Sialidase enhances spinal axon outgrowth *in vivo*

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Communicated by Saul Roseman, The Johns Hopkins University, Baltimore, MD, June 5, 2006 (received for review March 16, 2006)

The adult CNS is an inhibitory environment for axon outgrowth, severely limiting recovery from traumatic injury. This limitation is due, in part, to endogenous axon regeneration inhibitors (ARIs) that accumulate at CNS injury sites. ARIs include myelin-associated glycoprotein, Nogo, oligodendrocyte-myelin glycoprotein, and chondroitin sulfate proteoglycans (CSPGs). Some ARIs bind to specific receptors on the axon growth cone to halt outgrowth. Reversing or blocking the actions of ARIs may promote recovery after CNS injury. We report that treatment with sialidase, an enzyme that cleaves one class of axonal receptors for myelin-associated glycoprotein, enhances spinal axon outgrowth into implanted peripheral nerve grafts in a rat model of brachial plexus avulsion, a traumatic injury in which nerve roots are torn from the spinal cord. Repair using peripheral nerve grafts is a promising restorative surgical treatment in humans, although functional improvement remains limited. To model brachial plexus avulsion in the rat, C8 nerve roots were cut flush to the spinal cord and a peroneal nerve graft was inserted into the lateral spinal cord at the lesion site. Infusion of *Clostridium perfringens* sialidase to the injury site markedly increased the number of spinal axons that grew into the graft (2.6-fold). Chondroitinase ABC, an enzyme that cleaves a different ARI (CSPGs), also enhanced axon outgrowth in this model. In contrast, phosphatidylinositol-specific phospholipase C, which cleaves oligodendrocyte-myelin glycoprotein and Nogo receptors, was without benefit. Molecular therapies targeting sialoglycoconjugates and CSPGs may aid functional recovery after brachial plexus avulsion or other nervous system injuries and diseases.

axon regeneration | brachial plexus injury | chondroitinase ABC | gangliosides | spinal cord injury

The injured CNS is a highly inhibitory environment for axon regeneration, severely limiting functional recovery (1, 2). This limitation is due, in part, to axon regeneration inhibitors (ARIs), specific molecules that accumulate at injury sites. ARIs include myelin-associated glycoprotein (MAG), NogoA, and oligodendrocyte-myelin glycoprotein (OMgp) on residual myelin (3), and chondroitin sulfate proteoglycans (CSPGs) on astrocytes of the glial scar (2). Some of these ARIs bind to complementary receptors on axon growth cones and signal them to halt. Reversing ARI action may enhance axon outgrowth and recovery after CNS injury (4).

In contrast to the CNS environment, peripheral nerve sheathes support axon outgrowth (5), making peripheral–central nerve grafts an appealing therapeutic target for ARI blockers. Enhancement of CNS axon growth into peripheral nerve grafts is expected to translate into enhanced target innervation and function. One therapeutic application of peripheral nerve graft implantation into the CNS is in the treatment of brachial plexus avulsion (6).

Brachial plexus (nerve root) avulsion is characteristic of >70% of all traumatic brachial plexus injuries (7). Often associated with young adults in motor vehicle accidents, recovery is typically poor, resulting in lifelong disability from loss of significant motor and sensory function of the affected limb (8). Upon nerve root avulsion, the microscopic environment of the severed axons within the CNS is highly inhibitory, as evidenced by characteristic terminal retraction balls on axon pathways between the ventral horn and the pia mater (9).

Table 1. Axon regeneration inhibitors

ARI	Source	Axonal receptor	Enzyme modifier
NogoA	Residual myelin	NgR family	PI-PLC
OMgp	Residual myelin	NgR family	PI-PLC
MAG	Residual myelin	NgR family	PI-PLC
MAG	Residual myelin	Sialoglycoconjugates	Sialidase
CSPG	Reactive astrocytes	Unknown	Chondroitinase ABC

Regaining any sensorimotor function after avulsion, once thought to be impossible, was first accomplished by palliative nerve transfers to provide biceps function and shoulder stability (10). Recently, implantation of avulsed spinal nerve roots or peripheral nerve grafts into the spinal cord to bridge the CNS to the peripheral nervous system has led to functional reconnection in patients, providing a method for restorative surgical treatments (6, 11, 12). Although these microsurgical techniques are promising treatments for brachial plexus avulsion injuries, functional improvement is limited (6, 13). If ARI blockers enhance axon outgrowth from the spinal cord into implanted peripheral nerves, a combination of surgical reconnection and pharmacological agents may encourage the wider use of restorative surgical procedures for brachial plexus avulsion injury.

