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Axonal Regeneration Induced by Blockade of Glial Inhibitors Coupled with Activation of Intrinsic Neuronal Growth Pathways

Xingxing Wang¹, Omar Hasan¹, Alexander Arzeno¹, Larry I. Benowitz², William B. J. Cafferty¹, and Stephen M. Strittmatter^{1,*}

¹Cellular Neuroscience, Neurodegeneration and Repair Program, Yale University School of Medicine, New Haven, CT USA

²F.M. Kirby Neurobiology Center and Labs for Neuroscience Research in Neurosurgery, Children's Hospital; Harvard Medical School, Boston, MA USA

Abstract

Several pharmacological approaches to promote neural repair and recovery after CNS injury have been identified. Blockade of either astrocyte-derived chondroitin sulfate proteoglycans (CSPGs) or oligodendrocyte-derived NogoReceptor (NgR1) ligands reduces extrinsic inhibition of axonal growth, though combined blockade of these distinct pathways has not been tested. The intrinsic growth potential of adult mammalian neurons can be promoted by several pathways, including pre-conditioning injury for dorsal root ganglion (DRG) neurons and macrophage activation for retinal ganglion cells (RGCs). Singly, pharmacological interventions have restricted efficacy without foreign cells, mechanical scaffolds or viral gene therapy. Here, we examined combinations of pharmacological approaches and assessed the degree of axonal regeneration. After mouse optic nerve crush injury, NgR1^{-/-} neurons regenerate RGC axons as extensively as do zymosan-injected, macrophage-activated WT mice. Synergistic enhancement of regeneration is achieved by combining these interventions in zymosan-injected NgR1^{-/-} mice. In rats with a spinal dorsal column crush injury, a preconditioning peripheral sciatic nerve axotomy, or NgR1(310)ecto-Fc decoy protein treatment or ChondroitinaseABC (ChABC) treatment independently support similar degrees of regeneration by ascending primary afferent fibers into the vicinity of the injury site. Treatment with two of these three interventions does not significantly enhance the degree of axonal regeneration. In contrast, triple therapy combining NgR1 decoy, ChABC and preconditioning, allows axons to regenerate millimeters past the spinal cord injury site. The benefit of a pre-conditioning injury is most robust, but a peripheral nerve injury coincident with, or 3 days after, spinal cord injury also synergizes with NgR1 decoy and ChABC. Thus, maximal axonal regeneration and neural repair is achieved by combining independently effective pharmacological approaches.

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*To whom correspondence should be addressed at Stephen M. Strittmatter, Yale University School of Medicine, Program in Cellular Neuroscience, Neurodegeneration and Repair, P.O. Box 9812, New Haven, CT 06536-0812 USA, stephen.strittmatter@yale.edu.

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DISCLOSURE

Stephen Strittmatter is a co-Founder of Axerion, Inc., which seeks to develop NgR antagonists to improve neurological function after injury.

Keywords

Spinal cord injury; axon regeneration; preconditioning; chondroitin sulfate proteoglycan; nogo; nogo receptor; myelin; optic nerve regeneration

INTRODUCTION

After axotomy in the adult mammalian CNS, regeneration of cut fibers is nil, and hence neurological recovery is highly restricted. Aguayo and colleagues demonstrated that multiple classes of CNS axons are capable of regeneration in an altered environment (Benfey and Aguayo, 1982, David and Aguayo, 1981, Richardson, et al., 1980). Identified inhibitors of axonal growth include products of oligodendrocytes, such as Nogo (Chen, et al., 2000, GrandPre, et al., 2000), MAG (McKerracher, et al., 1994, Mukhopadhyay, et al., 1994), OMgp (Wang, et al., 2002), ephrinB3 (Benson, et al., 2005), Semaphorin 6A (Runker, et al., 2008), RGM (Hata, et al., 2006, Schwab, et al., 2005) and netrin (Low, et al., 2008). In addition, CSPGs from reactive astrocytes and in perineuronal nets are inhibitory for axonal growth (McKeon, et al., 1991, Snow, et al., 1990).

CSPG-mediated axon outgrowth inhibition can be overcome by digesting the sugar side chains on core proteins with the bacterial enzyme Chondroitinase ABC (ChABC). CNS delivery of ChABC improves neurological recovery after spinal cord hemisection, pyramidotomy, or dorsal rhizotomy (Alilain, et al., 2008, Bradbury, et al., 2002, Cafferty, et al., 2008, Cafferty, et al., 2007, Caggiano, et al., 2005, Pizzorusso, et al., 2002, Steinmetz, et al., 2005). Myelin inhibition from Nogo, MAG and OMgp is mediated by receptor complexes including NgR1 (Fournier, et al., 2001) or NgR2 (Venkatesh, et al., 2005) or PirB (Atwal, et al., 2008), and can be blocked by soluble NgR1 decoy (Barton, et al., 2003, Fournier, et al., 2002, Robak, et al., 2009, Zheng, et al., 2005). NgR1(310)ecto-Fc treatment increases axonal growth and recovery after spinal hemisection, spinal contusion, dorsal rhizotomy or stroke injury (Harvey, et al., 2009, Ji, et al., 2005, Lee, et al., 2004, Li, et al., 2004, MacDermid, et al., 2004, Wang, et al., 2006, Wang, et al., 2011). While myelin inhibitor blockade promotes axon growth and sprouting of several motor pathways, no studies have reported improved regeneration of somatosensory tracts ascending long distances in the spinal cord (Harvey, et al., 2009, Oudega, et al., 2000). Furthermore, combination approaches targeting myelin inhibitors and astrocyte-derived proteoglycans have not been combined previously.

