Glial Cell Line–Derived Neurotrophic Factor and Chondroitinase Promote Axonal Regeneration in a Chronic Denervation Animal Model

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Abstract

Functional recovery following nerve injury declines when target re-innervation is delayed. Currently, no intervention exists to improve outcomes after prolonged denervation. We explored the neuroregenerative effects of glial cell line–derived neurotrophic factor (GDNF) and chondroitinase (CDN) in a chronic denervation animal model. A fibrin-based sustained delivery method for growth factors was optimized *in vitro* and *in vivo*, and then tested in our animal model. GDNF, CDN, and GDNF+CDN were injected into the denervated stump at the time of nerve repair. Histomorphometry and retrograde labeling were used to assess axonal regeneration. The mechanisms promoting such regeneration were explored with immunofluorescence. Five weeks after repair, the GDNF+CDN group had the highest number and maturity of axons. GDNF was noted to preferentially promote axonal maturity, whereas CDN predominantly increased the number of axons. GDNF favored motor neuron regeneration, and upregulated Ki67 in Schwann cells. CDN did not favor motor *versus* sensory regeneration and was noted to cleave inhibitory endoneurial proteoglycans. Early measures of nerve regeneration after delayed repair are improved by activating Schwann cells and breaking down the inhibitory proteoglycans in the distal nerve segment, suggesting a role for GDNF+CDN to be translated for human nerve repairs.

Key Words Glial cell line–derived neurotrophic factor (GDNF) · chondroitinase · chronic denervation · nerve regeneration · Schwann cells

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Introduction

Peripheral nerve injury can lead to dismal functional outcomes when regeneration is sub-optimal. Despite substantial advancements in surgical techniques and in neuro-pathophysiology over the past 50 years, recovery of motor and sensory functions following nerve repair remains poor [1–3]. The consequences of these outcomes (including impaired motor function, chronic pain, dysesthesia, autonomic dysfunction, disability) are oftentimes disastrous and may lead to loss of limb function. This not only results in noticeable declines in patients' quality of lives [4], but also carries dire socioeconomic consequences as most of these injuries affect the military and the working individuals [5].

Many factors determine the success of surgical repair after nerve injury. These include the type and timing of repair, the surgical technique used, the location of the lesion, as well as other elements related to the intrinsic ability of the peripheral axons to regenerate and of the distal nerve segment to support regeneration [6]. Using advanced microsurgical techniques, clinical outcomes after distal nerve injuries are usually satisfactory [2]. However, with more proximal lesions (when axons must regenerate over much longer distances) or when nerve repair is delayed, optimal functional recovery is rarely achieved [7]. This is attributed to states of chronic axotomy in which axons lose their ability to regenerate in the absence of target innervation, but more importantly, to states of chronic denervation, in which Schwann cells in the distal nerve senesce and can no longer sustain regeneration [8, 9]. The underlying causes of this regenerative deficit are likely multiple. They include i) atrophy of Schwann cells and loss of their basal lamina in the distal nerve segment, in part due to a decrease in growth-promoting molecules such as glial cell line–derived neurotrophic factor (GDNF) and ii) increase in molecules that inhibit axonal growth in the endoneurium such as chondroitin sulfate proteoglycans (CSPGs) [10]. In this regard, therapies aiming at providing a controlled release of GDNF to the Schwann cells in the distal nerve while also degrading the proteoglycans that impede axonal growth might carry a potential in improving nerve regeneration after prolonged denervation.

In this paper, we assessed the effects of local delivery of GDNF, chondroitinase (CDN), and a combination of both in promoting axonal regeneration in a chronic denervation animal model. We utilized a preclinical nerve regeneration model, well known to recapitulate the regenerative challenges seen in human nerve repair. We first constructed a fibrin-based growth factor delivery method to provide an optimal and sustained delivery of GDNF. The release kinetics of our delivery method were fine-tuned *in vitro* and *in vivo*. We then tested it in our chronic denervation model. Finally, we investigated the underlying mechanisms through which these interventions promote axonal regeneration in chronic denervation.

Methods

Fibrin Gel Construction and *In Vitro* Release Kinetics Study

Fibrin gels (80 μ l total volume) were built by mixing equal parts of fibrinogen (75–115 mg/ml, 40 μ l) and thrombin (500 IU/ml, 40 μ l) from a Tisseel kit (Baxter Healthcare, Deerfield, IL). Fibrin gels were loaded with GDNF by mixing 5 μ g of GDNF into the thrombin solution before it was reacted with fibrinogen to form a gel.