Several ARIs and their axonal receptors have been identified (Table 1), providing potential molecular therapies to reverse ARI action (1, 3, 4). The transmembrane myelin proteins NogoA and MAG and the glycosylphosphatidylinositol (GPI)-anchored myelin protein OMgp are postulated to act by binding to a GPI-anchored family of receptors on axons, the Nogo receptors (NgRs) (14–16). MAG is also a member of the Siglec family of sialic acid-binding lectins (17) and has been proposed to inhibit axon regeneration by binding to axonal sialoglycoconjugates, including gangliosides GD1a and GT1b (18–21). Although the axonal receptor for CSPGs is unknown, its glycosaminoglycan chains are required for inhibiting axon outgrowth (22). These findings provide opportunities to block ARI function by infusing enzymes to injury sites. Chondroitinase ABC digests the glycosaminoglycan chains of CSPGs (22), phosphatidylinositol-specific phospholipase C (PI-PLC) removes NgRs from the axon surface and OMgp from myelin (23, 24), and sialidase destroys the glycan-binding determinant of MAG (18, 21, 25). We report the effect of each of these enzymes on spinal axon outgrowth into peripheral nerve grafts *in vivo*.

Conflict of interest statement: No conflicts declared.

Abbreviations: ARI, axon regeneration inhibitor; CSPG, chondroitin sulfate proteoglycan; MAG, myelin-associated glycoprotein; NgR, Nogo-66 receptor; OMgp, oligodendrocyte-myelin glycoprotein; PI-PLC, phosphatidylinositol-specific phospholipase C.

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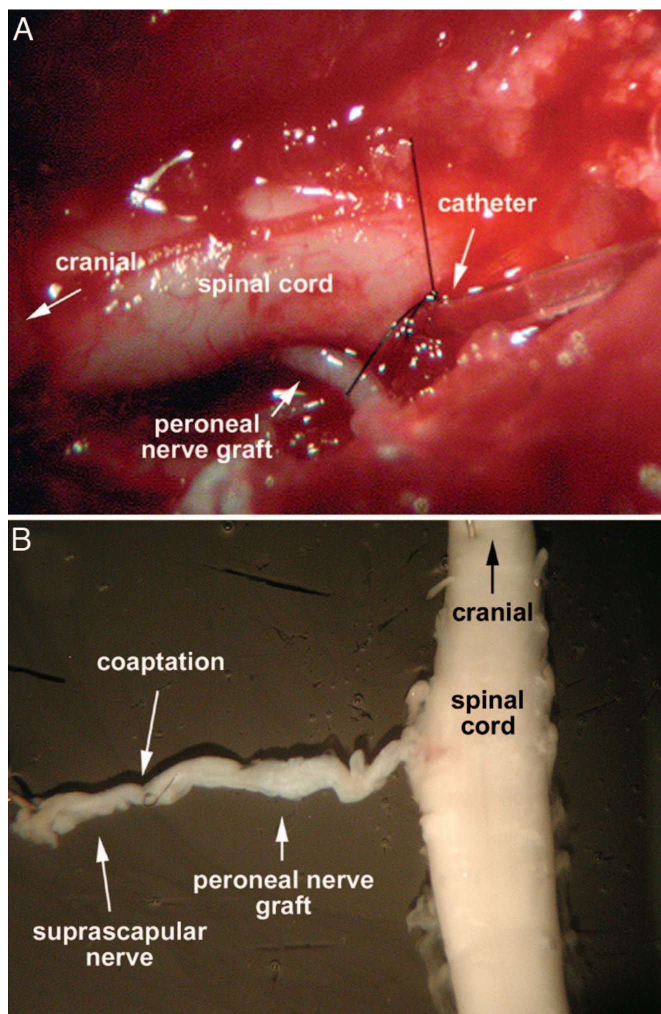