Cell-autonomous factors also have a substantial role in determining axonal regeneration. For DRG neurons, a growth program can be initiated by pre-conditioning peripheral nerve injury (Neumann and Woolf, 1999, Richardson and Issa, 1984). The combination of conditioning injury with ChABC is reported to enhance local sprouting in the spinal cord after rhizotomy (Steinmetz, et al., 2005), but has not been combined with myelin inhibitor blockade. For RGCs, intrinsic growth programs are enhanced by macrophage infiltration into the vitreous (Leon, et al., 2000). This process is augmented by zymosan (Yin, et al., 2003) and is mediated by oncomodulin (Yin, et al., 2009, Yin, et al., 2006). Zymosan alone achieves substantial, but not maximal, induction of cell autonomous growth pathways, which is synergistic with cAMP elevation and PTEN gene deletion (Kurimoto, et al., 2010). Neuronal PTEN deletion stimulates considerable regenerative growth (Liu, et al., 2010, Park, et al., 2008), but pharmacological inhibition has action on multiple cell types and more limited benefit (Lai, et al., 2007, Nakashima, et al., 2008). Viral overexpression of dominant negative NgR1 protein partially blocks myelin inhibition and leads to limited optic nerve regeneration that is substantially augmented by zymosan (Fischer, et al., 2004). For cortical neurons, inosine activates an intrinsic growth program and promotes recovery from stroke

and pyramidotomy (Benowitz, et al., 1999, Chen, et al., 2002). The NgR1 antagonist, NEP1-40, blocks a fraction of NgR1-dependent signaling and generates additive sprouting and behavioral recovery from stroke in rat (Zai, et al., 2011). More complete blockade of myelin inhibition has not been examined in combination with a treatment to enhance intrinsic growth potential.

Here, we utilized optic nerve and spinal cord dorsal column crush to assess the synergy between blocking glial inhibitors and promoting neurons' intrinsic growth state. The combination of NgR1 deletion and zymosan supported a degree of optic nerve regeneration greater than either intervention alone. For ascending dorsal column primary afferents, conditioning injury, ChABC or NgR1(310)ecto-Fc were equally effective in promoting axon growth after injury, but triple treatment yields axonal regeneration well past the lesion site. Thus, a combination of targeted interventions without foreign cells, scaffolds or gene therapy can achieve long distance axonal regeneration within the CNS.

MATERIALS AND METHODS

Mouse Optic Nerve Injury

C57BL/6 mice (11-12 weeks, Charles River Laboratories) and NgR1^{-/-} mice backcrossed with C57BL/6 mice for >10 generations (Kim, et al., 2004) were used in this experiment. Mice were anesthetized via intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg) and subsequently placed in a stereotactic head holder. 2% Lidocaine was applied to the surface of the eyeball and the right optic nerve was exposed intraorbitally. The optic nerve was then crushed with a jeweler's forceps (Dumont #5, Fine Science Tools) for 10 sec, 1 mm distal to the optic disc (Leon, et al., 2000). Nerve injury was verified by clearing under visual inspection of the injury site. Care was taken to not disturb the blood supply to the eye, specifically sparing the ophthalmic artery during the procedure.

For animals treated with Zymosan, an intraocular injection was made immediately following optic nerve injury at the posterior aspect of the eyeball using a 5 μ l Hamilton syringe attached to a 33 gauge needle. Three μ l of intravitreal fluid was withdrawn, and then 3 μ l of Zymosan A, a yeast cell wall suspension that is a potent macrophage activator (12.5 μ g/ μ l in normal saline) (Sigma, St. Louis, MO), was injected (Yin, et al., 2003). The Zymosan was preincubated in a 90°C incubator for 10 minutes prior to injection.

Two weeks after injury, animals were deeply anesthetized with an overdose of isoflurane and underwent transcardial perfusion with PBS followed by 4% paraformaldehyde in PBS. The optic nerves and retinas were carefully dissected and placed in 4% paraformaldehyde for post-fixation for 48 hours. They were then transferred to a 30% sucrose solution for cryoprotection, frozen and sectioned using a microtome. Longitudinal sections of 16 μ m thickness of the optic nerves were collected and subsequently immunostained for GAP-43, 1:2500 antibody, followed by a fluorescent tagged donkey anti-sheep IgG (primary antibody prepared in sheep) (Yin, et al., 2003).

Four longitudinal optic nerve sections were selected from each animal. The number of GAP-43 positive axons that crossed transverse lines at 0.5-3.5 mm distal to the injury site was counted. We then measured the cross-sectional width of the nerve where the axons were counted, we converted axon counts into axon crossings per unit nerve width (axons per mm) and obtained the average of these over four sections $\bar{a}_d$. Then the total number of axons extending a distance d in a nerve having a radius of r was estimated by summing over all sections of thickness t , as described (Yin, et al., 2003).

Immunohistological analysis of NgR1 and Nogo-A localization utilized paraformaldehyde fixed section of retina or optic nerve with the following primary antibodies: anti-NgR1(1:1000; R&D Systems), anti-Nogo-A (1:1000, as described (Wang, et al., 2002)) and anti- β III-tubulin(1:1000; Covance) antibodies.

Rat Dorsal Column Crush Injury and Sciatic Nerve Preconditioning

We utilized female Sprague-Dawley rats (11-12 weeks, 250-270 g) in this experiment. In order to evaluate the effect of combining treatment with NgR1(310)ecto-Fc protein, peripheral nerve injury, and Chondroitinase ABC (ChABC) animals were separated into ten different treatment groups (Supplemental Table S1). Animals underwent dorsal crush spinal cord injury at T7 with or without sciatic nerve injury, and were treated intrathecally with either NgR(310) or rat IgG (control). The sciatic injury was created 7 day before the SCI (PCI) or at the time of SCI (D0), or 3 days after SCI (D3). A subset of these animals were also treated with intracerebroventrically infused ChABC thus totaling eight different treatment groups: no sciatic injury and rat IgG, no sciatic injury and NgR(310), no sciatic injury with rat IgG and ChABC, no sciatic injury with NgR(310) and ChABC, PCI sciatic injury with rat IgG, PCI sciatic injury with rat IgG and ChABC, PCI with NgR(310), PCI with NgR(310) and ChABC, D0 with NgR(310) and ChABC, and D7 with NgR(310) and ChABC (Supplemental Table S1).

For sciatic nerve injury separate from SCI, rats were anesthetized by inhalation of isoflurane (5% induction/1-2% maintenance) seven days prior to (PCI) or 3 days after (D3) the spinal cord injury. An incision was made over the left mid thigh. The left sciatic nerve was exposed and transected with full separation of the cut ends at this level.

