Published in final edited form as:
Muscle Nerve. 2013 February ; 47(2): 213–223. doi:10.1002/mus.23490.

Nerve Allografts Supplemented with Schwann Cells Overexpressing GDNF

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Abstract

Introduction—We sought to determine if supplementation of acellular nerve allografts (ANAs) with Schwann Cells overexpressing GDNF (G-SCs) would enhance functional recovery following peripheral nerve injury.

Methods—SCs expanded *in vitro* were infected with a lentiviral vector to induce GDNF overexpression. Wild type-SCs (WT-SCs) and G-SCs were seeded into ANAs used to repair a 14mm nerve gap defect. Animals were harvested after 6 and 12 weeks for histomorphometric and muscle force analysis.

Results—At 6 weeks, histomorphometry revealed that ANAs supplemented with G-SCs promoted similar regeneration compared to the isograft at midgraft. However, G-SCs failed to promote regeneration into the distal stump. At 12 weeks, ANAs with G-SCs had lower maximum and specific force production compared to controls.

Discussion—The combined results suggest that consistent overexpression of GDNF by G-SCs trapped axons in the graft and prevented functional regeneration.

Keywords

Peripheral nerve injury; Neurotrophic factor; Schwann cells; GDNF; Lentiviral vector; Nerve regeneration

INTRODUCTION

The current gold standard for peripheral nerve repairs is a direct end-to-end coaptation. However, this is not always possible due to tension on the repair that would ultimately impair nerve regeneration [1]. In these instances, it may be necessary to use a nerve graft or conduit to bridge the gap defect. Although studies have proposed several options for this type of repair, the use of an autologous nerve graft remains the current gold standard. While the benefits are many, there are also disadvantages that make the repair with an autologous nerve graft less than ideal. For example, autologous nerve allografts require increased

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surgical time, are not readily available, and may leave the patient with sensory or motor deficits at the donor site [2,3].

The use of processed nerve allografts may be a promising substitute for the drawbacks of autografts. Advantages of using processed nerve include no donor site morbidity, an unlimited supply of donor nerves with the ability to match for size and functional specificities, decreased surgical time and a potential "off-the-shelf" alternative [4–6]. They may also be favored over nerve conduits, as they preserve the Schwann cell (SC) basal laminae and extracellular matrix (ECM) of the nerve. However, they are not without their own limitations. In addition to preserving structure and integrity to the graft, the processing techniques decellularize the allografts and result in acellular nerve allografts (ANAs). The lack of viable SCs limits the use of these ANAs to gaps of up to 3 cm. In addition, studies have also demonstrated inferior nerve regeneration at distal sites and suboptimal functional outcome with the use of ANAs when compared to the autograft model [7–16]. Current research has investigated the idea of enhancing nerve regeneration and functional recovery by supplementing alternatives to autoallografts, albeit ANAs or nerve conduits, with SCs and neurotrophic agents through various techniques [17–20].

Neurotrophic agents such as nerve growth factor (NGF), glial cell line derived neurotrophic factor (GDNF) and brain derived neurotrophic factor (BDNF) have been previously reported to aid in the support and maintenance of peripheral nervous system axons [21,22]. During injury, these growth factors are upregulated to provide cues to promote the migration of SCs to the injury site and to proliferate and secrete additional factors to guide the regeneration of injured axons [23–28]. In particular, GDNF has been shown to promote survival of motor neurons and has been found to increase during expression of GDNF in SCs and the number of GDNF receptors in motor neurons during injury [29,30]. This suggests that increased expression of GDNF may promote increased motor axon regeneration and enhance functional recovery.

Given the limitations of ANAs and the supportive characteristics of SCs and GDNF, we sought to evaluate the effects of supplementing ANAs with SCs that overexpress GDNF (G-SCs) on nerve regeneration and functional recovery. Specifically, we analyzed the extent of nerve regeneration using histology, histomorphometry, electron microscopy, and motor function recovery via muscle force testing of the extensor digitorum longus (EDL) muscle.

MATERIALS AND METHODS

Animals

A total of 56 adult (225–250 g) male Lewis (Harlan Sprague-Dawley, Indianapolis, IN) and 34 adult (225–250 g) male Sprague-Dawley rats were used in this study. Animals were maintained in a central housing facility for the duration of the study. All procedures were approved and conducted in strict accordance with regulations set forth by the Animal Studies Committee of Washington University.

Experimental Design

Fifty-six male Lewis rats (225–250 g) were randomized into 4 groups corresponding to the type of sciatic graft that was used to repair a 14 mm sciatic nerve gap. We used 28 adult male Sprague-Dawley rats as donors for nerve allografts and 2 adult male Lewis rats for SC expansion. The first group served as the positive control (Isograft, n = 14) and received a 14 mm reverse isograft repair obtained from a littermate. The second group received a 14 mm ANA injected with cultured Wild type-SCs expanded from the sciatic nerve at a concentration of 1×10^6 cells/5 μL (WT-SCs+ ANA, n = 14). The third group received an ANA injected with G-SCs (G-SCs+ ANA, n =14) at the same concentration as the WT-SCs

+ANA group. The final group served as the negative control, receiving a 14 mm cold-preserved ANA (ANA, n = 14). Of the 14 animals in each group, 6 underwent functional testing, and 8 were evaluated for histomorphometry.

Isolation and expansion of SCs

SC cultures were prepared as previously described [31,32]. Briefly, the rat sciatic nerve was harvested from adult rats and placed in Leibovitz L-15 medium (Invitrogen, Carlsbad, CA). Collagenase I (1%) (Fisher, Pittsburgh, PA) and trypsin (2.5%) (Invitrogen) were added to the fascicles and incubated for 30 min at 37°C. After centrifugation at 130 × g for 5 min, the pellet was washed with Dulbecco modified Eagle medium (DMEM, Invitrogen) supplemented with 10% heat-activated fetal bovine serum (FBS, Sigma-Aldrich, St. Louis, MO) and 1% antibiotic antimycotic (ABAM, Invitrogen). The cells were then seeded on 10 cm tissue culture dishes coated with poly-L-lysine (pLL) (Sigma-Aldrich). Tissue culture dishes were prepared by coating with 10 mL 0.01% pLL in sterile water and washing twice with sterile water. On day 2 of culture, 10 μM Cytosine-beta-arabino furanoside hydrochloride (Sigma-Aldrich), was added to cultures along with the media containing DMEM, FBS and ABAM. On day 6, the fibroblasts were complement-killed using an anti-Thy 1.1 antibody (1:40 dilution in media, Serotec, Raleigh, NC) and rabbit complement (1:4 dilution in media, Sigma-Aldrich). On subsequent days, the culture media was supplemented with 2 μM forskolin (Sigma-Aldrich), and 20 μg/mL pituitary extract (Biomedical Tech, Inc., Stoughton, MA). At the second passage in collagen type I-coated (Sigma-Aldrich) 75-cm^3 flasks, SCs were further purified with P75 NGF receptor primary antibody (Abcam, Cambridge, MA) using CELLection™ Pan Mouse IgG Kit (Invitrogen). Cultures were maintained at subconfluent levels in the same incubation conditions and passaged as necessary.