Fig. 1. Rat model of brachial plexus nerve avulsion injury with peripheral nerve graft. (*A*) Surgical preparation before closure. The spinal cord in the region of C8 is revealed, with the peroneal nerve graft extending from its insertion site in the ventrolateral aspect of the spinal cord toward its coaptation with the suprascapular nerve (not visible). A catheter extending from an osmotic pump is anchored, by a suture, to the dura just caudal to the graft insertion site. (*B*) Fixed (demonstration) preparation. After perfusion–fixation of the rat, the spinal cord, peroneal nerve graft, and coapted suprascapular nerve were dissected, allowing visualization of the bridging graft. In experiments using retrograde tracer (data not shown), 4 weeks after implantation the peroneal nerve graft was recut 7 mm distal to its spinal cord insertion site and sealed in a microreservoir of Fluoro-ruby dye.

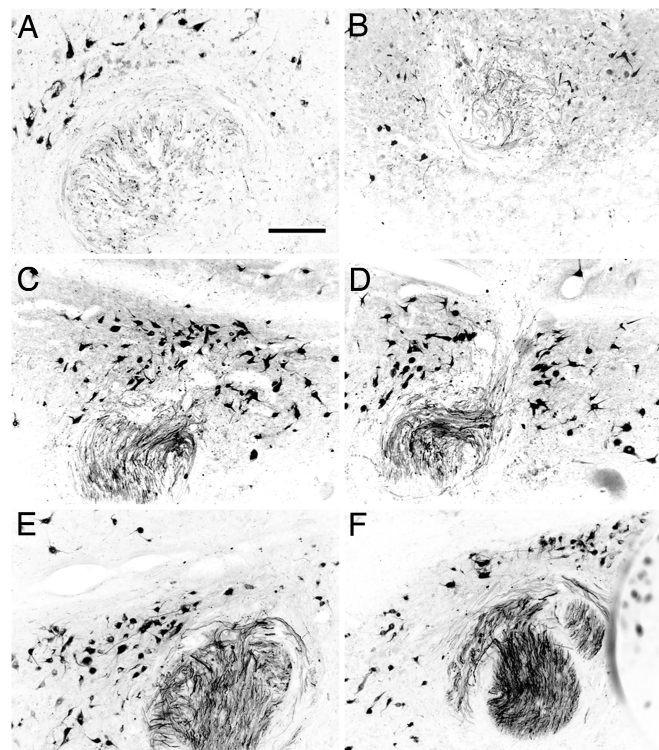

Fig. 2. Spinal neurons retrogradely labeled via a peroneal nerve graft. Horizontal sections of the spinal cord are shown in the area surrounding the peripheral nerve graft. The graft is visible as a roughly circular cross section containing labeled spinal axons, surrounded by retrogradely labeled spinal neurons. Red fluorescent images (indicating Fluoro-ruby retrograde staining) are presented as reverse grayscale for clarity. (*A–F*) Control (saline)-treated animals display some retrogradely labeled neurons (*A*). Animals treated with PI-PLC (*B*) (20 units/ml) appear similar to controls, whereas sialidase (*C* and *D*) (0.38 units/ml) and chondroitinase ABC (*E* and *F*) (0.5 units/ml) display significantly greater numbers of stained axons and spinal neurons. Most of the retrogradely labeled neurons are adjacent to the graft (but see Fig. 4). (Scale bar, 200 μm.)

Results

The C8 brachial plexus was lesioned adjacent to the spinal cord in rats, and an autologous peroneal nerve graft was inserted into the spinal cord at the same site (Fig. 1*A*). This model is analogous to human brachial plexus avulsion injury combined with therapeutic implantation of a peripheral nerve graft into the spinal cord with coaptation of the distal end of the graft to a local (suprascapular) peripheral nerve. Three enzymes that interrupt the actions of different ARIs, PI-PLC, sialidase, and chondroitinase ABC, were delivered to the graft insertion site with a loading dose and then for 14 days via a catheter attached to an osmotic pump. The concentrations of chondroitinase ABC (0.5 and 5.0 units/ml) equal and exceed those effective in prior *in vivo* studies (26–28). The concentrations of PI-PLC (2 and 20 units/ml) and sialidase (0.1 and 0.38 units/ml) equal and exceed those effective *in vitro* (18, 23, 24).