For spinal cord injury, animals were anesthetized with an intraperitoneal injection of ketamine (60 mg/kg) and xylazine (10 mg/kg). An incision was made over T7 and a laminectomy was performed to expose the spinal surface. Lidocaine (2%) was applied to the exposed spinal surface and a small incision was made in the dura matter. A jeweler's forceps (Dumont #7, Roboz, with 0.5 mm separation of the two tines) was inserted into the spinal cord at a depth of 1.5 mm and held together in order to complete the crush injury. The forceps were held together for 10 seconds before removal. Muscle and skin layers were sutured with 4.0 Vicryl. This dorsal crush injury is intended to completely sever axons in the dorsal columns. The D0 sciatic nerve injury group underwent sciatic nerve transection, as described for the PCI and D3 groups above, immediately after the spinal cord injury during the same surgical session.

To verify the completeness of the dorsal column injury in the PCI+NgR+ChABC group, transverse sections of the brainstem containing the dorsal column nuclei were examined from each rat for the presence of CTB-labelled fibers. No uncut fibers were detected from any of the PCI+NgR+ChABC rats.

Intrathecal and ICV cannulation surgery

After spinal cord injury, osmotic pumps (Alzet 2ML4; 2 ml volume, 2.5 μ l/hr, 28 day delivery) were filled with either 1.2 mg of rat IgG (Sigma) in PBS or 1.2 mg of NgR(310)ecto-Fc protein (Lee, et al., 2004, Li, et al., 2004, Wang, et al., 2006, Wang, et al., 2011). Animals were anesthetized by inhalation of isoflurane (5% induction/1-2% maintenance) and a pump was placed subcutaneously in the lower back. A catheter connected to the outlet of the pump was inserted into the intrathecal space of the spinal cord at the level of T8. Muscle and skin layers were closed with 4.0 Vicryl.

ChABC was delivered via an intracerebroventricular (ICV) cannula. Animals were placed in a stereotactic frame immediately after spinal cord injury while still under anesthesia. An

incision was made in the scalp and the underlying skull exposed. A right-sided burr hole was drilled and a cannula (Alzet brain infusion kit II; Alza Scientific Products) introduced into the right lateral ventricle at coordinates 0.6mm posterior and 1.2 mm lateral to bregma and 4.0 mm deep to the pial surface. The cannula was connected to a 30-mm-long flexible silicon tube. The cannula was glued in place externally between the eyes with cyanoacrylate, and the skin was sutured using 4.0 Vicryl. Immediately after the spinal cord crush lesion, 6 μ l (10U/ml) of high-purity, protease-free ChABC (Seikagaku) was injected into the cannula followed by a 6 μ l saline flush. Control groups received 6 μ l of normal saline instead of ChABC followed by 6 μ l saline flush. ChABC or control solution was delivered on alternate days for 10 days after lesion under isoflurane anesthesia. The total amount of ChABC received by each treated rat was 36 μ l (0.36 Units).

CTB retrograde tracing

Four weeks after SCI, animals were injected with 5 μ l of cholera toxin β -subunit (CTB; 10 mg/ml; List Biological Laboratories, California). The rats were re-anesthetized by isoflurane inhalation (5% induction/1-2% maintenance) and an incision was made approximately over the left mid thigh. The proximal portion of the left sciatic nerve was exposed and injected with CTB utilizing a 10 μ l Hamilton syringe.

Studies in unlesioned rats demonstrated that 4-7 days was sufficient for transport of CTB tracer through the dorsal columns to brainstem gracile nucleus termination (Suppl. Fig. S2A).

Spinal Cord tissue processing and immunostaining

One week after CTB injection (5 weeks post SCI), animals were euthanized via transcardial perfusion. The animals were deeply anesthetized and perfused initially with PBS followed by 4% paraformaldehyde (PFA) in PBS solution. The lesioned spinal cord was dissected from the perfused animals and placed into vials containing 4% PFA. The tissue was post-fixed for 72 hours at 4°C and then transferred to vials containing PBS with 0.01% sodium azide and stored at 4°C until ready to section. The rat spinal cord from 10 mm rostral to the injury site to 10 mm caudal to the injury site was embedded in 10% gelatin, and cut horizontally (40 μ m slices) on a vibrating microtome. Ten serial sections from each animal were pre-incubated for 1 hr in 10% normal donkey serum (Jackson ImmunoResearch) containing 0.3% Triton X-100 (Sigma) and then incubated with goat anti-CTB (1:2000, List Biological Laboratories) overnight at 4°C. After washing, sections were immunostained with donkey anti-goat FITC (1:500, Invitrogen) and anti-GFAP-Cy3 (1:700, Sigma) for 2 hr at room temperature. Sections were mounted onto slides and visualized with either epifluorescent illumination at 5-20X magnification or laser scanning confocal visualization at 40-63X magnification (Carl Zeiss Ltd).

Ten serial horizontal sections from each animal were measured for CTB-labeled fibers at various distances rostral and caudal to the lesion center as described (Bradbury, et al., 1999). The lesion center was defined as the rostral-caudal site with the greatest degree of increased GFAP expression plus cystic cavitation formation. The total number of labeled CTB fibers at 5 mm caudal to the lesion was similar in all groups, averaging 67 ± 15 per rat (mean $\pm$ std. dev.; Suppl. Fig. S2B).

A subset of sections was immunostained for the CSPG degradation product, chondroitin-4-sulfate using the 2B6 antibody (1:1000, Chemicon), or intact CSPG using CS-56 antibody (1:50; Sigma). Some sections were doubled stained for CTB and anti-GAP-43 (1:500; Sigma). All fluorescence reactions were visualized with appropriate secondary antibodies conjugated to AlexaFluor 488 and 568 (1:500; Invitrogen).

Damaged tissue was identified as the combination of increased GFAP expression and cystic cavitation formation. The lesion extent was measured at specific rostral/caudal distances using transverse right/left lines and did not differ between groups (Suppl. Fig. S1). The extent of damaged tissue in the spinal cord was measured in 10 serial anti-GFAP stained horizontal sections from each rat.