To test the release of GDNF from fibrin, 80- μ l gels with 5 μ g of GDNF in microcentrifuge tubes were constructed (Fisher Scientific, ON, Canada). The release time course was evaluated by incubating the fibrin gels in 1 ml phosphate-buffered saline (PBS) containing 1% bovine serum albumin at 37 °C under constant gentle agitation by vortex. The PBS was collected and replaced over 15 days, and samples were kept at –20 °C. ELISAs were then performed to measure the quantity of released GDNF.

Animals

Fifty-two 5-week-old to 6-week-old female Sprague-Dawley rats (185–200 g, Charles Rivers Laboratories) were used for this study. Figure 1 illustrates the experimental design. All surgeries (including peri-operative care) followed the protocols of the Johns Hopkins University Animal Care and Use Committee according to the guidelines established by National Institutes of Health and American Association for the Accreditation of Laboratory Animal Care. All animals were kept in a central animal care facility with a 12-h light/12-h dark cycle and provided with food and water ad libitum.

Surgical Procedure

A chronically denervated rat tibial nerve model was used in this study [11] (Fig. 2). At the time of initial surgery, after anesthetizing the animals with inhaled isoflurane and under aseptic conditions, the right tibial nerve was transected immediately distal to the sciatic trifurcation. The distal tibial stump was reflected away from the ligated proximal stump and sutured to the nearby muscle. The transection of the tibial nerve was confirmed by measuring the compound motor action potentials (CMAPs) in the intrinsic foot muscles at the plantar surface using subdermal needle electrodes, before and after the procedure. A PowerLab signal acquisition setup (ADInstruments) was used. Stimulation was done using also subdermal needle electrodes placed near the sciatic nerve at the sciatic notch. A grounding needle electrode was placed in the tail.

Three months later, 32 of the 42 denervated animals were randomized to 4 groups (1, GDNF; 2, CDN; 3, GDNF+CDN; 4, negative control; $n = 8$ per group) for the evaluation of nerve

Fig. 1 Experimental design and animal groups. Alveolar click (‡) symbol indicates that group 6 includes 12 animals (both hindlimbs were tested, hence $n = 24$). 10 of these 12 animals were denervated, and 2 were used as

positive control. Asterisk (*) symbol indicates that the positive control group consists of immediate nerve transfer without denervation. GDNF = glial cell line–derived neurotrophic factor; CDN = chondroitinase

regeneration (by histomorphometry and retrograde labeling). The remaining of the chronically denervated animals were used to test the *in vivo* release kinetics of GDNF and the mechanisms underlying axonal regeneration in the GDNF- and CDN-treated animals. An additional 10 animals were used as positive control, 8 for the histomorphometry/retrograde labeling experiments (group 5, positive control; $n = 8$), and 2 for the immunofluorescent staining (subgroup E, positive control; $n = 4$). In this positive control group, the tibial nerve was transected and cross-sutured immediately to a freshly cut peroneal nerve without the 3-month delay.

The absence of axonal growth into the distal tibial stump in the denervated animals at 3 months (before nerve transfer)

was confirmed by measuring the CMAPs in the foot muscles. The tibial nerve was then exposed, and the connective tissue around the distal stump cleared. All groups (except for the positive control) received 5- μ l injections into the distal tibial nerve using a 33-gauge needle attached to a 10- μ l Hamilton syringe. Group 1 received GDNF embedded in fibrin gel, group 2 received CDN dissolved in PBS, group 3 received a combination of both, and group 4 (negative control) received fibrin gel only; the different injections used in the experiments investigating the *in vivo* release kinetics of GDNF and the mechanism of regeneration in these treated animals are detailed below. The needle was advanced through the dissected stump of the distal tibial nerve until its tip was 5 mm distal to

Fig. 2 Chronic denervation model and therapeutic interventions. (A) At the initial surgery, the tibial nerve is transected, and its distal stump attached to the neighboring muscle to prevent regeneration. (B) After

3 months of denervation, the tibial nerve is cross-sutured to the freshly transected common peroneal nerve and injected with the corresponding solution.

the transection site. After the injection, the needle was kept in place for 2 min before it was slowly withdrawn. The peroneal nerve was then transected distal to the trifurcation, and the proximal end was cross-sutured to the chronically denervated tibial stump using 10-0 sutures. Finally, the thigh muscles were closed with 4-0 absorbable sutures and the skin with 3-0 non-absorbable suture. The animals were allowed to recover, and then returned to animal housing.

Retrograde Labeling of Motor Neurons (Ventral Horn Cells) and Sensory Neurons (Dorsal Root Ganglia)

Five weeks following nerve transfer, the incision was reopened under general anesthesia. The tibial nerve was transected 8 mm distal to the coaptation site. A parafilm patch was placed underneath the nerve stump to protect the underlying tissue from contact with the tracer in case of inadvertent leak during labeling. The proximal nerve stump was then introduced into a silastic tube containing 4% Fluoro-Gold. After a 1-h incubation, the proximal tibial nerve stump was removed from the tube, rinsed with saline, and cleaned with a swab to remove any excess tracer. At the same time as labeling the proximal stump, the distal stump was harvested for histology and nerve histomorphometry. The incision was then sutured, and the animals allowed to recover.