Enzyme efficacy *in vivo* was confirmed by immunohistochemistry (see Fig. 5, which is published as supporting information on the PNAS web site): Sialidase cleaved terminal sialic acids, as evidenced by the loss GT1b and the gain of GM1 immunostaining, PI-PLC released glycosylphosphatidylinositol-anchored proteins, as evidenced by loss of Thy-1 immunostaining, and chondroitinase ABC cleaved CSPG chains, as evidenced by the gain of immunostaining of the lyase product. When infused *in vivo*, the enzymes did not result in overt toxicity based on the behavior of treated animals over the course of the experiment. Histological examination of fixed spinal cords revealed that none of the treatments caused tissue damage except for 5 units/ml chondroitinase ABC, which induced tissue deterioration and was discontinued.

Four weeks after graft implantation, the number of spinal axons extending well into the peroneal nerve graft was determined by retrograde labeling. Uniform, reproducible, and complete retrograde labeling of the peripheral nerve graft was accomplished by transecting the graft 7 mm distal to its insertion into the spinal cord and then immersing and sealing the proximal end into a microreservoir filled with Fluoro-ruby dye. Three days later, rats were killed, and the number of retrogradely labeled spinal neurons was determined microscopically by investigators blind to the treatment group. Labeled neurons, which had extended axons well into the peripheral nerve graft, were primarily observed in the ventral horn near the site of the

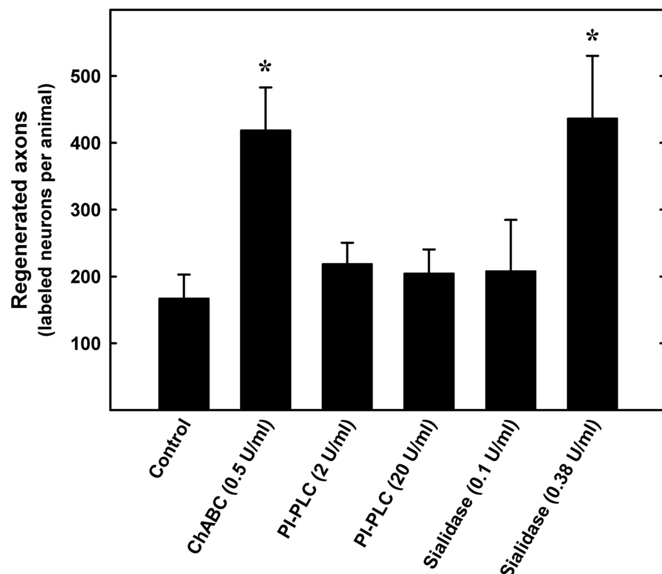

Fig. 3. Quantification of retrogradely labeled spinal neurons in control and enzyme-treated animals. Average total retrograde-labeled neurons in spinal cord sections from animals treated with saline (Control, $n = 12$), chondroitinase ABC (ChABC, $n = 11$), PI-PLC ($n = 6$ at each concentration), and sialidase ($n = 6$ at each concentration) are shown (mean ± SEM). Treatment with chondroitinase ABC (0.5 units/ml) and sialidase (0.38 units/ml) each resulted in significantly greater peripheral nerve graft innervation compared with the saline-treated control (*, $P \leq 0.005$ by Student's t test).

implant (Fig. 2). To rule out staining by leaked dye, control animals were used in which the graft was sutured in place but not inserted into the spinal cord. When these grafts were fitted with microreservoirs, no spinal neurons were stained, increasing confidence that stained spinal neurons were retrogradely labeled only via graft innervation.

Control (saline-treated) animals displayed some innervation of the graft (Fig. 2*A*), consistent with the clinical use of peripheral nerve grafts for brachial plexus avulsion injury, albeit with limited efficacy. Graft innervation in animals treated with PI-PLC (2 or 20 units/ml) was similar to that of controls (Fig. 2*B*). Notably, many more spinal neurons were retrogradely labeled in animals treated with sialidase (0.38 units/ml, Fig. 2 *C* and *D*) or chondroitinase ABC (0.5 units/ml, Fig. 2 *E* and *F*). Quantitative analyses confirmed this conclusion (Fig. 3). ANOVA indicated highly significant differences among the test groups ($P < 0.005$). Comparison of the sialidase-treated to the saline-treated group revealed that innervation increased 2.6-fold ($P = 0.005$). Treatment with chondroitinase ABC (0.5 units/ml) resulted in a similar, 2.5-fold increase in innervation ($P = 0.002$). In contrast, treatment with a lower concentration of sialidase (0.1 units/ml) or with PI-PLC (2 or 20 units/ml) resulted in small increases in innervation that were not significantly different from controls ($P > 0.4$).

