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Adjuvant neurotrophic factors in peripheral nerve repair with chondroitin sulfate proteoglycan-reduced acellular nerve allografts

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Abstract

Background—Acellular nerve allografts are now standard tools in peripheral nerve repair due to decreased donor site morbidity and operative time savings. Preparation of nerve allografts involves several steps of decellularization and modification of extracellular matrix to remove chondroitin sulfate proteoglycans (CSPGs), which have been shown to inhibit neurite outgrowth through a poorly understood mechanism involving RhoA and ECM-integrin interactions. Chondroitinase ABC (ChABC) is an enzyme that degrades CSPG molecules and has been shown to promote neurite outgrowth following injury of the central and peripheral nervous systems. Variable results following chondroitinase ABC treatment make it difficult to predict the effects of this drug in human nerve allografts, especially in the presence of native extracellular signaling molecules. Several studies have shown cross-talk between neurotrophic factor and CSPG signaling pathways, but their interaction remains poorly understood. In this study, we examined the adjuvant effects of nerve growth factor (NGF) and glial cell line-derived neurotrophic factor

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(GDNF) on neurite outgrowth post-injury in CSPG-reduced substrates and acellular nerve allografts.

Materials and Methods—E12 chicken DRG explants were cultured in medium containing ChABC, ChABC + NGF, ChABC + GDNF or control media. Explants were imaged at 3 d and neurite outgrowths measured. The rat sciatic nerve injury model involved a 1-cm sciatic nerve gap that was microsurgically repaired with ChABC pre-treated acellular nerve allografts. Prior to implantation, nerve allografts were incubated in NGF, GDNF or sterile water. Nerve histology was evaluated at 5d and 8wk post-injury.

Results—The addition of GDNF *in vitro* produced significant increase in sensory neurite length at 3 d compared to ChABC alone ($P < 0.01$), while NGF was not significantly different from control. *In vivo* adjuvant NGF produced increases in total myelinated axon count ($P < 0.005$) and motor axon count ($P < 0.01$), while significantly reducing IB4+ nociceptor axon count ($P < 0.01$). There were no significant differences produced by *in vivo* adjuvant GDNF.

Conclusions—This study provides initial evidence that CSPG-reduced nerve grafts may disinhibit the pro-survival effects of NGF *in vivo*, promoting motor axon outgrowth and reducing regeneration of specific nociceptive neurons. Our results support further investigation of adjuvant NGF therapy in CSPG-reduced acellular nerve grafts.

Keywords

Chondroitinase; NGF; GDNF; Neurotrophic; Nerve Repair; CSPG; Chondroitin; Axotomy; Sciatic; P75

Introduction

The peripheral nervous system has an impressive resilience to injury, in part, due to the ability of peripheral neurons to regenerate axons following injury. After transection, proximal segments of injured axons form growth cones that respond to neurotrophic and chemotropic extracellular signals to direct neurite outgrowth. Supportive endoneurial cells, such as Schwann cells and fibroblasts, secrete growth factors that participate in complex signaling pathways with regenerating nerve fibers, and ultimately promote either neuron survival or cell death. Extracellular matrix components also provide growth cues, either stimulatory or inhibitory, in addition to serving as a guiding substrate for nerve regeneration. For example, chondroitin sulfate proteoglycans (CSPGs) are well known inhibitors of neurite elongation and their degradation by the bacterial enzyme chondroitinase ABC has been studied extensively as a potential treatment for spinal cord injury.(1)

Theoretically, all peripheral nerve injuries with intact spinal roots and ganglia have the potential for near-complete functional recovery. However, in high-grade nerve injuries this is rarely the case due to several impeding conditions – aberrant neurite growth and neuroma formation, physical barriers to innervation, anterograde degeneration and insufficient rates of neurite outgrowth. The rate of neurite outgrowth at only 1-2 mm/d is an important determinant of functional outcomes, because proximal injuries have an extended period of target muscle denervation and increased muscle atrophy.(2)

There are numerous reports on the topic of enhanced peripheral nerve regeneration using various experimental therapeutics, nerve grafts and nerve conduits. Experimental pharmacotherapeutics have mainly targeted the neurotrophic signaling pathways, using recombinant human neurotrophic factors, such as NGF, BDNF and GDNF.(3, 4) Additionally, significant progress has been made toward the development of biomimetic nerve scaffolds, using materials such as poly-L-lactic acid, electrospun nanofibers and Schwann cell seeded poly-ε-caprolactone.(5-7) Total regenerating axon counts has been the primary outcome for most of these studies, reflecting changes in the regenerative potential of all neurons in the injured nerve. Less effort has been made to understand the specific characteristics of the nerve fibers that are targeted by these regenerative therapeutics, or the molecular events involved in promoting axon elongation and survival *in vivo*.