Five days later, the animals were re-anesthetized and perfused through the left ventricle with cold saline followed by 300 ml of 4% paraformaldehyde in 0.1 M PBS (pH 7.40). During dissection, the sciatic nerves were traced back to the spinal cord. The dorsal root ganglia (DRG) were confirmed as being L4, L5, and L6 by counting caudally from the first dorsal roots (C2) of the intact spinal cord. The spinal cord between L1 and S2 and the L4–L6 DRG were harvested. They were postfixed for 24 h in the same fixative at 4 °C, and then transferred into 30% sucrose in 0.1 M PBS. The L4–L6 spinal cord segments and harvested DRG were placed in TissueTek O.C.T. Compound (Saint Louis, MO), and snap-frozen in liquid nitrogen. Serial longitudinal 50- and 20- μ m frozen sections from the spinal cord and DRG, respectively, were cut and mounted on coated slides. O.C.T. was removed from the air-dried sections by rinsing in PBS before the slides were finally mounted with DPX mounting media (Sigma–Aldrich, 06522).

Microscopic Analysis and Stereological Examination

Fluorescent-labeled neurons in the prepared spinal cord and DRG sections were identified using a fluorescent microscope, Zeiss Axio Imager-2 (Carl Zeiss Microimaging LLC, Thornwood, NY) equipped with the corresponding single-band pass filters (Semrock, Rochester, NY). Both transverse plane and rostro-caudal views were obtained.

For an unbiased estimation of their number, labeled neuron populations in spinal cord section and every fifth DRG section for each animal were sampled using the optical dissector method [12]. This method has previously been validated for frozen tissue [13]. To compensate for the problem of uneven distribution of labeled neurons within a spinal cord and DRG tissue, a set sampling path was followed from a random starting point of each section sampled. A neuron was counted only if its nucleus could be clearly identified. Counts were expressed as mean $\pm$ standard deviation (SD).

Histology and Morphometric Evaluation of Nerves

To quantify the number of regenerating axons and assess their maturity, nerve tissue taken 8 mm distal to the coaptation site was studied. After harvest, it was fixed in 2% glutaraldehyde, then postfixed in 1% osmium tetroxide, dehydrated in ethanol, and finally embedded in Araldite 502 (Polysciences, Inc., Warrington, PA). Thin (0.6- μ m) sections were obtained and stained with 1% toluidine blue for examination under light microscopy. The slides were qualitatively evaluated for overall nerve architecture and quality of regenerated fibers. At $\times$ 1000 overall magnification (using Olympus BX51WI), the entire nerve cross section was captured and evaluated with image analysis software (Image J, NIH) using custom-designed plugins, based on a previous methodology [14]. Morphometric measurements obtained from the entire cross section included 1) average axon number (N); 2) average fiber diameter (μ m); 3) fiber diameter distribution; and 4) average myelin sheath thickness (μ m). A minimum of 300 fibers per nerve were randomly sampled and measured. All values of morphometric measures were shown as mean $\pm$ SD.

In Vivo Release Kinetics and Mechanistic Studies

Co-immunofluorescence studies were performed in the 12 animals of group 6 (using both hindlimbs, i.e., $n = 24$) to investigate the *in vivo* release kinetics of GDNF from fibrin gel, as well as the mechanisms through which GDNF and CDN promote axonal regeneration in a chronically denervated environment. There were 5 subgroups: A, GDNF (4 animals, $n = 8$); B, CDN (2 animals, $n = 4$); C, GDNF+CDN (2 animals, $n = 4$); D, negative control (2 animals, $n = 4$); and E, positive control (2 animals, $n = 4$) (Fig. 1). After 3 months of denervation (subgroups A–D), the distal tibial nerve stump was injected with the factor(s) being tested (or with empty gel), and then cross-sutured to a freshly transected common peroneal nerve, as outlined above. Animals were sacrificed at different time points depending on the experimental assay.

For the studies evaluating the mechanisms through which GDNF and CDN contributed to axonal regeneration (2 animals from each group, or $n = 4$), rats were sacrificed 3 days

after nerve transfer. Schwann cell activation and CSPG degradation were assessed in all 5 subgroups.

Schwann cell activation was defined by the expression of Ki67 on S100 cells [15]. Slides were co-stained with anti-S100/anti-Ki67 antibodies. For quantitative analysis, ~200 cells that stained positive for S100 were identified from 6 to 8 random 0.1 mm² microscope fields. Of those cells, the ones that co-stained for Ki67 were counted, and the percentage of Ki67 expression among S100 cells was computed and compared among the 5 subgroups.