In sialidase- (0.38 units/ml) and chondroitinase ABC-treated (0.5 units/ml) animals, most retrogradely labeled neurons were near the graft implant site (Fig. 2 *C*–*F*), suggesting that enhanced axon outgrowth was locally stimulated. However, labeled neurons were also found in the contralateral ventral horn and adjacent to the central canal (Fig. 4 *A* and *B*, arrows).

The total number of labeled neurons in all horizontal sections of grafted animals (averaging ≈170 in control and >400 in sialidase- and chondroitinase ABC-treated animals, Fig. 3) is consistent with the intense staining of axons exiting the spinal cord in the initial graft segment, especially in animals receiving effective treatment (Figs. 2 and 4). We infer that most of the

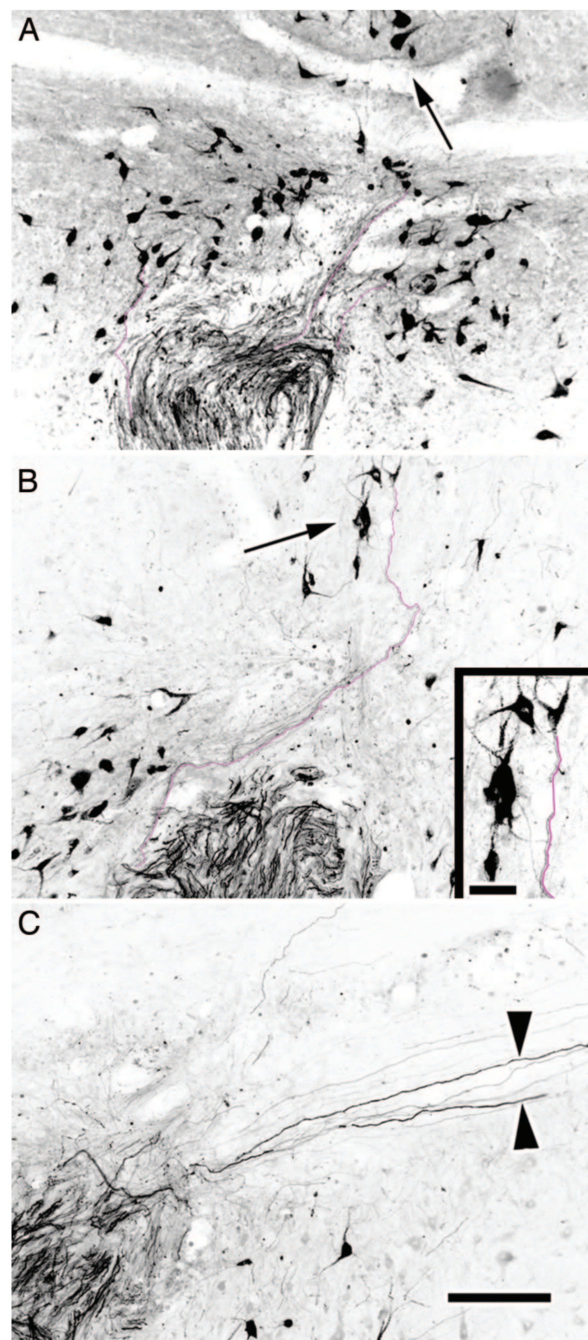

Fig. 4. Innervation of peripheral nerve grafts by remote neurons. Although most of the retrogradely labeled neurons are adjacent to the graft (see Fig. 2), it was not uncommon to find more distant labeled neurons near the central canal or on the contralateral side (*A* and *B*, arrows; *B Inset*). In some sections, the continuity of axons was traced from distant labeled spinal neurons to the graft (*A* and *B*, color-enhanced). Some axons spanned multiple segments in the horizontal sections (*C*, arrowheads). Treatments were: 0.38 units/ml sialidase (*A*) and 0.5 units/ml chondroitinase ABC (*B* and *C*). (Scale bar, 200 μm.) [Scale bar (*Inset*), 50 μm.]

labeled axons exiting at the initial graft segment emanate from cell bodies that reside in horizontal sections dorsal and ventral to the insertion site. Nevertheless, examples of the continuity of labeled axons with their corresponding cell bodies within a single horizontal section were not uncommon (color-highlighted, Fig. 4 *A* and *B*), and occasional labeled axons spanned multiple segments cranial or caudal to the site of implantation (Fig. 4*C*).