Neurotrophic factors, such as nerve growth factor (NGF) and glial cell line-derived neurotrophic factor (GDNF), have well characterized individual signaling pathways *in vitro*. NGF has two primary receptors – the p75 low affinity neurotrophic factor receptor and TrkA high affinity tyrosine kinase receptor. P75^{NTR} binds a wide range of neurotrophins and is known to play a major role in apoptotic signaling through activation of JNK and caspase pathways.(8) While TrkA is the primary NGF pro-survival receptor, p75^{NTR} has also been found to activate the MAPK pathway and the NF-κB transcription factor, resulting in anti-apoptotic effects.(9, 10) In addition to promoting survival of sensory neurons *in vitro*, NGF has been shown to protect injured neurons from apoptosis *in vivo* in transected rat sciatic nerves.(11, 12) In contrast, GDNF binds to GFRα1 and GFRα2, which both interact with the RET tyrosine kinase receptor to activate multiple pro-survival, intracellular signaling pathways.(13, 14) In addition to promoting sensory and motor neuron survival, GDNF has been found to be protective against injury-induced degeneration of motor neurons *in vivo*.(15-17)

Chondroitin sulfate proteoglycans (CSPG) are commonly discussed in the context of spinal cord injury and CNS plasticity, but they are also highly expressed in the extracellular matrix (ECM) of peripheral nerves and have been found to be up-regulated after peripheral nerve injury.(18) In the PNS, Schwann cells lining the basal lamina of endoneurial compartments produce CSPGs that are believed to interfere with the neurite-promoting activity of ECM-integrin interactions.(19) Chondroitinase ABC, an enzyme that degrades CSPG, has been reported to promote elongation of neurites when injected directly into acellular peripheral nerve grafts in rodent nerve injury models.(20) In fact, CSPG degradation is now a standard practice during the decellularization of cadaveric nerve in commercially available nerve allografts.(21)

Interestingly, neurotrophic factors have been reported to overcome the inhibitory signaling of CSPGs and restore neurite outgrowth.(22) However, this effect is likely reduced *in vivo* as shown in previous reports of CSPG-induced RhoA activation causing down-regulation of NGF-induced neurite outgrowth.(23, 24) Due to this complex regulatory cascade of neurite growth mediated by neurotrophic factors and ECM interactions, it is difficult to estimate the effects of specific neurotrophic factors following CSPG inhibition *in vivo*.

In this study, we attempt to measure the effects of neurotrophic factors, specifically nerve growth factor and glial-cell derived neurotrophic factor, on neurites regenerating into chondroitinase ABC pre-treated substrates. We began by evaluating the effects of each neurotrophic factor independently in the presence of chondroitinase ABC in a dorsal root ganglion (DRG) explant model. DRG neurite outgrowth is well known to be inhibited by CSPG and promoted by NGF and GDNF. This model thereby provided an initial measurement of neurotrophic factor bioactivity at optimal concentrations in the presence of a CSPG-reduced substrate and absence of uncontrollable *in vivo* factors, such as endogenous growth factors and variable pharmacokinetics. After this initial analysis, we investigated the adjuvant effects of NGF and GDNF in acellular nerve grafts using a rat sciatic gap nerve injury model. Acellular nerve grafts were pre-treated with ChABC to mimic commercially available nerve grafts that have reduced CSPG content.

Materials and Methods

All experimental procedures were approved by and performed in accordance with the standards set forth by the Institutional Animal Care and Use Committee at Vanderbilt University.