RESULTS

Optic nerve regeneration supported by zymosan plus NgR1 deletion

Certain motor tracts, such as the raphespinal system, are capable of axon regeneration when the NgR1 gene is deleted (Kim, et al., 2004). For other tracts, such as corticospinal fibers, collateral sprouting and growth from uninjured fibers occurs in NgR1^{-/-} mice, but frank regeneration of axotomized axons does not occur (Cafferty and Strittmatter, 2006, Kim, et al., 2004, Zheng, et al., 2005). For sensory fibers, the optic nerve provides a homogenous set of unidirectional fibers accessible to crush injury. We examined NgR1 deletion in this system to provide focused assessment of the role of this gene in sensory axon regeneration *per se*. Immunohistochemistry demonstrates that NgR1 is expressed in adult retinal ganglion cells before and after optic nerve crush injury (Fig. 1A), and that Nogo-A is detected in the central segment of the crush optic nerve (Fig. 1B) where it might act to inhibit the growth of NgR1-expressing axons.

Previous optic nerve regeneration work by one of us has demonstrated that virally mediated expression of a dominant-negative NgR1 (dn-NgR1) produces a slight degree of regeneration, and synergizes with intraocular inflammation that enhances the intrinsic growth state of retinal ganglion cells (Fischer, et al., 2004). We hypothesized that NgR1 deletion would block the effects of inhibitory signals more fully than expression of dn-NgR1 in WT mice, which leaves a fraction of RGCs untransfected and all RGCs with intact wild-type NgR1. Indeed, NgR1^{-/-} mice show substantial optic nerve regeneration (Fig. 2A, B, G), and this is as extensive as that elicited by zymosan injection alone (Fig. 2C, G). Regenerating fibers central to the lesion site can be detected by immunostaining for the presence of the growth-associated protein, GAP-43 14 days after axotomy. The number of regenerating fibers at 0.5-3.5 mm central to the crush site for either intervention is significantly greater ($P < 0.01$) than the extremely limited growth in untreated wild type mice (Fig. 2A, G). The deletion of NgR1 expression did not alter retinal ganglion cell survival after axotomy (data not shown). Thus, NgR1 has a direct role in limiting regeneration of adult sensory neurons in a CNS environment.

To examine the interaction between extrinsic and cell-autonomous determinants of axonal regeneration, we administered zymosan to NgR1^{-/-} mice in conjunction with optic nerve crush injury. Optic nerve regeneration is most robust in the dual intervention animals (Fig. 2D). At high magnification, many regenerating fibers are observed greater than 1 mm central to the crush site (Fig. 2E, F). Axonal regeneration is significantly greater ($P < 0.01$) in the zymosan plus NgR1^{-/-} group than in any of the other three groups (Fig. 2G). There is a significant positive interaction between NgR1 disruption and macrophage activation ($P < 0.05$ by two-way ANOVA) especially at sites more than 2 mm distal to the crush site. We conclude that these interventions are synergistic with regard to the regeneration of the retinal ganglion cell axons in adult mice.

Dorsal column axon growth supported by monotherapy with preconditioning, ChABC or NgR1 decoy

Next, we turned our attention to the dorsal column projection. Sensory axons regenerating within the damaged spinal cord are required to negotiate areas of dense astrogliosis.

Previous work had demonstrated synergy between activating the intrinsic growth state of the DRG neuron and digestion of CSPGs in promoting growth of axons through the dorsal root entry zone, the boundary between the peripheral and central nervous system environments (Steinmetz, et al., 2005). The conditioning lesion response is limited in the mouse but robust in the rat (Neumann and Woolf, 1999), so we utilized the latter species for these studies. Due to the use of rats, myelin perturbation was achieved with NgR1(310)ecto-Fc infusion rather than NgR1 genetic disruption. The dorsal column crush model provides the opportunity to study a second type of sensory fiber, and to examine the interaction between intrinsic growth state, myelin inhibition and the astroglial scar.

In order to deliver the NgR decoy or ChABC or both, we utilized two separate cannulae implanted in the CNS (Fig. 3A, B). ChABC protein is relatively unstable and needs to be administered by repeated bolus injections, whereas the NgR decoy is stable and can be loaded into an implanted minipump. To provide repeated access to the CNS for ChABC, we externalized an intracerebroventricular cannula. This approach led to the digestion of CSPGs throughout the neuraxis, as reported previously (Carter, et al., 2008). The 2B6 antibody detected CS degradation products above and below a mid-thoracic dorsal column crush site in the ChABC injected rats (Fig. 3C), and the presence of CSPG detected by CS-56 antibody was reduced after ChABC treatment (Fig. 3D). Higher magnification of the lesion zone demonstrates the extent of local digestion (Fig. 3C, D, right panels). Unilateral conditioning sciatic nerve transections were made one week prior to spinal cord injury, and axonal tracing was achieved by CTB injection 4 weeks later (Fig. 3A). Because the pre-conditioned sensory fibers were both transected peripherally and crushed centrally, we were not able to correlate functional recovery with anatomical evidence of regeneration in this model.

In a first set of comparisons, rats received no intervention or a single intervention plus control treatment (Fig. 4). Histological examination of injured rats with no intervention exhibited the expected pattern of failed regeneration (Fig. 4A). CTB-labeled ascending fibers project from the sciatic nerve rostrally through the dorsal column but terminate several millimeters caudal to the center of the injury site. No fibers are seen at the injury site, which exhibits cavitation and increased GFAP immunoreactivity.

None of the single treatments alter the degree of tissue cavitation or astrogliosis (Fig. 4B-D; Suppl. Fig. S1). As reported, preconditioning (PCI) allows a percentage of axons to grow into the lesion site but fibers do not extend into the intact spinal cord beyond the lesion site (Fig. 4B, 5A). The midpoint of the axon index curve, the site reached by 50% of the axons, is shifted from 2.8 mm caudal to the lesion center to 0.5 mm caudal to the lesion center (Fig. 5A; Suppl. Fig. S3). The length of the longest axon in the PCI group is 0.30 ± 0.10 mm rostral to the injury as compared to 1.20 ± 0.20 mm caudal in the control group.

The phenotype of NgR(310)ecto-Fc or the ChABC group is indistinguishable from the PCI group. In each treatment group, CTB-labeled axons reach into the injury site (Fig. 4C, D). The axon index midpoint is 1.5 mm caudal in the NgR group and 1.0 mm in the ChABC group versus 2.8 mm caudal in the control, and both distributions are significantly rostral-shifted compared to control rats (Fig. 5B, C; Suppl. Fig. S3). The average termination of the longest axon is 0.25 ± 0.20 mm caudal in the NgR group and 0.35 ± 0.30 rostral in the ChABC group (Fig. 5E). These findings demonstrate similar efficacy for PCI, NgR and ChABC treatments in preventing die-back or in promoting the rostral regeneration of ascending sensory axons into a spinal cord injury site. Alone, none of the interventions support axon growth beyond the lesion into the rostral intact spinal cord.