RNA preparation and quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was isolated using an acid-phenol extraction (TRIzol, Invitrogen) and purified using an RNeasy Mini Kit (Qiagen). Spectrophotometric and electrophoretic analyses were used to assess the mass, purity and integrity of the RNA. Purified RNA was subsequently reverse-transcribed into cDNA (QuantiTect Reverse Transcription Kit, Qiagen). GDNF and housekeeping gene β-actin transcripts (QuantiTect primer assays, Qiagen) were quantitated using real-time PCR with the SYBR Green reporter dye using an Applied Biosystems 7000 Real-Time PCR thermocycler. The C_t values of G-SC and WT-SC triplicates were obtained and analyzed using the comparative delta crossover threshold (C_t) method [33].

Plasmids and lentivirus production

Full-length rat GDNF cDNA was cloned into FUIV, a lentiviral expression vector in which gene expression is driven by the ubiquitin promoter, and coexpression of Venus fluorescent protein is enabled by an internal ribosomal entry site (IRES) element (Araki et al., 2004). An FUIV plasmid lacking the rat GDNF cDNA was used as control. Lentiviruses expressing GDNF or control plasmids were produced in HEK293T cells as previously described (Sasaki et al., 2006).

SC lentiviral infection

Established SC cultures were transduced at an early passage (<passage 3) with the appropriate lentiviral vectors. Efficiency of infection was confirmed by visualization of expression of GFP fluorescent reporter in the FUIV lentiviral vector in almost 100% of cells. GDNF overexpression in infected cells was confirmed with *qRT-PCR* and was quantified as a 6.15 ± 3.3 fold increase over normal SCs.

Nerve Allograft Processing

We used a cold-preservation technique to create ANAs. Briefly, explanted sciatic nerve segments from donor Sprague-Dawley rats were immediately transferred into sterile six-well plates with 10 mL of a solution containing UW solution [34] (15 ml; NPBI International BV, Emmer Compascuum, The Netherlands), penicillin G (200,000 U/L), regular insulin (40 U/L), and dexamethasone (16 mg/L). The solution was changed weekly in a sterile hood for 7 weeks and stored at 4°C as described previously [35].

Donor Graft Harvest

Donor animals were anesthetized with a subcutaneous injection of ketamine (75 mg/kg, Ketaset®, Fort Dodge Animal Health, Fort Dodge, IA) and medetomidine (0.5 mg/kg, Dormitor®, Orion Corporation, Espoo, Finland). Under aseptic conditions, both hind limbs were prepped and shaved. Using a number 15 blade, we made a 3 cm skin incision from the top of the femur towards the kneecap and separated the gluteal muscles to expose the sciatic nerve. Using a dissecting microscope, a 30–35 mm sciatic nerve segment was excised bilaterally and used for immediate isograft repair, nerve graft processing, or SC expansion. The animals were subsequently euthanized with intracardiac injection of Euthasol® (150 mg/kg, Delmarva Laboratories, Des Moines, IA).

Graft Implantation

As previously described, experimental animals were anesthetized, and the right hind limb was prepped and shaved. After the sciatic nerve was exposed and neurolysed, it was sharply transected 5 mm proximal to the trifurcation. The reversed 14 mm isograft (Isograft) or ANA was then sutured to the proximal and distal stumps with one 10-0 nylon suture at each end and secured with fibrin sealant. For groups that received SC treatments (WT-SC+ANA, G-SC+ANA), delivery of cells was completed after the ANA was securely fastened to the proximal stump as previously described [36]. Briefly, with a 27-gauge Hamilton™ syringe (Hamilton Company, Reno, NV) a concentration of 1×10^6 SCs/5 μL was injected longitudinally underneath the epineurium of the graft. Wounds were subsequently irrigated, and muscles and skin were reapproximated with interrupted 6-0 Vicryl (Ethicon, Somerville, NJ) and 4-0 nylon sutures, respectively. Animals were recovered with a subcutaneous injection of atipamezole HCl (1mg/kg, Antisedan®, Orion Corporation) and placed on a warming pad post-operatively. Following surgery and post-operative care, animals were returned to the central housing facility and closely monitored for infection, distress and other morbidities.

Graft Explantation

At 6 weeks post-operatively, animals were re-anesthetized and prepped to expose the right sciatic nerve and graft as previously described. Once identified, the graft was explanted with 5 mm portions of the proximal and distal nerve stumps and put in 3.5% glutaraldehyde fixative and stored in 4°C for histomorphometric analysis. Animals were euthanized with intracardiac injection of Euthasol® (150 mg/kg, Delmarva Laboratories, Des Moines, IA) immediately following harvest.

At 12 weeks post-operatively, sciatic nerve function was assessed by measuring the evoked motor response in reinnervated EDL muscle upon electrical stimulation of the repaired sciatic nerve. Animals were immobilized in an automated functional assessment station (FASt System, Red Rock Laboratories, St. Louis, MO) with the distal portion of the EDL muscle fixed to a 5 N load cell. Cathodic, monophasic electrical impulses (duration=200 ms, frequency = single–200 Hz, burst width = 300 ms, amplitude = 0–1000 μA) were applied to the sciatic nerve proximal to the interposed nerve graft via silver wire electrodes, while

resulting force production in the EDL was recorded using custom data acquisition software (RRL V.1.0, Red Rock Laboratories).

Elicited twitch contractions were utilized to determine the optimal stimulus amplitude (V_o) and optimal muscle length (L_o) for isometric force production in the EDL muscle. All subsequent isometric force measurements were made at V_o and L_o. Single twitch contractions were recorded, and maximum twitch force (F_t) was calculated. Tetanic contractions were recorded at increasing frequencies of stimulation (5–200 Hz), allowing 2 min intervals between stimuli to prevent muscle fatigue. Maximum isometric tetanic force (F_o) was automatically calculated from the resulting sets of recorded force traces.

The physiological cross-sectional area (PCSA) of the EDL muscle was calculated using the following equation:

$$PCSA = \frac{(M * \cos\theta)}{(\rho * L_o * 0.44)}$$

where the *PCSA* is the physiological muscle cross-sectional area (cm^2), M is the EDL muscle mass (g), $\cos\theta$ is the Angle of pennation of the EDL muscle (~0^o), ρ is the density of mammalian skeletal muscle (1.06 g/cm^3), L_o is the optimal muscle length (cm), and 0.44 is the ratio of fiber length to muscle length (L_f/L_m) in rat EDL muscle. Maximum specific isometric force was calculated as the maximum isometric force normalized to muscle PCSA. Values were reported relative to measurements taken from healthy, unoperated animals. Following assessment, both denervated/reinnervated and healthy, unoperated EDL muscles were harvested and weighed. Animals were euthanized as described above.