Dorsal root ganglia explant cultures

Chicken dorsal root ganglia (DRG) neurons were used in the initial *in vitro* assessment. There were two primary considerations in the choice of chick DRG neurons – first, chick DRG explants have fewer surrounding non-neuronal cells than rodent DRG that can complicate neurite measurements, and second, DRG substrate preferences have not been found to be species dependent.⁽²⁵⁾ E12 white leghorn chicken embryos of both sexes without regard to sex were dissected in ice-cold L-15 medium under high-magnification. Dorsal root ganglia were isolated, plated on collagen(Sigma)/CSPG(Millipore)-coated 35mm culture dishes in neuronal culture medium [MEM (Gibco) with 10% fetal bovine serum (Gibco), D-glucose 4g/L (Sigma) and L-glutamine 2mM (Sigma)] and incubated at 37C with 5% CO₂ overnight. Adherent DRG explants were treated the following day with Neurobasal medium [Neurobasal A (Mediatech) with 2% B27 supplement (Gibco), D-glucose 4g/L (Gibco) and L-glutamine 2mM (Gibco)] supplemented with ChABC, ChABC + NGF (50ng/ml; Proteintech), ChABC + GDNF (50ng/ml; Sigma) or control Neurobasal medium. Explants were photographed with a Nikon AZ100M fluorescent microscope and Nikon DS-Ri1 camera after 3 days of incubation. Cell membranes were live-cell stained prior to imaging with the Neurite Outgrowth Staining Kit (Molecular Probes A15001). Images were analyzed for neurite outgrowth area and neurite length using ImageJ (Fiji Distribution, National Institute of Health, Bethesda, MA), as shown in Figure 1.^(26, 27)

Rat sciatic nerve injury model

In vivo neurotrophic factor supplementation to CSPG-reduced nerve allografts was evaluated in this study using a rat sciatic nerve injury model repaired microsurgically. Twelve donor animals were sacrificed for nerve graft harvesting. Two experimental groups (n=8) received ChABC pre-treated nerve allografts impregnated with either nerve growth factor or glial cell line-derived neurotrophic factor. The control group (n=8) received a

ChABC pre-treated nerve allograft impregnated with sterile water. Subsets of each group (n=4) were sacrificed at 5 days post-injury for acute histologic analysis during the period of neurite outgrowth. The remaining animals were sacrificed at 8 weeks post-injury. One rat in the GDNF group was excluded from analysis due to illness and untimely death.

Animal procurement and housing

Thirty-six (12 donors and 24 recipients) adult female Sprague Dawley rats weighing 250 to 350 g were housed 2-3 per cage and maintained in a 12/12h light-dark cycled room with *ad libitum* access to food and water. Animals used in this study were cared for in accordance with the guidelines of the Institutional Animal Care and Use Committee of Vanderbilt University.

Graft preparation

Sciatic nerves were harvested from donors on the day prior to graft implantation as described in Surgical Procedures. The harvested nerves were trimmed to 20 mm in length and aberrantly bifurcated nerves were discarded. The trimmed ends were collected and frozen for C4S analysis. Immediately following harvest, nerves were placed in a sterile cryogenic vial and decellularized with repeated freeze-thaw cycles in liquid nitrogen and thawing in a 37° C water bath. Decellularized grafts were incubated in chondroitinase ABC for 16 h at 37° C, as described by Neubauer(20). The grafts were then rinsed in Lactated Ringers solution and incubated in sterile H₂O, nerve growth factor solution (10 ug/ml) or glial cell line-derived neurotrophic factor solution (20 ug/ml) for 1 h at room temperature. Processed grafts were washed repeatedly in Lactated Ringers and stored on ice until implantation.

Surgical Procedures

Donor graft harvest—Donor rats were euthanized at age 20 to 24 weeks by CO₂ inhalation with cervical dislocation. Immediately following sacrifice, the hindlimbs were shaved with clippers and prepped aseptically. Sciatic nerves were excised bilaterally from the sciatic notch to the distal trifurcation, with careful attention paid to dissecting perineural tissue and minimizing injury to the nerve. The harvested nerves were immediately rinsed in sterile Lactated Ringers (Hospira; Lake Forest, IL) and placed in sterile cryogenic vials to be processed as previously described.

Interposition graft implantation—Female Sprague-Dawley rats were anesthetized with inhaled isoflurane and the left hindlimb shaved with clippers and prepped aseptically. A 1-cm incision was made parallel, and just caudal, to the femur. Using sharp dissection, the cephalad border of the biceps femoris was freed to allow for caudal retraction and exposure of the left sciatic nerve. The exposed nerve was then dissected free of perineural tissue using sharp dissection and minimal retraction.