Triple combination therapy provides long distance axonal regeneration

Having established an assay system capable of detecting the pro-regenerative effects of PCI or NgR decoy or ChABC, we sought to test combinations of these interventions. Each pairwise double treatment paradigm was tested in the T7 dorsal column crush injury model. The degree of spinal cord damage reflected by cavitation and GFAP immunoreactivity is indistinguishable between the double-treated rat spinal cord and that observed in untreated rats or in single-treated rats (Figs. 4, 6). Moreover, axonal regeneration, as measured by the rostral growth of the leading axon or the midpoint of the entire population of regenerating axons, is similar in single- and double-treated rats (Fig. 6, 9A-D). Both the single- and double-treated animals show significantly greater ($P<0.01$) axon regeneration than do control untreated SCI rats.

Although the rostral extent of CTB-traced axonal fibers is similar in all of these groups, the PCI plus ChABC group exhibits a different pattern of axonal growth. In this group, there is an increased density of fibers within the lesion proper. This is detectable as an axon fiber index greater than one near the lesion site for the PCI plus ChABC group (Fig. 9B).

In contrast to the double-treatment groups, the triple-treatment rats exhibit a markedly different phenotype. The size and morphologic characteristics of the lesion are again unaltered (Fig. 6E). Axon regeneration is significantly greater in rats treated with PCI plus NgR plus ChABC than in any other group (Fig. 6E, 7, Fig. 9A, $P<0.01$). A subset of CTB-labeled sensory fibers grow many millimeters beyond the rostral termination of the lesion site in multiple different rats (Fig. 7). Camera lucida reconstructions of multiple sections from four different triple-treated animals illustrate the extensive fiber growth into the intact rostral spinal cord (Fig. 8A-D). Such growth is not detected in untreated control animals or in PCI only animals (Fig. 8E-G). The longest fiber in the triple-treated group extends on average 2.30 ± 0.70 mm rostrally beyond the lesion center, in contrast to halting 1.20 ± 0.20 mm caudal in the control group (Fig. 9A). The distribution of axonal regeneration in the triple-treated group shows significant numbers of fibers between one and 5 mm rostral to the injury site, which is not seen in any of the other groups (Fig. 9E). Thus, the combination of PCI plus NgR plus ChABC allows a subset of primary afferent fibers to regenerate several millimeters within the spinal cord extending well beyond the lesion site.

Time window for conditioning effect

Pretreatment with PCI is clearly of limited clinical applicability. To extend the relevance of the PCI plus NgR plus ChABC observations, we examined rats in which the peripheral nerve lesion was created shortly after the dorsal column crush during the same surgical session (D0), or in a separate surgery three days after the spinal cord injury (D3). These post-SCI peripheral conditions were combined with NgR(310)ecto-Fc and ChABC treatment (Fig. 10A). Sensory axon growth was assessed with CTB tracing as for the preconditioned triple therapy above. The length of the longest axon in the D0 and D3 triple treatment groups was compared to PCI triple treatment and the NgR+ChABC without conditioning groups. In the D0 group, the longest axon extends 0.6 ± 0.2 mm rostral to the injury and, in the D3 group, 0.6 ± 0.1 mm, both significantly greater than the -0.05 ± 0.1 mm value for the NgR+ChABC group but significantly less than the 2.3 ± 0.7 mm in the PCI triple condition ($P<0.05$, ANOVA, Fig. 10B). The distribution of all regenerating CTB fibers for the D0 and D3 groups are similar to one another, but extension is significantly more rostral than for the NgR+ChABC group and significantly less rostral than for the PCI triple condition ($P<0.05$, RM-ANOVA, Fig. 10C, D). Images of regenerating CTB-labelled fibers in the D3+NgR+ChABC group illustrate the extensive branching of the regenerating dorsal column fibers (fig. 10E, F). Thus, post-injury triple treatment yields rostral regeneration of sensory axons after spinal dorsal column crush injury.

Characteristics of Regenerating Growth Cones

Peripheral nerve injury is known to induce a group of intrinsic regeneration-associated genes that may promote the ability of axons to regenerate in hostile environments. Amongst these genes is GAP-43 (Skene, 1989). We assessed the morphology and GAP-43 expression of regenerating axons in the D3+NgR+ChABC group as compared to control IgG injured CTB-labeled fibers (Fig. 11). For the D3 group, the morphology of CTB labeled fibers revealed growth cone morphologies with GAP-43 immunoreactivity co-localized near the extending tips (Fig. 11A-F). Not every sensory fiber exhibiting GAP-43 immunoreactivity is CTB-labeled due the partial efficiency of the CTB-labeling procedure. In contrast to the GAP-43-positive growth cone morphology of regenerating fibers in the D3+NgR+ChABC group, non-regenerating CTB-labeled axons in the IgG control group showed no GAP-43 expression and frequent endings with the appearance of retraction balls (Fig. 11G-J). Thus, the D3 conditioning induced the expression and distal localization of at least one protein known to play a role in growth cone motility.

DISCUSSION

Pharmacological therapies targeting single pathways have yielded limited axonal regeneration after spinal cord injury. Combinatorial treatments, where effective, have included the introduction of foreign cells, biomaterials or recombinant viruses. Here, we provide robust evidence that combinations of molecularly defined interventions can support axonal regeneration. In the optic nerve model, both NgR blockade and macrophage activation each enhanced optic nerve regeneration. These actions are independent and synergistic, with substantially greater axon regeneration in mice lacking NgR1 and treated with zymosan. In the spinal cord injury model, PCI, the NgR decoy, and ChABC each supports growth of primary afferent fibers into a lesion site but not beyond, even when any two are combined. However, when all three are combined, injured fibers are able to grow several mm rostrally beyond the site of a spinal cord injury. These data provide evidence that combinations of defined interventions without cell transplantation or biomaterial scaffolds or viral gene therapy can be effective means to achieve long-distance axon regeneration. The length of axon regeneration is at least as great as that achieved with an approach including cell transplantation and recombinant virus injection (Kadoya, et al., 2009). The triple combination has detectable but reduced efficacy when the peripheral conditioning injury occurs after the central injury.