Discussion

Delivery of sialidase or chondroitinase ABC to a brachial plexus injury site enhanced innervation of peripheral nerve grafts >2.5-fold compared with animals that received saline alone or PI-PLC. Whereas chondroitinase ABC had been reported to enhance axon regeneration after brain and spinal cord injuries in the rat (26, 27), leading to its evaluation as a therapy for spinal cord injury (28), these data demonstrate a potential therapeutic benefit of sialidase in CNS injury. These findings add to growing evidence that blocking ARIs can enhance axon regeneration *in vivo* and promote recovery after CNS injury (4).

The mechanism by which sialidase enhances axon outgrowth *in vivo* has yet to be established. One possibility is that sialidase destroys axonal receptors for MAG, such as gangliosides GD1a and GT1b (18, 20, 21, 29, 30). The *Clostridium perfringens* sialidase used in this study cleaved the terminal sialic acids from gangliosides at the site of enzyme infusion (see Fig. 5). The data are consistent with the interpretation that enhanced axon outgrowth after sialidase treatment was due to destruction of GD1a and GT1b, resulting in loss of MAG-mediated inhibition. However, *C. perfringens* sialidase acts on gangliosides, sialoglycoproteins, and polysialic acid (31), any one of which might impact axon outgrowth. Furthermore, the product of sialidase action on major brain gangliosides, GM1, may have protective or trophic effects independent of MAG (32). Therefore, additional studies are needed to distinguish which sialoglycans play roles in axon outgrowth and the mechanism of sialidase enhancement of recovery from nerve injury.

Whichever sialoglycoconjugates are involved, axon outgrowth enhancement induced by sialidase is likely to be mechanistically distinct from that induced by chondroitinase ABC. The sugar chains of CSPG are unaffected by sialidase, and sialoglycoconjugates are unaffected by chondroitinase ABC. No evidence implicates a functional link between sialoglycans and CSPGs in the inhibition of axon regeneration. Further experiments will be needed to test whether combining sialidase and chondroitinase ABC will result in yet greater enhancement of axon outgrowth.

The lack of axon outgrowth enhancement by PI-PLC implies that NgR and OMgp are not, by themselves, significant inhibitors of spinal axon outgrowth into peripheral nerve grafts in this model. However, quantitative removal of NgR and OMgp by PI-PLC was not established, leaving the possibility that residual NgR or OMgp were inhibitory. Nevertheless, the data are consistent with experiments in NgR-null mice, in which axon regeneration remains inhibited in the corticospinal tract (33). OMgp-null mice are worthy of further evaluation (34).

The concept that inhibition of axon regeneration in the injured CNS is due to a single ARI (35) or a single ARI receptor (15) are not supported by genetic experiments (33, 36). Although modest or pathway-specific enhancements of axon regeneration have been reported in mice engineered to lack NogoA, MAG, or NgR (37–40), depleting any single ARI or ARI receptor failed to produce robust and widespread axon regeneration. Given these findings, it seems prudent to deplete different ARIs and ARI receptors, individually and in groups, to determine which are most important in each nerve pathway targeted for therapy. Although the current studies used enzymes to delete ARIs and their receptors, other anti-ARI therapies may be effective, including function-blocking antibodies (41), ARI immunization (42), function-blocking peptides (43), and modulators of downstream signaling molecules such as RhoA (44) and cAMP (45).