Histomophometry

All harvested nerves were post-fixed with 1% osmium tetroxide, serially dehydrated in ethanol and embedded in Araldite 502 (Polyscience Inc., Warrington, PA). Tissues were cut into thin 1μm cross-sections using an ultramicrotome and consequently stained with 1% toluidine blue in preparation for light microscopy imaging and qualitative analysis. Using a semi-automated program previously described [37], an observer blinded to experimental groups measured total nerve fiber number, fiber width (μm) and percent neural tissue (100 × neural area/intrafascicular area) at mid-portions of the graft and at areas 3–5 mm distal to the graft.

Electron Microscopy

We performed electron microscopy on randomly selected nerve segments of groups supplemented with SCs (WT-SCs+ANA, G-SCs+ANA) to evaluate the neural ultrastructure of regenerating fibers. Ultrathin sections were cut using a microtome and subsequently stained with uranyl acetate-lead citrate. Electron micrographs were then taken of each specimen with a Zeiss 902 electron microscopy (Zeiss Instruments, Chicago, IL). An observer blinded to the randomly selected nerve segments within the 2 groups evaluated the electron micrographs for the quality of myelination and relative prevalence of unmyelinated fibers.

Statistical Analysis

Statistical analyses were run using SigmaStat 3.0 (Systat Software, San Jose, CA). Multiple groups were compared with a one-way analysis of variance (ANOVA) if conditions of normality (assessed with the Kolmogorov-Smirnoff normality test) and equal variance (assessed with the Levene Median test) were met. If ANOVA returned a statistically

significant *p* value, a *post-hoc* Student-Newman-Keuls test was used to isolate significant differences between groups with correction for multiple comparisons. Significance was set at $\alpha=0.05$ ($p<0.05$). All results are reported as mean ± standard error of the mean.

RESULTS

Histology of nerve mid-graft and segments distal to the graft was used to assess nerve regeneration qualitatively. In the mid-graft cross-sections, there were myelinated nerve fibers present through the allografts, thus qualitatively demonstrating adequate nerve regeneration in all groups (Figure 1). In segments distal to the allografts however, there were apparent differences between the groups. The group supplemented with G-SCs (G-SCs +ANA) regenerated very few myelinated fibers distally (Figure 2) and appeared to have more debris present. This is in striking contrast to the isograft, WT-SCs+ANA and ANA group, in which there were abundant myelinated fibers in distal segments. Overall, there was robust regeneration in mid-graft sections and distal segments of the positive control isograft, WT-SCs+ANA and negative control ANA groups. The group treated with G-SCs+ANA demonstrated a pronounced difference between the qualitative appearance of nerve regeneration at mid-graft sections and distal segments (Figures 1 & 2).

Sections at mid-graft and areas 3–5 mm distal to the nerve graft were explanted and evaluated for histomorphometric analysis at 6 weeks post-operatively. To quantify the degree of fiber maturity and nerve regeneration, we analyzed parameters such as total myelinated fiber count, percent neural tissue, and fiber width. Total fiber number at mid-graft and distal segments may be used as a marker of nerve regeneration. On sections taken from mid-graft, nerve regeneration was enhanced in nerve allograft groups supplemented with (WT-SC+ANA, total fiber count 9908 ± 7988) and without GDNF-infection (G-SCs +ANA, 7371 ± 5052) to a level not statistically different from the positive control isograft group (11462 ± 1850). The negative control ANA group was statistically inferior to the 3 other groups (5880 ± 1907) (Figure 3).

Notably, in segments distal to the graft, the total number of regenerating fibers was significantly reduced in animals treated with ANAs supplemented with G-SCs (405 ± 589) when compared to all other groups. In addition to the positive control isograft (8,016 ± 1528) and WT-SCs+ANA (4573 ± 2645), the negative control ANA (3764 ± 1852) also demonstrated better regeneration distally than those treated with G-SCs+ ANAs. The Isograft demonstrated a significantly greater number of nerve fibers distally compared to all groups (Figure 3). For reference, the average number of fibers in a normal rat sciatic nerve is approximately 7200 ± 3300 [38].

In the mid-graft sections, the percentage of neural tissue was significantly greater in the isograft positive control (20.53 ± 4.87) than in the other groups (WT-SCs+ANA 12.89 ± 9.98; G-SCs+ANA 9.18 ± 5.09; ANA 10.02 ± 4.74) (Figure 4). While there was no statistical difference in the percentage of neural tissue at mid-graft between groups treated with WT-SCs+ANA or G-SCs+ANA and the negative control ANA group, interestingly, this was not true with regards to the percentage of neural tissue in distal segments. Groups treated with G-SCs+ANA had significantly less percent neural tissue (0.32 ± 0.49) distally than all other groups (isograft 13.98 ± 4.53; WT-SCs+ANA 6.23 ± 4.46; ANA 4.54 ± 2.56), implying a poorer quality of nerve regeneration distally when ANAs were supplemented with G-SCs. In addition, the isograft group also regenerated a significantly greater percentage of neural tissue distally than all other groups (Figure 4).

Fiber width was quantified to assess fiber maturity in treated animals. While the mid-graft myelinated fiber widths of all groups were approximately 3 μm, there was a small but

significant difference between groups (Figure 5). Distally, the isograft group (2.90 ± 0.18) had thicker fibers on average than all other groups (WT-SCs+ANA 2.66 ± 0.15; G-SCs +ANA 1.68 ± 1.16; ANA 2.53 ± 0.11). The average regenerated fiber was thinner when treated with G-SCs+ANAs than those with WT-SCs+ANA and in the negative control ANA group (Figure 5). The width of the unrepaired sciatic nerve is approximately 6.5 μm [38].

Electron microscopy studies were performed to further investigate the differences seen on histological images and histomorphometric analysis. The light microscopy images and total fiber counts of the group treated with G-SCs+ANA demonstrated robust regeneration at mid-graft but poor regeneration distally. Based on these observations, we hypothesized that the overproduction of GDNF by transplanted SCs was acting as a chemo-attractant that trapped axons within the mid section of the graft. Therefore, we used electron microscopy studies to test this hypothesis and identified bundling of axons not apparent in the other groups (Figure 6). The high density of axons bundled together appeared to be random regeneration of axons within a heightened trophic environment.

Measurement of evoked force production in the EDL muscle upon electrical stimulation of repaired sciatic nerve was performed to assess the degree to which regenerating motor axons crossed the imposed defect and reinnervated distal motor targets. Maximum isometric tetanic force measurements obtained 12 weeks post-operatively demonstrated that EDL muscles innervated by sciatic nerves repaired with fresh nerve isografts and WT-SCs+ANA experienced the greatest degree of functional recovery (Figure 7A). Both the isograft and the WT-SCs+ANA treated groups demonstrated restoration of force 32.65 ± 1.52 % and 30.65 ± 1.51 % of unoperated control force production (1.23 ± 0.06 N and 1.16 ± 0.06 N). In contrast, the G-SCs+ANA and ANA groups resulted in a significant decrease in force production in reinnervated EDL muscles (11.43 ± 1.20 %, 21.99 ± 0.79 % of unoperated control force production) compared to nerve isografts.