The efficacy of a peripheral conditioning lesion as part of combination therapy decreased, but was not eliminated, when administered at the time of spinal injury as compared to 7 days before. The D0 efficacy of conditioning lesion in the presence of ChABC plus NgR-Fc is consistent with the observation that DRG gene expression induced by peripheral nerve injury are as great after spinal cord injury as before (Blesch, et al., 2011). The experiments here do not assess functional outcomes. The deficit created by the peripheral lesion as well as the central lesion renders any behavioral studies of unclear significance. While the extent of growth with the triple combination treatment is dramatically different from control, it is also far from complete restoration of circuitry. Only a small percentage of fibers regenerate several millimeters past the lesion, and none come close to their physiological targets several centimeters away in the medulla. Nonetheless, combination therapy supporting substantial axon growth may be of functional benefit when new polysynaptic connections can restore function (Courtine, et al., 2008, Raineteau, et al., 2002).

For myelin inhibitors and NgR1, the data extend previous observations in several directions. First, the optic nerve studies demonstrate that the absence of NgR1 is sufficient to elicit significant axon regeneration in this system. In a prior study, infection of RGCs with a virus expressing a dominant-negative form of NgR elicited only minor regeneration after optic

nerve injury in rats, leading the authors to conclude that overcoming inhibitory signals mediated via NgR1 was insufficient to promote optic nerve regeneration (Fischer, et al., 2004). However, RGCs in that study continued to express wild-type NgR1, and therefore the ability of axons to overcome inhibitory signals reflected a competition between wild-type and dominant-negative receptors for ligand binding. Here, in the complete absence of wild-type NgR1, axon regeneration was appreciable. The dorsal column studies provide the first evidence for the role of NgR1 in the increased growth of ascending sensory axons after SCI; previous studies had described enhanced axonal growth in descending motor pathways in the spinal cord (Cafferty and Strittmatter, 2006, Kim, et al., 2004, Lee, et al., 2004, Li, et al., 2004) or sensory fibers at the segmental level (Harvey, et al., 2009). As predicted from their separate modes of action, NgR1 interruption has additive effects with manipulations that increase the intrinsic growth status and that remove astrocyte-derived CSPGs. Our previous study of spinal contusion combined rolipram, a phosphodiesterase inhibitor that elevates cAMP, with NgR1 decoy but found no benefit over NgR1 decoy alone, likely due to the non-significant effect of rolipram alone (Wang, et al., 2006). In a more recent study, we combined the NgR1 antagonist, NEP1-40, with inosine to promote the intrinsic growth state of cortical motor neurons and observed additive effects on axonal growth and recovery after stroke (Zai, et al., 2011). Thus, multiple molecular pathways are synergistic with NgR-targeted interventions.

Although the PCI plus NgR plus ChABC combination for dorsal column crush show evidence for strong synergy in promoting growth beyond the lesion site, none of the double combinations were superior to single treatment in the current spinal cord model. One possible explanation for this result is that single manipulations provide substantial growth into the immediate vicinity of the lesion, and that there is a “ceiling” for greater growth in this region. Growth into more rostral regions of the spinal cord is a greater hurdle that requires all three interventions for this particular class of primary afferents. Since PTEN inhibition appears to enhance neuronal growth propensity more than PCI or zymosan (Kurimoto, et al., 2010, Liu, et al., 2010, Park, et al., 2008), even greater degrees of regeneration may be achieved by substituting pharmacological PTEN inhibition (Lai, et al., 2007, Nakashima, et al., 2008) for zymosan or PCI.

For the optic nerve crush injury, a combination of two interventions is effective in promoting greater regeneration. There are many differences between RGCs in optic nerve crush and DRGs in dorsal column crush, so it does not seem surprising that in one case two treatments synergize whereas in the other three treatments are required for synergy. Amongst the many differences is the fact that zymosan and conditioning peripheral axotomy are quite distinct techniques to enhance the intrinsic growth state. In addition, DRG neurons have a peripheral process that RGCs do not possess, and the central DRG axon injury in the spinal cord is much further from the cell body (nearly 5 cm) than is the optic nerve crush (<1 cm). The cavitation and reactive gliosis of the spinal cord injury are much greater than in the optic nerve crush. Finally, one experiment uses rat (spinal) and the other uses mouse (optic).

Chondroitinase ABC treatment has been shown to have additive effects with peripheral preconditioning. One study demonstrated increased growth at the dorsal root entry zone and at the segmental level in the spinal cord (Steinmetz, et al., 2005). Another showed ChABC enhancement of scaffold plus neurotrophin-supported growth in the spinal cord (Lee, et al., 2010). The present study demonstrates an additive effect of ChABC in long distance axonal regeneration within the spinal cord in the absence of cell transplantation, biomaterial scaffolds or viral gene therapy interventions. Thus, it is clear that ChABC can be synergistic with intervention to enhance neurons' intrinsic growth state. The double treatment, ChABC plus NgR1, did not have detectably different results from the single treatments. As described

above, this may be due to a threshold for growth beyond a lesion that was not met in these experiments, despite the independent action of these pathways.

Neurotrophins have been utilized in a number of published studies to promote neuronal survival, protection from injury, axonal growth, plasticity and neurological recovery through a range of cell-autonomous and non-autonomous actions. There is evidence that neurotrophins support limited neurological recovery after spinal cord or brain trauma (Bradbury, et al., 1999, Bregman, et al., 1997, Hagg, et al., 2005, Mitsui, et al., 2005, Schnell, et al., 1994, Tobias, et al., 2003, Tuszynski, et al., 2003, Ye and Houle, 1997), and neurotrophins augment benefit from several combination treatments (Alto, et al., 2009, Kadoya, et al., 2009). We have not altered neurotrophin signaling in the studies reported here, but there is reason to believe that neurotrophins would enhance the PCI plus NgR plus ChABC combination. For example, separate studies of anti-Nogo antibodies have shown greater benefit with NT-3 administration (Schnell, et al., 1994) and ChABC treatment synergizes with cellular or viral neurotrophin delivery (Lee, et al., 2010, Massey, et al., 2008).