A 5 mm segment of sciatic nerve was then excised with iris scissors and the wound was irrigated with Lactated Ringers (Hospira; Lake Forest, IL). Using standard microsurgical techniques, the processed allograft was trimmed at each end, for a final graft length of 10mm, and was then sutured in place using 9-0 Ethilon (Ethicon, Somerville, NJ). The

trimmed ends of the chondroitinase ABC treated grafts were collected and frozen for C4S analysis. Proximal and distal ends were approximated in an end-to-end fashion using an interrupted suturing technique and microscopic magnification, followed by wound irrigation with sterile Lactated Ringers. All animals were then given a subcutaneous injection of ketoprofen (5 mg/kg) and allowed to emerge from anesthesia.

Recipient nerve harvest—Recipient rats were anesthetized with inhaled isoflurane and sacrificed via intracardiac injection of Fatal-Plus Solution (Vortech, Dearborn, MI) at 5 days or 8 weeks following graft implantation. The left sciatic nerve was harvested immediately after sacrifice with careful attention paid to maintaining nerve integrity with at least 5-mm margin. Excised nerves were stored in 10% neutral buffered formalin for tissue fixation prior to paraffin embedding.

Immunohistochemical analysis

Nerve immunohistochemistry—Immunohistochemical staining was performed using commercial antibodies specifically directed against myelin basic protein (MBP) (Abcam, Cambridge, MA), choline acetyltransferase (ChAc) (Santa Cruz Biotechnology, Santa Cruz, CA), and Chondroitin-4-Sulfate (MP Biomedicals). Isolectin GS-IB4, biotin-XX conjugate (IB4; Invitrogen) was also used to detect GDNF-dependent nociceptive sensory neurons.(28, 29)

For MBP, ChAc, and IB4 staining, formalin-fixed paraffin embedded tissues were sectioned at 5 μ m, placed on slides and warmed overnight at 60°C. Slides were deparaffinized and rehydrated with graded alcohols ending in Tris buffered saline (TBS-T Wash Buffer, LabVision, Fremont, CA). For MBP and IB4, heat mediated target retrieval was performed in 1 $\times$ Target Retrieval Solution (pH 6.0, DAKO, Carpinteria, CA). Endogenous peroxidases and non-specific background were blocked by subsequent incubations in 3% H₂O₂ (Fisher, Suwanee, GA) in TBS-T and serum-free Protein Block (RTU, DAKO). Primary antibody to MBP was applied at 1:800 dilution for 1 hr, followed by incubation in EnVision+ HRP Labelled Polymer (RTU, DAKO). For IB4 staining, Isolectin IB4, biotin-XX conjugate (10 μ g/ml) was applied for 1 hr, followed by incubation in SA-HRP (BD Pharmingen).

For ChAc staining, no target retrieval was required. Endogenous peroxidases were blocked as previously described. Non-specific background, secondary, and tertiary labeling of target was accomplished by use of Vector's ABC Elite Goat IgG kit (Vector Laboratories, Burlingame, CA). Primary antibody to ChAc was applied at 1:35 dilution for 1 hour.

For C4S staining, frozen tissues were embedded in OCT and sectioned at 5 μ m, and stored in the -80°C till ready to stain. Tissues were adhered to the slides by washing in ice-cold acetone, followed by air-drying at room temp. OCT was removed by washing in TBS-T. Endogenous peroxidases and non-specific background were blocked by subsequent incubations in 3% H₂O₂ (Fisher, Suwanee, GA) in TBS-T and serum-free Protein Block (RTU, DAKO). Primary antibody to C4S was applied at 1:4,000 dilution overnight at +4°C, followed the next morning by incubation in horse biotinylated anti-mouse secondary antibody (1:200), and finally incubated in SA-HRP.

For all targets, slides were rinsed with TBS-T between each reagent treatment and all steps were carried out at room temperature unless otherwise noted. Visualization was achieved with DAB+ chromogen (DAKO). Slides were counterstained with Mayer's hematoxylin, dehydrated through a series of alcohols and xylenes, and then coverslipped with Acrytol Mounting Media (Surgipath, Richmond, IL).