EDL muscles innervated by sciatic nerves repaired with fresh nerve isograft experienced the least amount of net muscle atrophy 12 weeks post-operatively, retaining 63.52 ± 1.87 % of unoperated control muscle mass (0.129 ± 0.004 g) (Figure 7B). Repair of sciatic nerve defects from the WT-SCs+ANA were similar in comparison with nerve isografts. EDL muscles innervated by sciatic nerves repaired with G-SCs+ANA demonstrated the greatest muscle atrophy (32.20 ± 1.16 % of unoperated control muscle mass) of any nerve allograft group.

Calculation of maximum specific force production provided a metric of functional capacity independent of muscle atrophy. Similar to maximum isometric force measurements, EDL muscles innervated by sciatic nerves repaired with fresh nerve isografts and WT-SCs+ANA demonstrated the greatest specific tetanic force production of any experimental group (Figure 7C). Isograft and WT-SCs+ANA repair of the imposed sciatic nerve defect were observed to restore 49.57 ± 1.42 % and 58.18 ± 1.55 of unoperated control specific force production (17.03 ± 0.49 N/cm^2 and 19.99 ± 0.53 N/cm^2) respectively. The G-SCs+ANA and ANA groups both resulted in decreased specific force production (31.31 ± 1.47 %, 35.16 ± 0.47 % of unoperated control specific force production) compared to nerve isografts.

DISCUSSION

Many studies have explored the delivery of the GFs, especially GDNF, at or near the injury site to promote axon growth [39–41,26,21]. Other studies have also explored the transplantation of SCs as a cell therapy to guide regenerating axons[42,18]. Due to the supportive nature of GDNF and SCs in the treatment of peripheral nerve injury, we designed a study to determine

if SCs can be used as vehicles for efficient delivery of GFs to the injury site. The effect of transplanting G-SCs at the injury site was assessed by histomorphometry at 6 weeks post transplantation into the ANAs and muscle force testing on the EDL 12 weeks post transplantation into the ANAs.

GDNF is a major component of the regenerative process after peripheral nerve injury. While GDNF plays an active role in promoting the survival of motor neurons in the central nervous system (CNS), these neurons do not express GDNF, [43–46] and following injury, the levels of GDNF in motor neurons is unchanged [47]. In contrast, GDNF is expressed in the periphery, and after nerve injury its expression is upregulated in the distal nerve stump and denervated muscles [48,49]. The expression of GDNF is higher in neuromuscular tissue distal to the site of injury, whereas minimal expression is observed in the proximal stump of the injured nerve [47,50]. The increased production of GDNF in the distal stump and denervated muscle following nerve injury occurs early after injury followed by a gradual decrease in expression with increasing time [51–53]. The pattern of GDNF expression following injury creates a temporary concentration gradient that increases with distance distal to the site of injury [48]. The temporary distal upregulation and secretion of GDNF is a signal that promotes motor nerve regeneration. The duration of the GNDF signal is often shorter than the time necessary for the regenerating axons to reach the end organ target. The levels of GDNF in the distal nerve after chronic denervation (>4 weeks in the rat) are not sufficient to stimulate robust axonal regeneration from the proximal stump [40,54]. Thus, before the nerve reaches its target, the signal stimulating it is gone.

SCs are critical for efficient axonal regeneration following peripheral nerve injury through production of GFs, extracellular matrix and cell adhesion molecules. Following peripheral nerve reconstruction with an ANA, it has been shown that migration of endogenous SCs into the graft leads to the onset of axonal regeneration[55].

It was anticipated that the inclusion of G-SCs in ANAs (G-SCs+ANA) would increase nerve regeneration and functional recovery over ANAs alone or supplemented with WT-SC. However, our results demonstrate that the ANA supplemented with G-SCs did not enhance regeneration distally. Rather, the G-SCs+ANA group produced less myelinated fibers with smaller width and less neural tissue at the distal end of the graft compared to the positive control isograft and the negative control, the ANA group. Interestingly, when we examined midline sections of the supplemented grafts, the G-SCs+ ANA group had similar myelinated fiber regeneration as the isograft group, which suggests that there was an ample amount of regeneration at mid-graft. Closer inspection using electron microscopy revealed that there indeed was an increased density of axon bundling compared to the WT-SCs+ANA group. Other studies have demonstrated that constant high levels of GDNF at the injury site have prevented motor axon regeneration by sequestering motor axons at the site of GDNF expression in the nerve, a phenomenon called the "*candy-store effect*"[56]. In the "*candy-store effect,*" artificially increased levels of GDNF in the nerve attract and trap regenerating axons and prevent them from extending distally to reinnervate the end organ. Although these results show that the G-SCs do not promote successful regeneration across the graft, they re-affirm the hypothesis that regenerating axons follow environmental cues during regeneration. The GDNF produced by G-SCs within the ANAs acts as a strong and constant directional cue for regenerating axons trapping them within the graft.

The discrepancy between mid-graft and distal sections within the G-SCs+ANA group was reinforced in the muscle force testing of the EDL. We assessed the level of functional recovery by measuring the maximum isometric force and maximum specific force on the EDL muscle. Both force measurements, maximum isometric tetanic force and specific tetanic force, were the lowest for the G-SCs+ANA group compared to the isograft and WT-

SCs+ANA groups. The maximum specific force can be roughly associated with the percentage of fibers that have reinnervated the muscle. It is interesting to note that the G-SCs+ANA and ANA groups have the lowest maximum isometric force production, but only the G-SCs+ANA group has a significantly lower EDL muscle mass. After nerve injury, the EDL muscle will atrophy until it is reinnervated by regenerating nerve fibers. The lower EDL mass in the G-SCs+ANA group implies that the axons regenerated at a lower rate than the other groups, possibly due to the "*candy-store effect.*" Thus, the specific force is attributed to the lower number of fibers that regenerated across the graft to reinnervate the EDL. Although the EDL muscle mass is higher for the ANA group without SCs (negative control), the force production in this group shows the importance that SCs are necessary to promote mature nerve fibers to reinnervate the muscle.

In the past, it has been shown that the addition of exogenous GDNF for the treatment of peripheral nerve injury can extend the regeneration window of peripheral nerve axons [40]. Temporary release of GDNF from conduits at the site of peripheral nerve injury has been shown to enhance regeneration of motor neurons in acute short gap models [39,41]. Others have utilized continuous release of GDNF via mini-osmotic pump at the site of injury and found it increased the number of axons that regenerate after prolonged denervation.[40] However, based on our results and others [56] the levels provided by the osmotic pump were below those needed to elicit the trapping effect seen in this study. Similarly, cells genetically altered to overexpress GDNF have been seeded into nerve conduits to enhance their typically poor regenerative performance[57,58]. In these studies, increased levels of GDNF within the conduits is credited with enhanced peripheral nerve regeneration. However, the study by Li et al makes no comparison to an isograft control and fails to show survival of transplanted cells within the artificial conduit. It is possible that low survival of transplanted cells decreased the therapeutic dose of GDNF below that necessary to induce axonal trapping but maintained levels that stimulated repopulation of the acellular graft with host regenerative support cells (i.e. host SCs). This increase in intra-conduit support cells may explain the increases in functional recovery. Further, it is difficult to place these increase in recovery in context, because of the lack of an isograft positive control. Peripheral nerve regeneration through nerve conduits is poor,[59,16] and the functional increases seen in the Li et al study may be marginal in comparison to the gold standard isograft. Even in this study, the G-SCs+ ANA group demonstrated some levels of functional recovery.