CSPG degradation was defined by the presence of the cleaved chondroitin 4-sulfate (C4S) epitope [16]. Slides were co-stained with anti-chondroitin 4-sulfate/anti-TUJ1 antibodies. For quantitative analysis, the fluorescence of Cy5 was measured (in pixels) in 4 random 0.1 mm² microscope fields and compared among the 5 subgroups.

For the *in vivo* release kinetics of GDNF from fibrin gel (involving 3 animals from subgroup A, or $n = 6$), there were 3 endpoints: day 1 ($n = 2$), day 3 ($n = 2$), and day 10 ($n = 2$) after nerve transfer. To note, slides from 1 of the 2 GDNF animals that was sacrificed at day 3 for the mechanistic studies were also used for the day 3 *in vivo* release assessment. Qualitative evaluation of Alexa594 fluorescence (in pixels) was performed to determine the presence of GDNF on days 1, 3, and 10 post-injection.

Whole animal transcardiac perfusion fixation technique was performed as described above, and the common peroneal/tibial nerves were harvested. They were postfixed for 24 h using the same fixative at 4 °C, and then transferred to 30% sucrose in 0.1 M PBS. They were then embedded into O.C.T. medium and snap-frozen in liquid nitrogen. Sections (10 µm) were cut using a cryostat and mounted on coated slides. A list of the antibodies that were used in these experiments is presented in Table 1. Briefly, the sections were initially treated with 0.2% Triton X-100 for 10 min at room temperature (permeabilization step). After rinsing in PBS, the sections were covered with 10% normal goat serum and incubated for 60 min. The slides were then washed in PBS and incubated overnight with the primary antibodies (diluted in PBS containing 5% normal goat serum) in a humidified chamber at 4 °C. Sixteen hours later, sections were washed in PBS and incubated for 1 h with the corresponding secondary antibodies in a humidified chamber at room temperature, and finally mounted using ProLong Gold Antifade Mountant containing DAPI (Life Technologies). Negative control slides were included in each of these experiments, using the exact staining steps but omitting the primary antibody. The nerves were imaged under fluorescent filtration with the use of a Zeiss Axio Imager-2. All images were obtained with matched exposure settings and in a blinded fashion.

Statistics

Statistical analysis was performed using the Kruskal–Wallis one-way analysis of variance by ranks. Provided significant

differences were detected, the Dwass–Steel–Critchlow–Fligner post hoc analysis method was applied for multiple pairwise comparisons. A p value < 0.05 was considered significant. SPSS Statistics version 23 (IBM, Armonk, NY) was used.

Results

In Vitro and *In Vivo* Release of GDNF from Fibrin Gels

In vitro, the dynamic release of GDNF from fibrin gel during the polymerization into PBS (pH 7.4, containing 0.1% bovine serum albumin) was followed for 4 days at 37 °C. Figure 3A demonstrates this release. The data are normalized to the recovered GDNF (free GDNF, 4.5 ± 0.1 µg). A statistically equivalent total amount of GDNF was released from the GDNF fibrin gel. No large initial burst of GDNF release from the fibrin gels was observed, probably due to the high concentration of fibrinogen within the fibrin gels (~50 mg/ml). The system delivered GDNF at levels ≥ 0.1 µg/day for at least 4 days.

In vivo, the repair site was found to be completely enveloped by GDNF-containing gel 1 day post-injection. GDNF remained in place for at least 3 days and was visibly degraded by day 10 (Fig. 3B).

Morphometric Measures of Nerve Regeneration

Five weeks following nerve transfer, cross sections were harvested 8 mm distal to the coaptation site for histological and histomorphometric analysis (Fig. 4A) to assess the extent of nerve regeneration.

Number of myelinated axons (Fig. 4B)

There was a statistically significant difference in the number of myelinated axons between the different groups, $\chi^2(2) = 34.53$, $p = 0.001$, with a mean rank of 36.50 for positive control, 25.5 for GDNF+CDN, 22.50 for CDN, 13.50 for GDNF, and 4.50 for negative control. Pairwise comparisons revealed that the positive control group had the highest number of regenerated axons. Next came both the GDNF+CDN and CDN groups (which were statistically similar), then the GDNF group, and finally the negative control group.