Quantification of myelinated axons—Anti-MBP stained sections of nerve distal to the interposition allograft were analyzed for quantification of myelinated axons. Myelinated axon number was determined in four random fields in the distal sciatic nerve at 40× magnification. Axon profiles were counted using semi-automated analysis with NIH *ImageJ* software, which has been previously validated for axon quantification.(30) Post-processing of images was performed with an *ImageJ* Color Deconvolution Plug-in(31) to isolate the DAB color channel followed by conversion to 8-bit grayscale images. A threshold was then applied to the grayscale image to produce a binary representation of axon profiles, with careful attention paid to accuracy of the resultant image. Axon count, area and Feret diameter was measured using the *ImageJ* particle analysis feature with constant selection thresholds.

Statistical analysis

All statistical analyses were performed using GraphPad Prism 5 for Mac OS × (GraphPad Software; San Diego, CA). For comparison of axon counts Student's t-test was used to compare specific groups. All p values were two-tailed and significance was determined at $p < .01$.

Results

Dorsal root ganglion explants were used to test the effects of neurotrophic factor supplementation on neurite outgrowth in CSPG-reduced substrate. Chondroitinase ABC (ChABC) was supplemented to degrade nascent CSPG produced by Schwann cells present in cultured dorsal root ganglia explants. Neurite outgrowth and axon length was evaluated after 3d incubation in supplemented neuronal growth medium. At 3d, similar maximum axon lengths was observed in control, ChABC and NGF + ChABC supplemented medium. Neurite length was significantly increased in GDNF + ChABC supplemented medium, in addition to increased axonal arbor density. ChABC and NGF + ChABC supplemented media showed a trend towards increased neurite outgrowth, but they did not reach significance. Table 1 lists the neurite outgrowth and maximum axon length for each condition. Representative photomicrographs of live-stained dorsal root ganglia with fiducial markers are shown in Figures 2A-D.

To study the *in vivo* effects of CSPG inhibition and neurotrophic factors on injured peripheral nerves, we treated acellular nerve grafts with H₂O, NGF or GDNF prior to engrafting into a rat sciatic nerve lesion. Acellular nerve allograft was incubated with chondroitinase ABC (ChABC) to cleave native chondroitin sulfate proteoglycans (CSPG), which are well-known extracellular inhibitors of nerve regeneration and plasticity. In Figure 3, anti-chondroitin-4-sulfate (C-4-S) antibody immunohistochemistry is shown as validation of CSPG digestion in the processed nerve allografts. Chondroitin-4-sulfate is the

proteoglycan stub that remains after enzymatic digestion of CSPG by ChABC. Following overnight incubation in ChABC solution, we observed significantly increased anti-C-4-S staining compared to untreated nerve allograft, confirming its proteolytic activity.

To assess total axon regeneration in the processed nerve allografts, we quantified the total number of myelinated axons in distal sections of the lesioned sciatic nerve using anti-myelin basic protein (MBP) immunohistochemistry. Figure 4 shows total myelinated axon counts at 8 weeks post-injury. Myelinated axons were not visible mid-graft at 5 days post-injury, as expected due to immaturity of nascent axons. Total myelinated axon counts were assessed using the semi-automated ImageJ analysis previously described. Nerve growth factor was found to significantly increase total myelinated axon count compared to control ($p < 0.001$). No significant difference was found with control and GDNF treated nerve allografts.

Under strictly controlled *in vitro* conditions nerve growth factor and glial cell line-derived neurotrophic factor affect only specific pools of neuronal cells; however, indirect signaling pathways and extrinsic factors, such as extracellular matrix components, may augment *in vivo* neurotrophic activity. To classify regenerated neurons in our experimental model we performed immunohistochemistry with choline acetyltransferase, predominantly expressed in motor neurons, to identify efferent motor neurons. Figure 5 shows representative sections of choline acetyltransferase stained axons mid-graft at 5 days post-injury. Significantly increased stained axons were found in the NGF treated allografts, representing increased motor axon outgrowth compared to control.

Additionally, isolectin B4 was used to classify GDNF-dependent (*IB4+*, *TrkA*-) nociceptors and NGF-dependent (*IB4*-, *TrkA*+) nociceptors. In GDNF seeded grafts, increased regenerating motor axons were found mid-graft at 5 days post-injury compared to control. *IB4+* neurons represent sensory afferents lacking the TrkA receptor and are dependent on GDNF for survival. Therefore, immunohistochemical analysis with isolectin B4 provides a measurement of NGF versus GDNF driven survival following injury. Figure 6 shows representative sections of mid-graft at 5 days post-injury stained for IB4. NGF allografts significantly decreased IB4-positive axon outgrowth compared to control and GDNF allografts, representing decreased outgrowth or increased apoptosis of GDNF-dependent (*IB4+*, *TrkA*-) nociceptors following injury.