While showing some benefits, administration of GDNF at the site of injury does not mimic the physiological gradient of GDNF that is seen following peripheral nerve injury. Typically, as the axons extend past the injury site, the GDNF concentration dips below the threshold for signaling, and the axons are no longer influenced by the signal. Studies of the constant over-expression of GDNF in the denervated end organ muscle[60] or capped nerve stumps[61] have demonstrated the ability of growth factors to enhance motor neuron survival and regeneration. The results of these studies are consistent with the results of our study in that constant expression of GDNF at the terminal site of regeneration (ideally the end organ muscle) increases motor neuron survival and attracts regenerating axons. In this study, the constant expression of GDNF in SCs located in the graft induced the *"candy store effec*t" to cause a bundling of axons in the mid-graft area and prevent regenerating axons from continuing through to reinnervate the end organ, the EDL.

Other factors that could have contributed to abnormal axonal regeneration in this study are lack of long term survival of the transplanted SCs, inflammatory response to the transplanted SCs, and abnormal extracellular matrix (ECM) deposition within the interposed nerve graft. SCs are essential for peripheral nerve regeneration, and in the setting of a nerve graft, the absence of SCs can negatively affect regeneration[12,13,62,63,55,64]. Electron micrographs from the harvested ANAs supplemented with G-SCs demonstrated the presence

of SCs throughout the harvested graft (Figure 6). It is possible that the transplanted G-SCs did not survive, and the observed SCs in the graft are endogenous host SCs that migrated into the graft to support regeneration. However, the drastic difference in regeneration pattern observed between G-SC and the WT-SC control group would suggest that the transplanted G-SCs survived and altered the regenerative environment. In all aspects other than viral transfection, the transplanted SCs (either WT or G) were cultured and expanded using identical methods. It is possible that the viral vectors from the *in vitro* transfection process induced an inflammatory response that resulted in significant augmentation of regeneration. However, the viral vectors used in this study were stripped of replication machinery as a safety precaution. This safeguard, in combination with subsequent cellular passage following the transfection event, significantly reduces the possibility that virus was transplanted with the G-SCs. Further, a significant inflammatory event in response to the viral vector would have resulted in an increase in immune infiltrate within the graft. Immune reactive cells (macrophages, neutrophils, etc) can be identified using the histomorphometric and electron microscopic analyses that were performed. If the transplanted G-SCs or accompanying viral vector were inducing a significant immune response, accumulation of these immune reactive cells would have been apparent during sample analysis. All of the groups demonstrated a complete lack of immune reactive cells within the grafts. Finally, changes in ECM deposition by transplanted cells could also account for a difference in axonal regeneration following peripheral nerve injury[65]. While we did not specifically assay for changes in ECM deposition, the arrangement and organization of collagen fibers within the nerve can be observed and assessed via electron microscopy. No differences in collagen deposition were observed between the experimental groups. These observations, however, do not account for the deposition of other ECM proteins, such as laminin and chondroitin sulfate proteoglycans, which could significantly alter regeneration within the graft.

In summary, this study demonstrates that SCs that are genetically altered to express GDNF can be used as delivery vehicles to treat peripheral nerve injury and nerve regeneration. ANAs seeded with G-SCs (G-SCs+ANA) to treat rat sciatic nerve injury trapped regenerating axons within the graft. This allowed only a few distal axons to regenerate, which contributed to poor functional recovery. Our results demonstrate that the increased expression of GDNF in the SCs indeed attracted the regenerating axons. However, due to the inability to reduce the GDNF signal, the axons continued to respond with local axonal elongation and failed to leave the area. This study has reinforced the idea that increased levels of GDNF can stimulate axonal regeneration, but it also shows the importance of turning off the GDNF signal to allow the axons to regenerate towards the denervated end organ. Future studies will be done to understand the timing and distribution of GDNF at the distal end of the injury site to promote increase regeneration and reinnervation of the end organ. Understanding the basics of growth factor cues and SC behavior will allow for better designed cell therapies to improve rate of peripheral nerve regeneration and function recovery post injury.

Acknowledgments

The authors were funded and supported by the National Institute of Health (**R01NS05170605**). We would also like to acknowledge the Hope Center for Neurological Disorders' Viral Vector Core at Washington University in St. Louis, MO, USA for funding, use of the qRT-PCR thermocycler, and Lenti Viral Vector production. We like to thank Dr Jeff Milbrandt for his assistance in GDNF vector design.

Abbreviations

EDL	extensor digitorum longus

GDNF	glial cell line-derived neurotrophic factor
LV	lentiviral vector
PCR	polymerase chain reaction
PBS	phosphate buffered saline
SCs	Schwann cells
ANA	acellular nerve allograft
G-SCs	GDNF transfected SCs
WT	wild type
CNS	central nervous system
GF	growth factors
ANOVA	one-way analysis of variance
V_o	optimal stimulus amplitude
Lo	optimal muscle length
Ft	maximum twitch force
Fo	maximum isometric force
GFP	green fluorescent protein
PLL	Poly-L-lysine
NGF	nerve growth factor
qRT-PCR	quantitative real time polymerase chain reaction