Myelin sheath thickness and fiber diameter (Fig. 4B)

There was a statistically significant difference in the myelin sheath thickness between the different groups, $\chi^2(2) = 32.75$, $p = 0.001$, with a mean rank of 35.81 for positive control, 27.50 for GDNF+CDN, 21.06 for GDNF, 12.50 for CDN, and 5.63 for negative control. Pairwise

Table 1 Immunofluorescence antibodies used for the *in vivo* release kinetics and mechanistic studies

Co-staining combinations		Source	Catalog no./clone	Dilution
Primary	Mouse anti-chondroitin 4-sulfate	Millipore	MAB2030	1:100
Secondary	Cy5 goat anti-mouse	Jackson labs	115-175-166	1:600
Primary	Rabbit anti-TUJ1	Covance	MRB-435P	1:2000
Secondary	Fluorescein goat anti-rabbit	Vector labs	FI-1000	1:800
Primary	Mouse anti-S100	Abcam	ab11179/ab14849	1:500/1:100
Secondary	Alexa488 donkey anti-mouse	Jackson labs	715-545-150	1:200
Primary	Rabbit anti-Ki67	Thermo Scientific	RM-9106-S0	1:400
Secondary	Alexa594 goat anti-rabbit	Jackson labs	711-585-152	1:500
Primary	Rabbit anti-GDNF	PeproTech	500-P81	1:500
Secondary	Alexa594 goat anti-rabbit	Jackson labs	711-585-152	1:500
Primary	Mouse anti-TUJ1	Promega	G7121	1:1000
Secondary	Alexa488 donkey anti-mouse	Jackson labs	715-545-150	1:200

comparisons revealed that the positive control group had the thickest myelin sheath. Then, came both GDNF+CDN and GDNF (which were statistically similar). CDN and negative control followed. Although CDN had a greater myelin thickness than negative control, the difference did not reach a customary level of significance ($p = 0.06$).

As for fiber diameter, there was a statistically significant difference between the different groups, $\chi^2(2) = 31.66$, $p =$

0.001, with a mean rank of 36.13 for positive control, 24.81 for GDNF+CDN, 23.94 for GDNF, 12.81 for CDN, and 4.81 for negative control. Pairwise comparisons revealed that the positive control group had the highest fiber diameter. Next came both GDNF+CDN and GDNF (which were statistically similar). CDN followed, and negative control was last (here, the difference between CDN and negative control was significant).

Fig. 3 *In vitro* (A) and *in vivo* (B) release kinetics of GDNF from fibrin gels. (A) *In vitro* release was followed for 4 days at 37 °C. No large initial burst of GDNF release was noted. Release levels reached ≥ 0.1 $\mu\text{g/day}$ for at least 4 days. (B) *In vivo* release was performed in a chronic denervation

model. Immunofluorescent longitudinal sections of the nerve distal to the repair site at days 1, 3, and 10 following injection of GDNF-loaded fibrin gel are shown. GDNF was present on days 1 and 3, and absent on day 10. Scale bars, 20 μm . Error bars represent standard deviations

Fig. 4 Histomorphometric assessment of nerve regeneration. The panel (A) shows representative toluidine blue cross sections of regenerated nerves taken from each group 5 weeks following nerve transfer. A minimum of 300 fibers per nerve were randomly sampled and measured. Panel (B) displays a quantification analysis of nerve morphometric parameters. Cross sections were analyzed for number of

axons, myelin sheath thickness, and fiber diameter. Panel (C) presents a subgroup analysis of fiber diameters. Statistical comparison was performed using the Kruskal–Wallis test, and pairwise comparison using the Dwass–Steel–Critchlow–Fligner method. Mean $\pm$ SD are shown; error bars represent standard deviations. * $p < 0.05$; alveolar click (†) symbol indicates approaching significance. Scale bars, 10 μ m

Fiber diameter distribution (Fig. 4C)

Subgroup analysis was performed to examine the distribution of fiber diameter in the 5 groups (positive control, GDNF+CDN, GDNF, CDN, negative control). Fiber diameter subgroups were defined as smallest ($< 1 \mu$ m), small (1–2 μ m), medium (2–3 μ m), large (3–4 μ m), and largest ($> 4 \mu$ m) diameter.

All 5 groups showed a statistically significant difference in the proportion of fibers of different sizes. $\chi^2(2)$ values were 33.14 (positive control), 34.52 (GDNF+CDN), 35.17

(GDNF), 29.54 (CDN), and 31.15 (negative control); all p -values were less than 0.001. Pairwise comparisons within each group revealed the following:

The distribution pattern of fiber diameter is similar in the untreated groups (positive and negative control). In these untreated groups, the proportion of medium and large fibers (as well as that of the smallest and small fibers) is similar. In the GDNF-treated groups, however (both GDNF+CDN and GDNF groups), the proportion of large fibers is significantly higher than that of medium fibers. This pattern is not observed in the CDN-treated group, in which the proportion of medium

and large fibers is still similar. This observation suggests that GDNF, in the setting of chronic denervation, potentiates the regeneration of large mature fibers.

On the other hand, in all treated groups (GDNF+CDN, GDNF, and CDN), the overall proportion of medium and large fibers is higher than that of negative control. This shows that the higher number of axons noted in the treated groups consists principally of mature fibers.