In the current model, using a peripheral nerve graft as a therapy for nerve root avulsion, sialidase and chondroitinase ABC each provided significant improvement. Avulsion occurs in >70% of brachial plexus injuries (7), and avulsion injury involving the ventral roots has a poor capacity for functional regeneration because of the physical separation of the axons and their nerve sheathes from their corresponding nerve cell bodies within the CNS (9). The mainstay of treatment is surgical and includes palliative surgery, such as nerve or muscle transfers, and restorative surgery, such as the implantation model used in this study (6, 10–13). Two types of spinal cord implantation can restore connections between ventral horn neurons and their peripheral targets: reimplantation of the avulsed roots into the spinal cord and implantation of grafts between the spinal cord and distal nerve stumps or muscles. Reimplantation, however, is not an option if the avulsed nerve roots retract, which commonly occurs during the delay between injury and surgery. Although grafts used in humans have ranged from artificial substrata to freeze-dried muscles, autologous nerve grafts are the most viable (10). Even with this surgical option, reinnervation of muscle does not significantly restore function unless the regenerated axons establish appropriate contacts in a timely fashion, a severe limitation to clinical efficacy because of the inherently slow rate of axon regeneration. Because graft implantation carries its own risks of spinal cord injury, nerve surgeons who perform this procedure have called for optimizing functional outcomes by using neurobiological strategies to improve regeneration (6, 10, 46). The findings reported here, that sialidase and chondroitinase ABC each enhance axon regeneration into peripheral nerve grafts implanted into the spinal cord, provide two potential therapeutic targets to improve regeneration.

Quantitatively, stimulation of axon outgrowth into the peripheral nerve graft by sialidase and chondroitinase ABC appears very robust. Fig. 3 indicates an increase in axons extending into the graft from ≈170 (control) to >400 (sialidase and chondroitinase ABC). In comparison, Jivan *et al.* (47) report that 440 motoneurons were labeled when the transected proximal end of the ventral branch of the C7 spinal nerve was immersed in Fast blue, a very efficient retrograde label. In the same report, retrograde labeling through a peripheral nerve graft (16 weeks after grafting) resulted in 170 labeled neurons.

Spinal cord neurons induced by sialidase and chondroitinase ABC to extend axons into peripheral nerve grafts are not strictly limited to those in the ventral horn adjacent to the graft insertion (Fig. 4). This finding is in agreement with prior studies on the effects of an acute implantation of an avulsed lumbar ventral root into the rat spinal cord, in which a few contralateral neurons reinnervated the implant (48). Likewise, neurons from multiple spinal cord segments have been shown to extend axons into a single reimplanted ventral root (49). The morphology of the retrogradely labeled neurons in the current study is consistent with their identification, predominantly, as motoneurons. However, the effects of sialidase and chondroitinase ABC treatment on different spinal cord neurons needs further study.

The results reported here establish that sialidase enhances axon outgrowth in an *in vivo* animal model. Whether sialidase, alone or in combination with other ARI blockers, will enhance axon outgrowth in other nerve injury models has yet to be determined. The finding that chondroitinase ABC independently enhances axon outgrowth in the same model holds promise that targeting ARIs, especially sialoglycans and CSPGs, will enhance, and thereby justify, restorative surgical treatment for brachial plexus avulsion injuries.

Materials and Methods

Animal care was administered by The Johns Hopkins University (accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International) and met requirements of U.S. federal laws and National Institutes of Health regulations.

Enzymes and Osmotic Pumps. The following enzymes were diluted in sterile Dulbecco's PBS (50), without calcium or magnesium, as follows: PI-PLC from *Bacillus cereus*, 2 or 20 units/ml (P-5542;

Sigma-Aldrich), sialidase from *Clostridium perfringens*, 0.1 or 0.38 units/ml (neuraminidase 480708; Calbiochem), and chondroitinase ABC from *Proteus vulgaris*, 0.5 or 5.0 units/ml (Seikagaku 100332; Associates of Cape Cod). Osmotic pumps (Alzet, 200 μl, 0.5 μl/hr, 14 days; Durect, Cupertino, CA) were equipped with 5 cm of PE-60 tubing secured with 2–0 silk, filled with sterile saline or enzyme solution, and preincubated in sterile saline at 37°C overnight to prime the pump.

Peripheral Nerve (Graft) Harvest. Male Sprague–Dawley rats weighing 200–250 g were subjected to continuous halothane anesthesia after induction. After sterilizing the skin, an incision was made over the line of the peroneal nerve and taken down to reveal the nerve, which was sharply dissected free of the surrounding tissues from the ankle to the takeoff from the sciatic nerve. A 2- to 3-cm segment was harvested and placed in sterile gauze moistened with saline until use.