Cellular transplantation, like neurotrophin treatment, may have multiple actions and be synergistic with the therapies utilized here. Amongst the various actions of stem or differentiated cell preparations are the release of neurotrophins, neuroprotection, scaffolding properties, replacement of the endogenous inhibitory environment and remyelination (Thuret, et al., 2006). For spinal cord injuries, head injury and stroke cellular transplantation has little role in replacing neurons lost after injury. Prior studies have shown a synergy between certain cell transplantations and ChABC treatment. Neural stem/progenitor cell migration and axonal growth from endogenous cells is enhanced by ChABC co-treatment (Ikegami, et al., 2005). Axonal growth and recovery can be supported by glial-restricted precursor cells in combination with ChABC and neurotrophins (Karimi-Abdolrezaee, et al., 2010). It is reasonable to speculate that cell transplantation strategies would also be synergistic with blockade of myelin inhibitors.

Most relevant for clinical translation is the combination of molecular interventions with physical therapy and training. Several studies have demonstrated that neurological recovery is enhanced by a combination of training with myelin inhibitors. Stroke recovery is greater with the NgR1 antagonist peptide, NEP1-40, and training, than with either intervention alone (Fang, et al., 2010). Motor deficits after cervical lateral hemisection of the spinal cord are lessened independently on different tasks by task-specific training versus NgR1 deletion (Harel, et al., 2010). However, an anti-Nogo antibody therapy was not synergistic with treadmill training in a partial spinal cord injury model (Maier, et al., 2009). ChABC treatment has been shown to provide a time window in which training enhances performance on specific tasks after spinal cord injury (Garcia-Alias, et al., 2009). Continued analysis of different training paradigms with various molecular interventions will help us better define the bases for interactive benefit.

In conclusion, the current study demonstrates substantial beneficial interactions between intrinsic limits on axonal growth, and inhibition of either astroglial or oligodendroglial derived growth inhibitors. In an optic nerve model, macrophage activation to support intrinsic growth potential coupled with NgR1 gene disruption led to substantial RGC axon growth. In a spinal cord injury model, the triple combination of PCI plus NgR plus ChABC allowed a subset of sensory afferents to regenerate for millimeters beyond a crush injury. Future combinations of independent means to enhance axonal growth with training may further enhance reparative axonal extension.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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HIGHLIGHTS

- > Ascending dorsal column axon regeneration was assessed.
- > Regeneration greater with preconditioning or CSPG digestion or Nogo Receptor blockade.
- > Combination of all 3 allows best regeneration, millimeters past a spinal cord injury.

Figure 1. NgR1 expression in the retina and Nogo-A protein in the injured optic nerve

(A) Transverse section of retina from adult WT or NgR1^{-/-} mice, with or without optic nerve crush injury 14 days previously, were stained with anti-NgR1 and anti-βIII-tubulin antibodies, as indicated. The arrows indicate retinal ganglion cells showing βIII tubulin colocalization with NgR1. Scale bar, 20 μm.

(B) Two weeks after optic nerve crush, the optic nerve was removed, sectioned and stained with anti-NogoA and anti-GFAP antibodies. The eye is the left and the brain to the right, the crush site is indicated by the asterisks. Scale bar, 50 μm.

Figure 2. Optic Nerve Regeneration in NgR1^{-/-} Mice Enhanced by Zymosan

(A-D) Mice of the indicated genotypes underwent optic nerve crush injury at 10-12 weeks of age with or without zymosan injection, and tissue was collected 14 days later for anti-GAP-43 immunostaining of growing fibers. Intact fibers close to the eye are visible at the far left, and regeneration past the lesion is detected in the bottom three panels. Scale bar, 250 μ m.

(E, F) Higher magnification view of the two areas boxed in red from D demonstrates regenerating fibers in NgR1^{-/-} mice treated with zymosan.

(G) The number of regenerating optic nerve fibers is presented as a function of distance central from the crush site and of genotype. Data are mean + sem for 5-11 mice per group.

*, $P < 0.01$, for zymosan and for NgR genotype increase in regeneration by two-way ANOVA with post-hoc Fisher's least-significant difference test;
#, $P < 0.05$ for an interaction between zymosan and NgR genotype, as well as $P < 0.01$ for zymosan and for NgR genotype single factor effects on axon regeneration, SPSS.

Figure 3. Experimental Design for Triple Intervention after Dorsal Column Crush

(A) Time line of experimental manipulation.

(B) Schematic of treatment delivery and lesion sites.

(C) Horizontal sections of spinal cord 4 weeks after dorsal column crush immunostained with the 2B6 antibody to detect the chondroitin-4-sulfate(C-4-S) product of enzyme digestion. Rats were treated with or without ICV delivery of ChABC to degrade CSPG. The micrographs are matched for exposure times and staining conditions. Higher magnification of the boxed area of the lesion is shown to the right.

(D) Horizontal sections of spinal cord 4 weeks after dorsal column crush immunostained with the CS-56 antibody to detect the CSPGs. Rats were treated with or without ICV delivery of ChABC to degrade CSPG. The micrographs are matched for exposure times and staining conditions. Higher magnification of the boxed area of the lesion is shown to the right.

Figure 4. Single Treatments Increase the Rostral Extent of Dorsal Column Sensory Axons
Rats with T7 dorsal column crush injury were untreated or treated singly with PCI or NgR(310)ecto-Fc or ChABC. Ascending fibers from the lumbar DRG were traced by CTB injection in the sciatic nerve. Representative images of horizontal sections of spinal cord illustrate CTB-labeled ascending sensory fibers near the injury site (*). Rostral is to the left. For untreated injured mice (A), all CTB-labeled fibers are caudal to the center of the injury site. In the PCI treated-group (B), the intrathecal NgR(310)ecto-Fc treated-group (C), and the ChABC treated-group (D), CTB-labeled ascending sensory axons grow into the lesion site, but not beyond. The low magnification insets (a-d) illustrate the distribution of CTB (green) relative to anti-GFAP (red) immunoreactivity. The area in the blue boxes from A-D

are magnified in a'-d'. The lesion centers are marked with astericks. Scale bars, 1000 μm in A-D and a-d; 100 μm in a'-d'.