It is worth noting here that, within the “> 4 μm (largest)” category, the majority of the fibers in the positive control group (66.49%) have a diameter of 7–8 μm , whereas the majority of the fibers in the other groups have a diameter of 4–4.5 μm (98.62% in GDNF+CDN; 98.23% in GDNF; 99% in CDN and 100% in the negative control group). This explains why the positive control group has the highest average of fiber diameter (Supplemental Figure 1).

Retrograde Labeling of Neurons Following Nerve Transfer

To quantify the percentage of motor and sensory neurons that regenerated their axons in response to the injected growth factors, Fluoro-Gold retrograde dye was applied to regenerated axons 8 mm distally from the coaptation site 5 weeks after nerve transfer. Labeled cells were characterized by red–yellow fluorescence. In the majority of cases, identification of neuronal nuclei was possible due to the bigger size of neuronal than glial cell nuclei and typical distribution of glial cells around the neurons. However, difficulties were encountered in performing unbiased identification of neuronal nuclei in areas with a high density of labeled cells. The histograms in Fig. 5 (bottom panel) display the raw motor and sensory neuron counts along with the proportion of neurons that regenerated their axons in each group (assuming positive control achieved 100% regeneration).

Motor Neurons

There was a statistically significant difference between the number of motor neurons that regenerated their axons among the different groups ($\chi^2(2) = 34.44$, $p = 0.001$), with a mean rank of 36.50 for positive control, 26.38 for GDNF+CDN, 22.63 for GDNF, 10.13 for CDN, and 6.88 for negative control. Pairwise comparisons revealed significant differences between positive control and GDNF+CDN, between GDNF and CDN, and between GDNF and negative control. GDNF+CDN was similar to GDNF. CDN and negative control were also similar.

Sensory Neurons

There was a statistically significant difference between the number of sensory neurons that regenerated their axons by

different groups ($\chi^2(2) = 20.24$, $p = 0.001$), with a mean rank of 36.50 for positive control, 20.69 for GDNF+CDN, 16.25 for GDNF, 14.13 for CDN, and 14.94 for negative control. Pairwise comparisons revealed a significant difference between positive control and GDNF+CDN. All other groups were similar.

Mechanisms Underlying GDNF and CDNs Induced Axonal Regeneration

The injection of GDNF-loaded fibrin gels in chronically denervated tibial segments was found to stimulate Schwann cells to upregulate the proliferation marker Ki67. The proportion of S100 cells expressing Ki67 increased from 12.94% $\pm$ 0.97 in the negative control group to 28.62% $\pm$ 1.71 in the GDNF group ($p < 0.05$), and to 27.57% $\pm$ 1.71 in the GDNF+CDN group ($p < 0.05$). This suggests enhanced activation of Schwann cells as a mechanism that supported and sustained axonal growth in chronic denervation (Fig. 5, top panel). To note, the proportion of S100 cells expressing Ki67 following CDN injection (11.82% $\pm$ 1.05) was similar to that of negative control. This implies that CDN injection had no effect on Schwann cell activation (Supplemental Figure 2).

CDN injection was found to result in positive staining for the cleaved chondroitin sulfate epitope C4S. The fluorescence of C4S increased from 99.68 $\pm$ 8.45 pixels in the negative control group to 349.88 $\pm$ 18.40 pixels in the CDN-treated group ($p < 0.01$), and to 353.26 $\pm$ 20.58 pixels in the GDNF+CDN group ($p < 0.01$). This confirms digestion of chondroitin sulfate side chains by chondroitinase, and subsequent loss of the growth inhibitory endoneurial molecules. This reduction in growth inhibitory compounds may be a mechanism for improved axonal growth in chronic denervation (Fig. 5, top panel). To note, C4S staining was not noted neither in the GDNF-treated group, 99.73 $\pm$ 9.83 pixels, nor in the positive control group, 97.34 $\pm$ 8.21 pixels (Supplemental Figure 3). This implies that GDNF treatment has no effect on CSPG degradation; these are digested following CDN injection.

Discussion

Peripheral nerve injury continues to pose unique challenges to both clinicians and patients. Whenever the injury occurs distally and is followed by immediate repair, axonal regeneration is still possible as the integrity of the Schwann cells' basal lamina is maintained. These cells provide the axonal cone with growth-promoting substrates and shield it from the growth inhibitory extracellular matrix components (namely CSPGs) [17].