Discussion

Nerve growth factor is a well-characterized neurotrophin that plays a critical role in sensory neuron development and regeneration. Typically, in the presence of NGF, primary cultures of dorsal root ganglion neurons show increased neurite elongation and reduced apoptosis following injury. However, this was not observed in this study, in which NGF was applied to dorsal root ganglion explants in the presence of chondroitinase ABC. Instead, we found that NGF produced no significant difference in sensory neuron outgrowth compared to control conditions. The effects of NGF on injured neurons involve an elaborate cytosolic signaling cascade initiated by its two membrane-bound receptors – TrkA and p75. TrkA is a tyrosine receptor kinase with high-affinity for nerve growth factor, whose upregulation after injury has been shown to be protective against apoptosis. The p75 neurotrophin receptor

binds with low-affinity to all neurotrophins, including NGF, BDNF and NT-3, and is known to play a significant role in apoptotic signaling in addition to having pro-survival effects via the NF- κ B pathway. While chondroitinase ABC would be expected to further enhance NGF-induced neurite growth, these effects may not be additive, as both pathways intersect at common secondary messengers and regulatory proteins, such as RhoA.

In addition to the neurotrophin family of proteins, glial cell line-derived neurotrophic factor is a highly conserved ligand that is important to neuronal regeneration and survival after injury, particularly in motor neurons and the IB4-positive, TrkA-negative subpopulation of nociceptors. In this study, dorsal root ganglion neuron outgrowth was found to significantly increase in the presence of GDNF and chondroitinase ABC. The GDNF/RET signaling pathway is completely independent of known pathways of CSPG inhibition and therefore may have additive effects with chondroitinase ABC in inducing neurite outgrowth.

Interestingly, the *in vivo* model found that early NGF supplementation of CSPG-reduced nerve allografts resulted in improved motor neuron survival and increased myelinated axon outgrowth. NGF is well known to enhance sensory neuron growth and survival, but is not expected to have these effects on motor neurons because they lack the pro-survival TrkA receptor. However, the low-affinity p75 neurotrophin receptor is present in motor neurons and also has the capacity to promote neuron survival and growth. These data support a possible role for chondroitinase ABC in disinhibiting the p75-NF κ B pro-survival pathway. This relationship has been discussed in prior *in vitro* studies of CSPG-induced activation of RhoA, which is thought to inhibit NGF-induced nerve outgrowth, but to our knowledge has not been shown with an *in vivo* model.

NGF also appears to promote apoptosis (or inhibit regeneration) of IB4-positive sensory neurons, causing a significant reduction of surviving nociceptive neurons in CSPG-reduced nerve allografts. While this could be explained by decreased outgrowth of all sensory neurons, our *in vitro* model shows no significant difference in total sensory neuron outgrowth between NGF and control conditions. This effect may be prevented with combined NGF/GDNF adjuvant therapy, but this was beyond the scope of the current study.

Additional limitations of this study include minor variation in microsurgical technique between surgeons and a single method of nerve graft processing. Since this was a study of adjuvant neurotrophic factor therapy in CSPG-reduced nerve allografts, all nerve grafts were pre-treated with Chondroitinase ABC. We did not include a group without Chondroitinase ABC pre-treatment, because all commercially available nerve grafts at time of publication have been similarly processed to reduce CSPG content. The benefits of CSPG-reduced nerve allografts are supported by several *in vitro* and *in vivo* studies, and are currently being evaluated in the AxoGen Ranger® multicenter clinical study. The goal of this study was to evaluate the benefits of adjuvant neurotrophic factor therapy and not to reassess the current standards in nerve allograft processing.

Conclusions

Pre-treatment with chondroitinase ABC is now standard practice in commercially available nerve allografts. The effects of ECM and integrin modifications in these conduits may not only provide a fertile substrate for nascent neurite elongation, but they may also produce significant alterations in the molecular effects of neurotrophic factors, such as NGF and GDNF. It is important for the surgical community to understand these overlapping molecular pathways, which may provide answers to the variable effects observed with chondroitinase ABC treatment in the peripheral nervous system, as well as in the spinal cord.