Figure 5. Quantification of Ascending Sensory Fiber Sprouting Into the Injury Site in Single-Treatment Groups

(A-D) CTB-labeled ascending fibers were assessed in 10 serial horizontal sections from each rat. The fiber index at various distances rostral and caudal to the lesion center was calculated by dividing the axons crossing a transverse line at a specific distance by the number of CTB-labeled axons counted 5 mm caudal to the lesion center. For the x -axis, a positive value is rostral to the center of the lesion, and a negative value is caudal to the center of the lesion. For clarity, two-way comparisons are provided in A-C and the same data are replotted together in D.

(E) The site of termination for the most rostral CTB-labeled ascending sensory axon from each rat was measured and averaged for each single treatment group.

All data are mean $\pm$ sem for $n = 8-10$ rats per group. *, $P < 0.01$ for the indicated comparisons by repeated-measures ANOVA, with LSD post-hoc tests (SPSS).

Figure 6. Combination Treatment of Thoracic SCI Increases Regeneration of Ascending Sensory Axons

Rats with T7 dorsal column crush injury were treated with combinations of PCI, NgR(310)ecto-Fc and ChABC and ascending fibers from the lumbar DRG were traced by CTB injection in the sciatic nerve. Representative images of horizontal section of spinal cord illustrate CTB-labeled ascending sensory axons (light in gray scale images) from different combination treatment groups. The low magnification insets illustrate the distribution of CTB (green) relative to anti-GFAP (red) immunoreactivity. Lesions are at the center and rostral is to the left.

(A, B) PCI plus NgR(310)ecto-Fc-treated rats.

(C) PCI plus chABC-treated rat.

(D) chABC plus NgR(310)ecto-Fc -treated rat.

(E) Triple combination of PCI plus NgR(310)ecto-Fc plus chABC. Long distance regeneration of axons is illustrated (arrows).

(A', B') High-magnification images from boxed regions of PCI plus NgR(310)ecto-Fc-treated rats in A and B illustrate CTB-labeled dorsal column ascending sensory axons extending into the lesion site.

The lesion centers are marked with astericks. Scale bars, 500 μm in A, a, E, and 100 μm in A' and B'.

Figure 7. Morphology of Sensory Axons Regenerating Rostral to Thoracic SCI after Triple Treatment

(A-C) Horizontal sections of CTB-labeled regenerating axons from three different rats in the PCI+NgR+ChABC group are illustrated. The boxed regions are shown at higher magnification. Panel B is from the same rat shown at lower magnification in Fig. 6E. (A'-C') High-magnification images from boxed regions from A-C show CTB-labeled dorsal column ascending sensory axons extending into the lesion site. The lesion centers are marked with asterisks. Scale bar in A is 500 μ m for A and C. Scale bar in B is 500 μ m.

Figure 8. Camera Lucida Reconstruction of Regenerating Sensory Axons in Triple-Treatment Rats

Camera lucida drawings are generated from 5 serial horizontal sections with CTB-labeled fibers from separate rats. Gray color represents fibroblast scar and cavitation at the lesion site.

(A-D) Separate PCI plus NgR(310)ecto-Fc plus ChABC rats.

(E) Control IgG rat.

(F-G) Preconditioned (PCI) rats.

Rostral is to the left. Scale bar, 1000 μ m.

Figure 9. Combination Intervention Supports Axon Regeneration past the Lesion Site

CTB-labeled ascending fibers were assessed 5 weeks after T7 dorsal column crush in 10 serial horizontal sections from each rat in the double and triple treatment groups.

(A) The site of termination for the most rostral CTB-labeled ascending sensory axon from each rat was determined and averaged for each combination treatment group. The single-treatment data from Fig. 4 are replotted for comparison to combination treatment.

(B-E) The regenerating fiber index rostral and caudal to the lesion center is presented for the indicated combination treatment groups. Rostral to the lesion center is plotted as a positive value on the x-axis, and caudal as negative. The data from B and D are replotted with an expanded scale in C and E, in order to illustrate the fibers extending rostral to the injury site.

All data are mean $\pm$ sem for n = 8-10 rats per group. *, $P < 0.01$ for the indicated comparisons by repeated measures ANOVA, with LSD post-hoc tests (SPSS).

Figure 10. Conditioning Injury Has Reduced But Detectable Benefit Three Days After Spinal Cord Injury

(A) A schematic of the experiment showing three different time points for sciatic nerve conditioning in combination with NgR(310)ecto-Fc and ChABC treatment.

(B) The site of termination for the most rostral CTB-labeled ascending sensory axon from each rat was determined and averaged for the D0 and D3 treatment groups. The IgG, NgR + ChABC and PCI+NgR+ChABC data from Fig. 9 are replotted for comparison. The indicated comparisons are significantly different; *, $P < 0.05$, ANOVA with LSD post-hoc tests (SPSS). Mean $\pm$ sem with n = rats per group.

(C, D) The regenerating fiber index rostral and caudal to the lesion center is presented for the D0 and D3 treatment groups. The data from C are replotted with an expanded scale in D. *, $P < 0.01$ for the indicated comparisons by repeated measures ANOVA, with LSD post-hoc tests (SPSS). Mean $\pm$ sem with n = rats per group.

(E, F) Photomicrograph showing regeneration one mm rostral (left) to the spinal cord injury in a horizontal section from a D3 triple treatment rat. The boxed region in E is magnified in F. CTB stain is green in E, white in F. Anti-GFAP is red in E. Scale bar in E, 1000 μ m.

Figure 11. Regenerating Axons Have GAP-43 Immunoreactive Growth Cones

Horizontal sections from rats with dorsal column injury in the D3+NgR+ChABC (A-F) or control IgG group (G-J) were immunostained for anti-CTB (green) and anti-GAP-43 (red). The injury occurred between the two large blue arrows in A and G. Rostral is to the left. The regions marked by boxes in A and G are shown at higher magnification in B-F and H-J. Growth cones immunoreactive for both CTB and GAP-43 are indicated by light blue arrows in B-F. Scale bar in A represents 1000 μ m in A, G; scale bar in J is 20 μ m in B-F and H-J.