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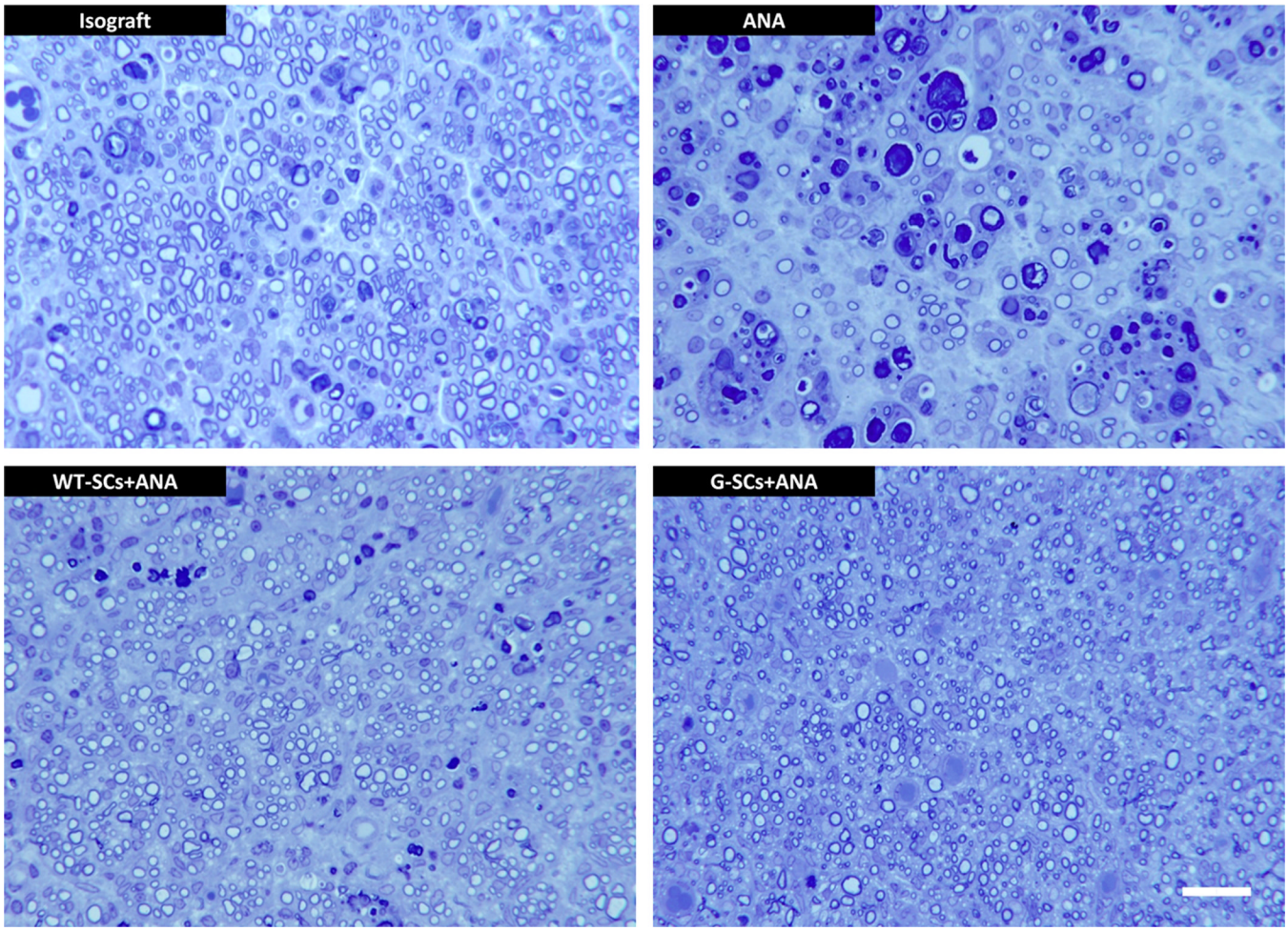

Figure 1. Light micrographs of nerve at the graft midline 6 weeks post-transplantation

All groups showed similar myelination of the axon fibers at the midpoint of the graft after 6 weeks. Qualitatively the ANA group appears to have fewer regenerating nerve fibers when compared to the Isograft. In contrast, the SCs+ANA and G-SCs+ANA look similar to the Isograft. Scale bar = 20 μm.

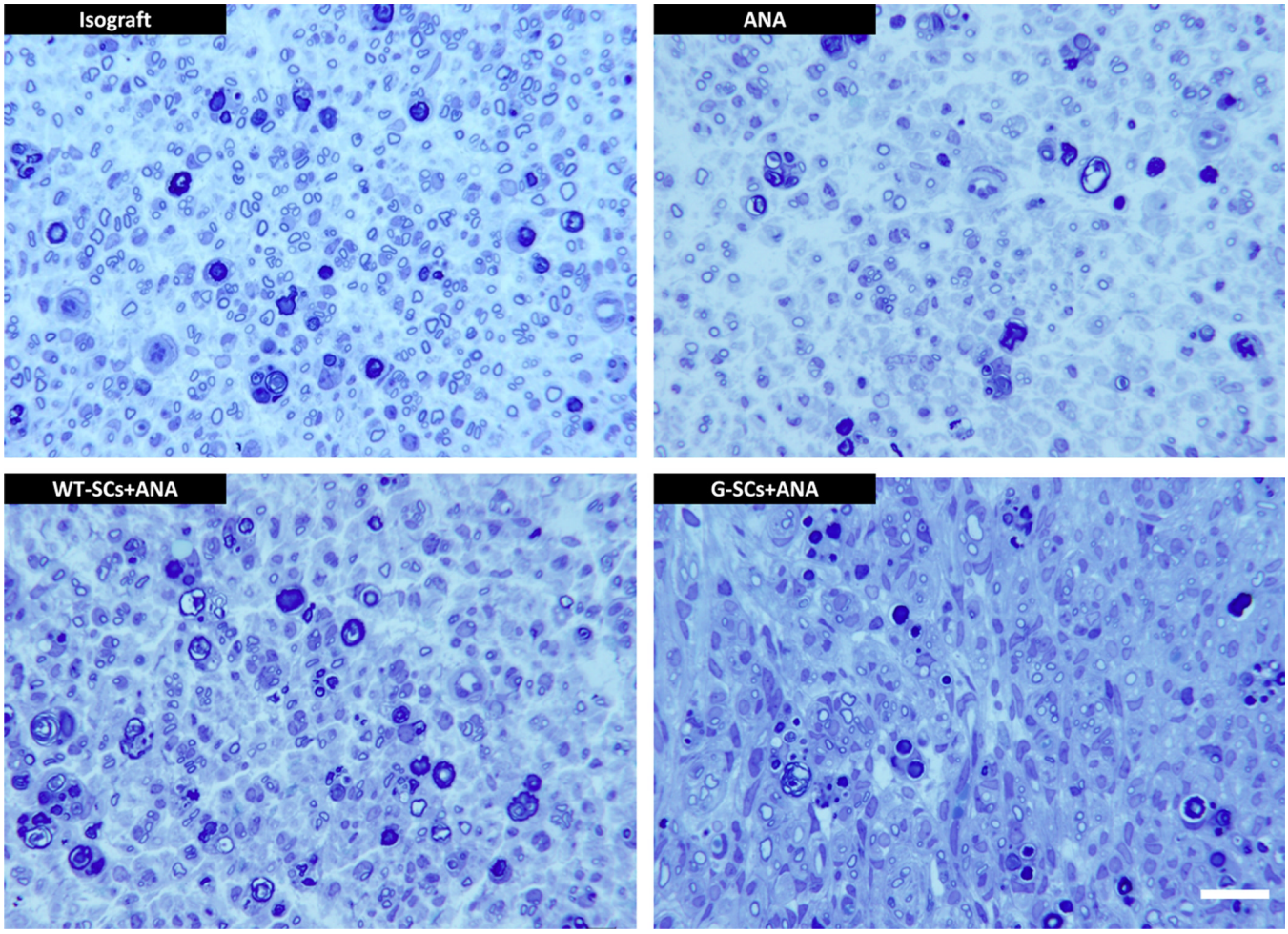

Figure 2. Light micrographs of nerve distal to the graft 6 weeks post-transplantation

The isograft group regenerated more myelinated nerve fibers than the ANA negative control group. The groups treated with WT-SCs+ANA closely approximate the myelination level and fiber number of the isografts. The G-SCs+ANA treated groups demonstrated less robust myelination distal to the nerve graft and qualitatively more closely resembled the ANA as opposed to the Isograft control. Scale bar = 30 μm.

