2009; 17:992–1002. [PubMed: 19240691]
- Liu X, Hu Y, Filla MS, Gabelt BT, Peters DM, Brandt CR, Kaufman PL. The effect of C3 transgene expression on actin and cellular adhesions in cultured human trabecular meshwork cells and on outflow facility in organ cultured monkey eyes. *Mol Vis.* 2005; 11:1112–1121. [PubMed: 16379023]
- Monnier PP, Sierra A, Schwab JM, Henke-Fahle S, Mueller BK. The Rho/ROCK pathway mediates neurite growth-inhibitory activity associated with the chondroitin sulfate proteoglycans of the CNS glial scar. *Mol Cell Neurosci.* 2003; 22:319–330. [PubMed: 12691734]
- Norton WT, Poduslo SE. Myelination in rat brain: method of myelin isolation. *J Neurochem.* 1973; 21:749–757. [PubMed: 4271082]
- Peng X, Zhou Z, Hu J, Fink DJ, Mata M. Soluble Nogo receptor down-regulates expression of neuronal Nogo-A to enhance axonal regeneration. *J Biol Chem.* 2010; 285:2783–2795. [PubMed: 19901030]
- Ramer MS, Priestley JV, McMahon SB. Functional regeneration of sensory axons into the adult spinal cord. *Nature.* 2000; 403:312–316. [PubMed: 10659850]
- Ramer MS, Bishop T, Dockery P, Mobarak MS, O’Leary D, Fraher JP, Priestley JV, McMahon SB. Neurotrophin-3-mediated regeneration and recovery of proprioception following dorsal rhizotomy. *Mol Cell Neurosci.* 2002; 19:239–249. [PubMed: 11860276]
- Snow DM, Lemmon V, Carrino DA, Caplan AI, Silver J. Sulfated proteoglycans in astroglial barriers inhibit neurite outgrowth in vitro. *Exp Neurol.* 1990; 109:111–130. [PubMed: 2141574]
- Wang KC, Koprivica V, Kim JA, Sivasankaran R, Guo Y, Neve RL, He Z. Oligodendrocyte-myelin glycoprotein is a Nogo receptor ligand that inhibits neurite outgrowth. *Nature.* 2002; 417:941–944. [PubMed: 12068310]
- Wang R, King T, Ossipov MH, Rossomando AJ, Vanderah TW, Harvey P, Cariani P, Frank E, Sah DW, Porreca F. Persistent restoration of sensory function by immediate or delayed systemic artemin after dorsal root injury. *Nat Neurosci.* 2008; 11:488–496. [PubMed: 18344995]
- Wu Z, Mata M, Fink DJ. Prevention of diabetic neuropathy by regulatable expression of HSV-mediated erythropoietin. *Mol Ther.* 2011; 19:310–317. [PubMed: 20924361]
- Wu Z, Mata M, Fink DJ. Prolonged regulatable expression of EPO from an HSV vector using the LAP2 promoter element. *Gene Ther.* 2011 In Press.
- Zhou Z, Peng X, Fink DJ, Mata M. HSV-mediated transfer of artemin overcomes myelin inhibition to improve outcome after spinal cord injury. *Mol Ther.* 2009; 17:1173–1179. [PubMed: 19293775]

Highlights

- An HSV vector expressing C3 transferase (vC3t) inhibits Rho GTPase activity
- Transfection with vC3t enhances axonal regeneration after cervical rhizotomy
- Axonal regeneration was accompanied by improved behavioral recovery

Figure 1.
Schematic of vector vC3t.

Figure 2.

Expression of C3t RNA (A) and protein (B) in HEK 293 cells noninfected control or infected with two HSV vector constructs carrying the C3t gene 111 and 121 and control vector vGFP at MOI of 1 for 24 hrs. 111 and 121 infection also increased the amount of β III tubulin protein expression (B) coincident with elaboration of processes by the cells (Tuj-1 immunostaining, 96 hrs after infection (C)). Bar = 100 μ m.

Figure 3.

Expression of C3t in primary DRG neurons noninfected control or infected with vC3t and control vector vGFP for 2 hrs at MOI of 1. C3t mRNA by RT-PCR (A). C3t protein by Western blot determination of HA tagged (B). Rho-A activity measured by G-LISA presented as ratio to control. (***) $P < 0.001$. (C). Induction of β III tubulin protein in DRG by vC3t 24 hrs after infection at MOI of 1 (D).

Figure 4. vC3t enhances neurite growth. β III tubulin (Tuj1 antibody) immunostaining of transfected P10 DRG neurons plated in the absence (A) or presence (B) of 3 μ g/well of purified CNS myelin. Graphs depict the total length of neurites measured in at least 200 cells per condition, presented as percent of the control (uninfected) neurons. Bar = 50 μ m; *** $P < 0.001$

Figure 5. Schematic representation of subcutaneous delivery of vector in left forepaw and retrograde transport of vector to DRG (A, B). C. Expression of C3t mRNA in DRG in vivo 1 wk after subcutaneous inoculation of vC3t or vGFP. D. Rho-A activity in DRG in vivo measured by G-LISA presented as ratio to control. *** $P < 0.001$

Figure 6.

Schematic representation of surgical approach and histological analysis from cervical DREZ and DH at the levels of injury (A) and dorsal column at C4 (B) and C3–C4 (C).

Immunostaining of DH for CGRP and DREZ for Tuj1 8 weeks after L C5-T1 root injury and injection of vZ or vC3t (A). The area of immunostaining was quantitated as a ratio of ipsilateral to contralateral (uninjured). * $P < 0.05$; ** $P < 0.01$ compared to vZ; Bar = 100 μm .

Figure 7.

A. Immunostaining of DREZ for CTB 8 wks after left C5-T1 root injury and injection of vZ or vC3t. The area of immunostaining was quantitated as a ratio of ipsilateral to contralateral (uninjured). ** $P < 0.01$ compared to vZ; Bar = 100 μm . B. Immunostaining for CTB 8 wks after injury and injection vZ or vC3t using DAB-HRP detection. Regenerating fibers seen in vC3t but not in vZ ascending in the ipsilateral C4–C5 dorsal columns at similar location as injury-induced Wallerian degeneration (arrows, cross-sections). C. Immunofluorescence detection of CTB containing axons in ipsilateral dorsal column of C3–C4 spinal cord in vC3t but not in vZ (longitudinal sections). Bar = 100 μm . Sections shown in A, B and C correspond to the regions as labeled in the schematic illustration in Figure 6.

Figure 8. Subcutaneous inoculation of vC3t results in improvement in behavioral function (proprioception) measured by foot faults on the grid walk test (A) and reaching measured by the cylinder test (number of left paw touches over total touches, B). $N = 10$ animals per group; for both behavior measurements, $P < 0.05$ by repeated measures analysis of variance; vC3t - open circles; vZ - filled circles.