However, in the clinically relevant situation of delayed nerve repair, the Schwann tubes are not intact anymore as they

Fig. 5 Five weeks following nerve transfer, retrograde labeling of neurons regenerating their axons was performed 8 mm distal to the coaptation site. Sensory neurons in the dorsal root ganglia (1; 20- μ m sections, every fifth section counted) and ventral horn cells in the spinal cord (2; 50- μ m sections, all sections counted) were counted to assess motor and sensory neuron regeneration, respectively. The distal stump (3) was sent for nerve histomorphometry. In addition (4), immunofluorescent staining was performed to understand the mechanisms through which GDNF and CDN promote axonal growth. The injection of GDNF increased the percentage of S100 cells co-expressing Ki67 almost twofold

(from $12.94\% \pm 0.97$ in the negative control to $28.62\% \pm 1.71$ in the GDNF group, and to $27.57\% \pm 1.54$ in the GDNF+CDN group). The injection of CDN resulted in the degradation of CSPGs as evidenced by an increase in the fluorescence of C4S (from 99.68 ± 8.45 pixels in the negative control to 349.88 ± 18.40 pixels in the CDN group and to 353.26 ± 20.58 pixels in the GDNF+CDN group). Statistical comparison was performed using the Kruskal–Wallis test, and pairwise comparison using the Dwass–Steel–Critchlow–Fligner method. Mean $\pm$ SD are shown; error bars represent standard deviations. * $p < 0.05$

shrink by 80–90% in the first 3 months [18, 19]. The regenerating axons are thus exposed to the growth-inhibiting

molecules of the extracellular matrix. There is also less growth-promoting molecules in this milieu. Such an

environment does not sustain effective nerve regeneration and may result in severe functional disabilities [2, 10].

In this regard, a modality that reactivates Schwann cells in the denervated segment to stimulate their secretion of neurotrophic factors might enhance the capacity for axonal regeneration following delayed nerve repair. To achieve optimal outcomes, this modality must also degrade the inhibitory molecules within the denervated segment to further augment axonal elongation.

In this paper, we developed a fibrin-based delivery method that releases a sustained and constant amount of GDNF. GDNF is one of the most prominent neurotrophic factors playing a role in the survival of motor neurons and their regeneration [20–23]. The effects of this modality on axonal regeneration were tested in a chronic denervation animal model. Along with GDNF, we also investigated the impact of CDN therapy on axonal growth. CDN is a glycosidase that deactivates CSPGs, which are known to be major inhibitory regulators of axonal regeneration after nerve injury [24, 25]. In our study, we chose a chronic denervation rat animal model, as this model accounts for the prolonged periods that are required for human nerve regeneration. It thus replicates similar changes in the human denervated nerve segments, reproducing the challenges to human nerve regeneration [26]. This makes our model favorable for testing translational therapeutic advances.

To our knowledge, this study is the first to examine the therapeutic potential of GDNF, CDN, and a combination of both in the setting of chronic denervation. In an acute denervation model, GDNF expression is known to upregulate in the distal stump 48 h after injury. This is to promote neurite outgrowth. However, as the duration of denervation is increased, GDNF expression is lost. This is mainly due to Schwann cell senescence and their inability to secrete GDNF [27]. Such changes in GDNF levels may explain the poor axonal regeneration seen in chronic denervation. Thus, we hypothesized that treating the distal stump with a modality that releases a sustained amount of GDNF will activate these senescent Schwann cells and stimulate them to secrete the necessary growth-promoting molecules that sustain axonal growth. Our data supported this hypothesis. We found that the GDNF-treated groups exhibited a higher number and maturity of regenerated axons. This was accompanied by a nearly two-fold increase in the expression of Ki67 on Schwann cells suggesting that their activation might have mediated the positive effects of GDNF.

There have been mixed reports with regard to whether a continuous supply of GDNF is required to elicit a significant neurotrophic response [28, 29], or if similar results can be obtained after a single dose [30, 31]. The consensus from these studies is that a single dose is enough to improve axonal regeneration, but a more prolonged release (achieved mainly with mini-osmotic pumps) leads to a greater benefit. Implanted osmotic pumps and infusion catheters, however,

whereas suitable for experimental analysis, are unlikely to be adopted for clinical use in human nerve repair. In our study, we decided on a compromise to use a fibrin-based system for delivery of GDNF over 4 days, as this method carries a greater translational potential in nerve repair (as compared with implanted catheters).