Preparation of the Graft Site. After sterilizing the skin, a midline posterior cervical incision was made to expose the cervical musculature. The lateral edge of the trapezius and the attachment of the trapezius to the spine of the scapula were also exposed. The attachment of the upper trapezius to the scapular spine was detached, revealing the attachment of the omohyoid to the region of the suprascapular notch. The suprascapular nerve approaching the suprascapular notch was visualized anterior to the supraspinatus muscle, dissected free of the surrounding connective tissues, and transected 1 cm anterior to the suprascapular notch.

Nerve Root Avulsion and Graft Implantation. After cervical laminectomy (C6–T1), the paraspinous muscles were transected in the line of graft implantation. The distal end of the graft was coapted to the recipient suprascapular nerve with 9–0 nylon suture (Fig. 1*B*). The PE-60 tubing from the osmotic pump was then tunneled parallel to the spine under 2 cm of paraspinous musculature and cut so that the end lay immediately caudal to the intended site of graft insertion. The tubing was anchored to the edge of the dura and adjacent muscle with 8–0 nylon so that the opening of the tubing lay intradural adjacent to the left side of the spinal cord (Fig. 1*A*). To model nerve avulsion, C8 dorsal and ventral rootlets were transected at the transitional zone. The proximal end of the peroneal nerve graft was then implanted 1.5 mm into the ventrolateral aspect of the C8 spinal cord by using a fine beveled syringe tip. The epineurium of the graft was secured to the dura with 9–0 nylon. Immediately after graft insertion, 50 μl of saline or enzyme solution (the same solution used to load the osmotic pump), was introduced intradurally to the operative site. The trapezius and paraspinous muscles were reapproximated with 4–0 silk suture and the skin closed with surgical staples.

Retrograde Labeling with Fluoro-Ruby. Four weeks after the initial operation, under continuous halothane anesthesia, rats underwent a second operation for retrograde labeling via the peripheral nerve graft. A 2-cm incision lateral to the prior cervical incision was made and taken sharply through the subcutaneous tissues. The sutures reapproximating the trapezius were cut to reveal the suprascapular nerve and its coaptation to the graft. The graft was traced medially until the suture securing the epineurium of the graft to the dura was visualized (marking the lateral edge of the spinal canal). The graft was transected 7 mm lateral (distal) to the suture, and the newly cut end was inserted into a microreservoir consisting of the 3-mm tip of a heat-sealed 200-μl micropipet tip containing 5 μl of 5% Fluoro-ruby dye (tetramethylrhodamine/lysine dextran, D1817; Invitrogen–Molecular Probes) in sterile water. Approximately 100 μl of Tisseel fibrin sealant (Baxter, Deerfield, IL) was added to seal the top of the reservoir and ensure continuity of the cut distal end of the graft with the enclosed retrograde tracer. The trapezius and paraspinous muscles were reapproximated with 4–0 silk suture and the skin closed with surgical staples.

Perfusion Fixation, Sectioning, and Microscopy. Three days after initiating retrograde labeling, under halothane anesthesia, saturated urethane in water (2 ml) was injected i.p. When the rat lacked spontaneous breathing, transcardial perfusion was initiated with saline and then 4% paraformaldehyde in saline. The spinal cord, in continuity with the peripheral nerve graft, was removed *en bloc* (see Fig. 1*B*) and postfixed in 4% paraformaldehyde overnight, followed by cryoprotection in 30% sucrose for 24 h. Horizontal sections (40 μm) were cut by using a freezing microtome and mounted on glass slides. Fluorescent images were obtained with a Sony charge-coupled device camera attached to a Nikon TE200 fluorescence microscope using rhodamine filters. Retrogradely labeled neurons in all sections were counted; to reduce the likelihood of double-counting, only neurons in which the nucleus was apparent were counted. Counting was performed by investigators blind to the treatment group. Statistical comparisons among groups was by ANOVA and between each enzyme-treated group and the saline control group by Student's *t* test.

Enzyme Efficacy *in Vivo*. Methods and results of experiments testing the efficacy of sialidase, PI-PLC, and chondroitinase ABC *in vivo* are presented in *Supporting Text*, which is published as supporting information on the PNAS web site.

We thank Esther J. Kim for providing technical expertise. This work was supported by National Institute of Neurological Disorders and Stroke Public Health Service Grant NS046669 (to R.L.S.), National Heart, Lung, and Blood Institute Grant HL16315 (to L.P.S.), and Croatian Ministry of Science Grant MZOŠ0219021 (to K.V.).

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