This study provides initial evidence that the pro-survival effects of NGF *in vivo* may be disinhibited in CSPG-reduced substrates (such as ChABC pre-treated nerve grafts), promoting motor axon outgrowth and reducing the outgrowth of a specific subpopulation of nociceptive neurons. Future studies of CSPG-reduced nerve allografts with adjuvant growth factor therapy in rodent and large animal models should be pursued to answer these questions.

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Highlights

- We investigated the adjuvant effects of NGF and GDNF in processed nerve grafts.
- We examine the complex relationship of ECM processing and neurotrophic signaling.
- NGF enhanced total myelinated axon and motor axon outgrowth in CSPG-reduced grafts.
- CSPG-reduced nerve grafts may disinhibit the pro-survival effects of NGF.

Figure 1.

Examples of (A) live-cell stained dorsal root ganglion explant and (B) measurement of a dorsal root ganglion explant using ImageJ. Blue shaded area is the outlined ganglion. Red outline is the axon border. Black lines are the minimum and maximum Ferret diameters of the ganglion and their intersection represents the ganglion's center of mass. Green line is the longest axonal Ferret diameter passing through the center of mass.

Figure 2.

Dorsal root ganglion explants at 3 days of incubation in (A) control medium, (B) ChABC, (C) NGF + ChABC and (D) GDNF + ChABC. Increased axonal arbor density is visible in the GDNF + ChABC conditioned group.

Figure 3.

Chondroitin-4-sulfate (C-4-S) antibody immunohistochemistry in processed nerve grafts following (A) ChABC and (B) vehicle treatment. Increased specific C-4-S antibody staining is visible in the longitudinal nerve fiber tracts in the ChABC-treated nerve grafts, confirming degradation of CSPGs. Epineurial staining is visible in vehicle-treated nerve grafts, possibly due to tissue edge artifact.

Figure 4.

Myelin basic protein antibody immunohistochemistry at 8 weeks post-injury of control, GDNF and NGF treated interposition allografts. Semi-automated counts of myelinated axons located in the sciatic nerve distal to the allograft. Total myelinated axon count is significantly increased in the NGF treated group. Asterisks indicate treatment group is significantly different from the control group (** $p < 0.005$, two-tailed t test).

Figure 5. Immunohistochemical analysis of choline acetyltransferase in the mid-graft at 5 days post-injury. Representative sections of (A) Control, (B) GDNF and (C) NGF treated nerve allografts. (D) Quantification of positively stained axons, representing predominantly regenerating motor neurons. Asterisks indicate treatment group is significantly different from the control group (* $p < 0.01$, two-tailed t test).

Figure 6.

Immunohistochemical analysis of IB4-positive neurons in the mid-graft at 5 days post-injury. Representative sections of (A) Control, (B) GDNF and (C) NGF treated nerve allografts. (D) Quantification of IB4-positive axons, representing GDNF-dependent nociceptive neurons. Significant decreased IB4-positive neuron survival was found in NGF supplemented nerve grafts. Asterisks indicate treatment group is significantly different from the control group (*p < 0.01, two-tailed *t* test).

Table 1
Measurements of dorsal root ganglion explants neurite outgrowth

	Neurite outgrowth (mm ²)		Neurite Length (μm)		<i>n</i>
	<i>Raw</i>	<i>Corrected</i>	<i>Raw</i>	<i>Corrected</i>	
Control	3.94 ± 0.80	4.25 ± 0.74	2780 ± 282	2075 ± 114	8
ChABC	5.70 ± 1.76	5.99 ± 1.68	3122 ± 457	2432 ± 413	8
ChABC + NGF	4.79 ± 0.58	4.79 ± 0.58	2870 ± 153	2286 ± 123	7
ChABC + GDNF	6.93 ± 1.86	6.93 ± 1.86	3745 ± 254*	3011 ± 161**	8

Asterisks indicate conditioned group is significantly different from the control group

* $p < 0.05$, two-tailed *t* test;

** $p < 0.01$, two-tailed *t* test.

Corrected values exclude areas of axonal arbor with no visible outgrowth by 3 days post-injury, most likely injured during tissue transfer.