Axonal regeneration is characterized by a complex interaction of growth-promoting and inhibitory molecules. In order to achieve optimal regenerative outcomes, we chose to supplement our GDNF treatment with another therapy that works through a different mechanism (degradation of growth inhibitory molecules, CSPGs). Although the presence and role of CSPGs in the CNS have been substantially studied [32], their impact on the regeneration of the acutely injured peripheral nerve is the subject of a considerable amount of research. Following acute peripheral nerve transection, neurite outgrowth inhibitory molecules (such as CSPGs) are upregulated within the distal stump [33, 34]. The growth inhibitory influence of CSPGs becomes detrimental as neural tissue attempts to regenerate [35]. Using the enzyme CDN, the inhibitory properties of CSPGs can be overcome. This was demonstrated both *in vitro* [36, 37] and *in vivo* [38–41] (in acute injury models). In these animal studies, a single injection of CDN into the distal stump at the time of nerve repair resulted in the digestion of CSPGs and a marked elevation of axonal growth. CDN therapy was however not investigated in a chronically denervated nerve stump. Because it is known that the gene expression pattern in delayed repair of rat sciatic nerves (as compared with immediate repair) is characterized by a noticeable increase in the expression of phosphacan and, to a lesser extent, versican (phosphacan and versican being CSPGs) [42], we hypothesized that CDN therapy would also promote axonal growth in the setting of chronic denervation. Our results supported this hypothesis. CDN therapy resulted in a significant increase in the number of regenerated axons. These effects were suggested to be mediated by the digestion of the growth inhibitory CSPGs, as demonstrated by the significant increase in their degradation product (C4S).

Although our model involved the deposition of a biomaterial loaded with GDNF directly into the denervated distal stump, it is worth noting that there are other nerve repair techniques that involve different modalities for local delivery of neurotrophic proteins. One of these is the use of synthetic conduits loaded with growth factors. This modality has been largely used in acute injury models, and rarely in chronic denervation. Barras et al. used it in chronically denervated rat facial nerves. In their study, they combined a nerve graft with a GDNF-releasing synthetic conduit (made from nonresorbable ethylene vinyl acetate) to repair the facial nerve after a 7-month period of delay. The GDNF-releasing channel was placed between the graft and the denervated distal stump. This modality resulted in an increase in the number and maturation of nerve fibers (as compared with nerve graft alone,

and to nerve graft with an empty channel) [43]. These results underline the critical importance of Schwann cell activation in the denervated distal stump to support axonal regeneration. Notwithstanding the possible differences between facial and sciatic nerve regeneration, we speculate that a useful synthetic conduit for a chronic denervation model would also address the other challenges seen in chronic denervation. Besides distal stump Schwann cell senescence, these also include the accumulation of growth inhibitory molecules. Thus, the conduit would provide the necessary factors (e.g., GDNF and CDN) to the denervated distal stump to overcome these challenges. In addition, refinements can be introduced in the fabrication of the conduit itself (fiber pore and size, etc.) to modulate the inflammatory response at the repair site towards a regenerative phenotype, as we have previously shown in an acute injury model [44]. Such bioactive synthetic scaffolds loaded with growth factors might hold promising avenues in chronic denervation models.

Our data suggest that GDNF is mainly contributing to fiber maturity (by enhancing fiber diameter and myelin sheath thickness), and that CDN is primarily promoting the number of regenerated axons (as evidenced by an increased axonal count). When used together, both modalities worked synergistically to enhance the number of regenerated axons and their maturity (i.e., myelin sheath thickness and diameter). Our hypothesis is that the GDNF-activated Schwann cells produced regeneration-promoting factors (like nerve growth factor (NGF), brain-derived growth factor (BDNF), GDNF [45–47]), and aligned to regular structures (Bands of Büngner) which stimulated axonal regeneration. Equally important, CDN shielded these regenerating axons from the endoneurial growth inhibitory molecules, CSPGs, and allowed them to reach the endoneurial tubes. Altogether, GDNF and CDN helped to establish a regeneration-permissive environment for the growing axons. Our findings show that this combination carries the potential to be translated for use in human nerve repair. Moreover, they also shed light on some of the basic changes that occur in the distal portion of nerves after a period of denervation, and that hinder regeneration.

It is important to mention that one of the limitations of our study is the lack of functional analysis. Our 5-week endpoint was not long enough for the regenerating axons to reach their target muscle; hence, we were unable to assess the sciatic function index and could not perform nerve conduction studies. Based on our data, we would anticipate a higher compound muscle action potential amplitude (i.e., increased number of axons connected to muscle fibers) in the CDN-treated groups, and a shorter latency (i.e., higher number of mature fibers reaching the muscle target) in the GDNF-treated groups. A longer-term endpoint (14–16 weeks) that allows a comprehensive assessment of functional recovery is essential to further support our conclusions.

Conclusion

In summary, early measures of nerve regeneration after delayed nerve repair are best improved by simultaneously targeting axonal growth (using GDNF) and degrading inhibitory molecules in the distal stump (using CDN).

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Required Author Forms Disclosure forms provided by the authors are available with the online version of this article.

Compliance with Ethical Standards

All surgeries (including peri-operative care) followed the protocols of the Johns Hopkins University Animal Care and Use Committee according to the guidelines established by National Institutes of Health and American Association for the Accreditation of Laboratory Animal Care.

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