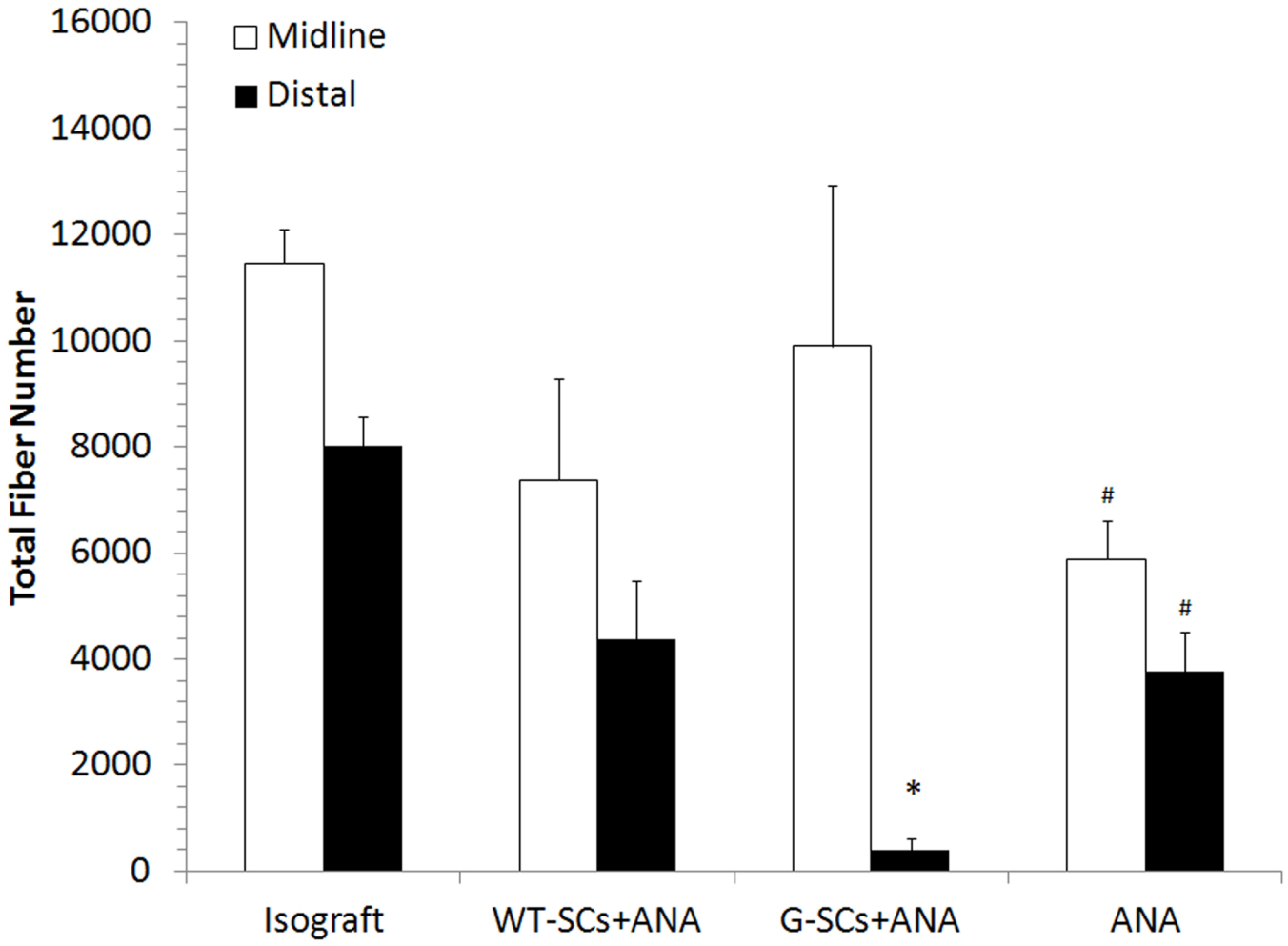

Figure 3. Total nerve fibers regenerated at mid-graft and segments distal to the nerve allografts 6 weeks post-transplantation

At the midpoint of the graft, the groups treated with WT-SCs+ANA and G-SCs+ANA regenerated a similar number of total nerve fibers when compared to the Isograft. All 3 groups (Isograft, WT-SCs+ANA, G-SCs+ ANA) had more regenerated nerve fibers than the ANA negative control group. Distal to the graft, the G-SCs+ANA group had significantly fewer fibers than the WT-SCs+ ANA, the isograft and the ANA groups. Error bars represent the standard error of the mean (n = 8). *# - $p < 0.05$ versus isograft at same location, * indicates $p<0.05$ versus WT-SCs+ANA and ANA; all differences are within the same location (i.e. midpoint or distal).*

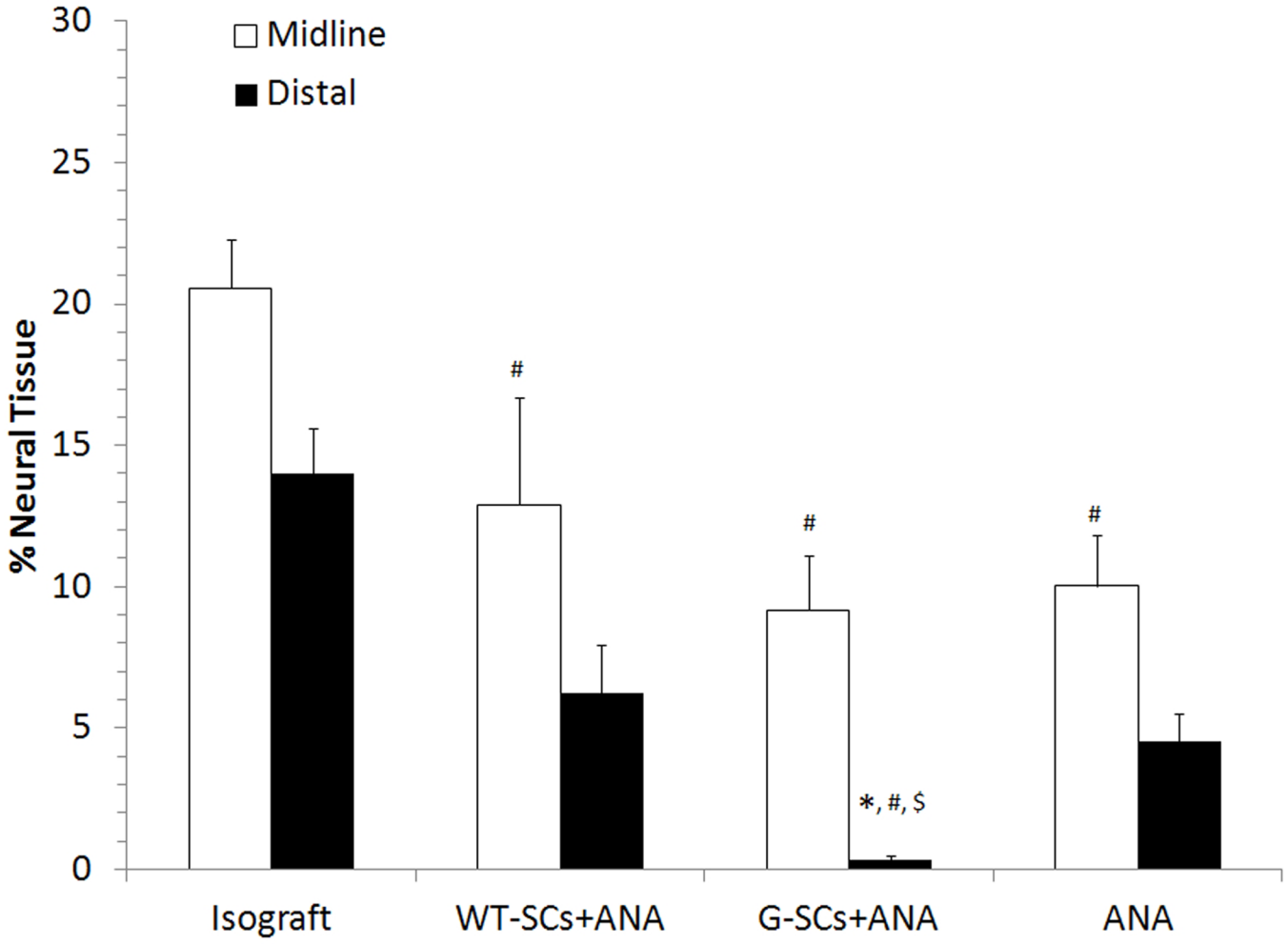

Figure 4. Percent neural tissue regenerated at mid-graft and segments distal to the nerve allografts 6 weeks post-transplantation

At the midpoint of the graft, more neural tissue was present in the isograft group compared to the other 3 groups. At the distal end of the graft, the G-SCs+ANA group demonstrated significantly fewer regenerated nerve fibers compared to the isograft. Error bars represent the standard error of the mean (n = 8). ** - denotes p<0.05 versus WT-SCs+ANA # - p < 0.05 versus isograft, $ - denotes p<0.05 versus ANA; all differences are within the same location (i.e. midpoint or distal).*

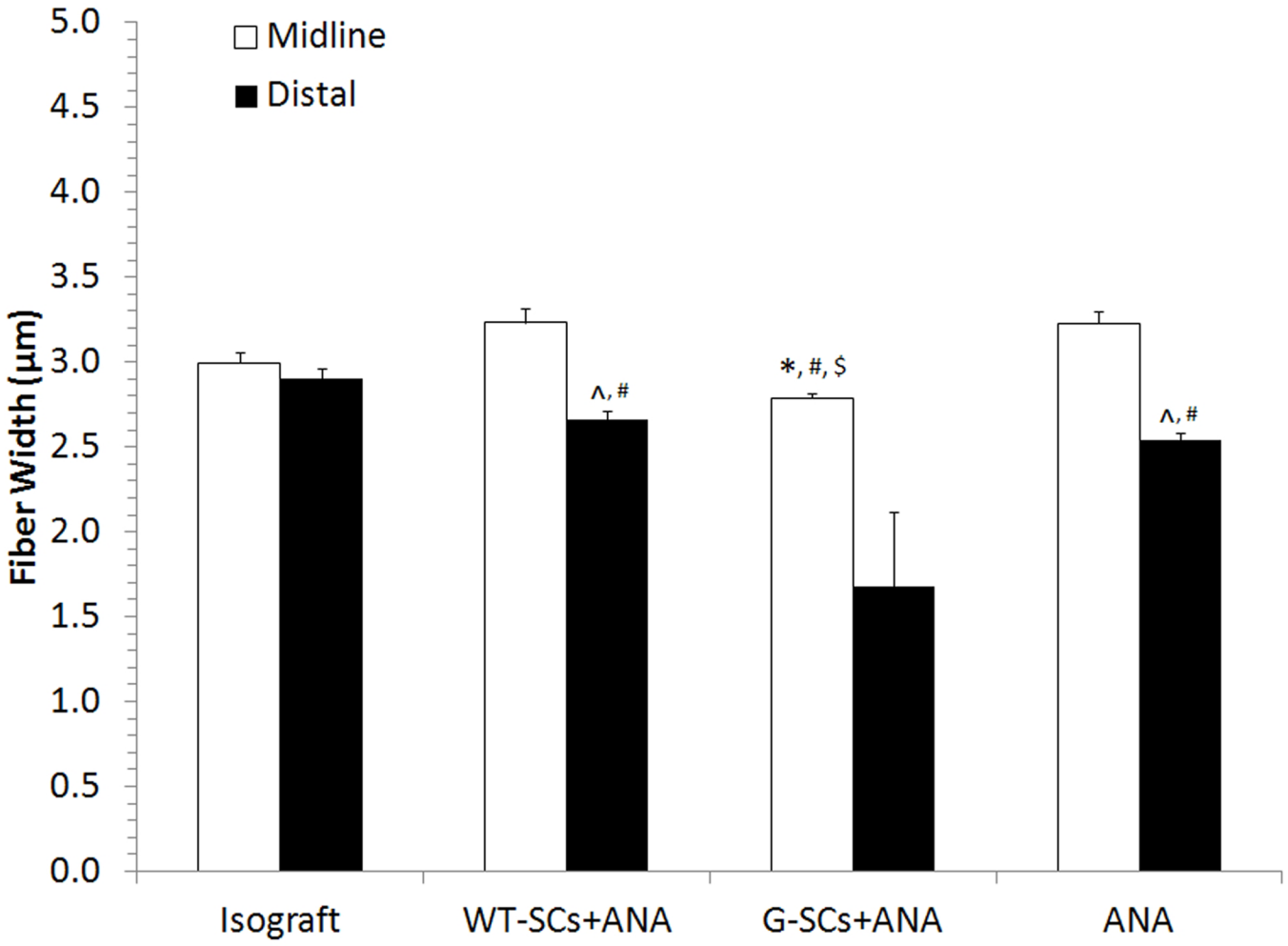

Figure 5. Fiber width of the myelinated fibers regenerated at mid-graft and segments distal to the nerve allografts 6 weeks post-transplantation

The fiber width of the G-SCs+ANA group was thinner than the fiber width of the isograft, WT-SCs+ ANA and ANA groups. Additionally, at the distal end, the isograft group had thicker fibers than all other groups. Error bars represent the standard error of the mean (n = 8). ** - denotes p<0.05 versus WT-SCs+ANA; # - p < 0.05 versus isograft; $ - denotes p<0.05 versusANAP; ^ - p<0.05 versus G-SCs+ANA; all differences are within the same location (i.e. midpoint or distal).*

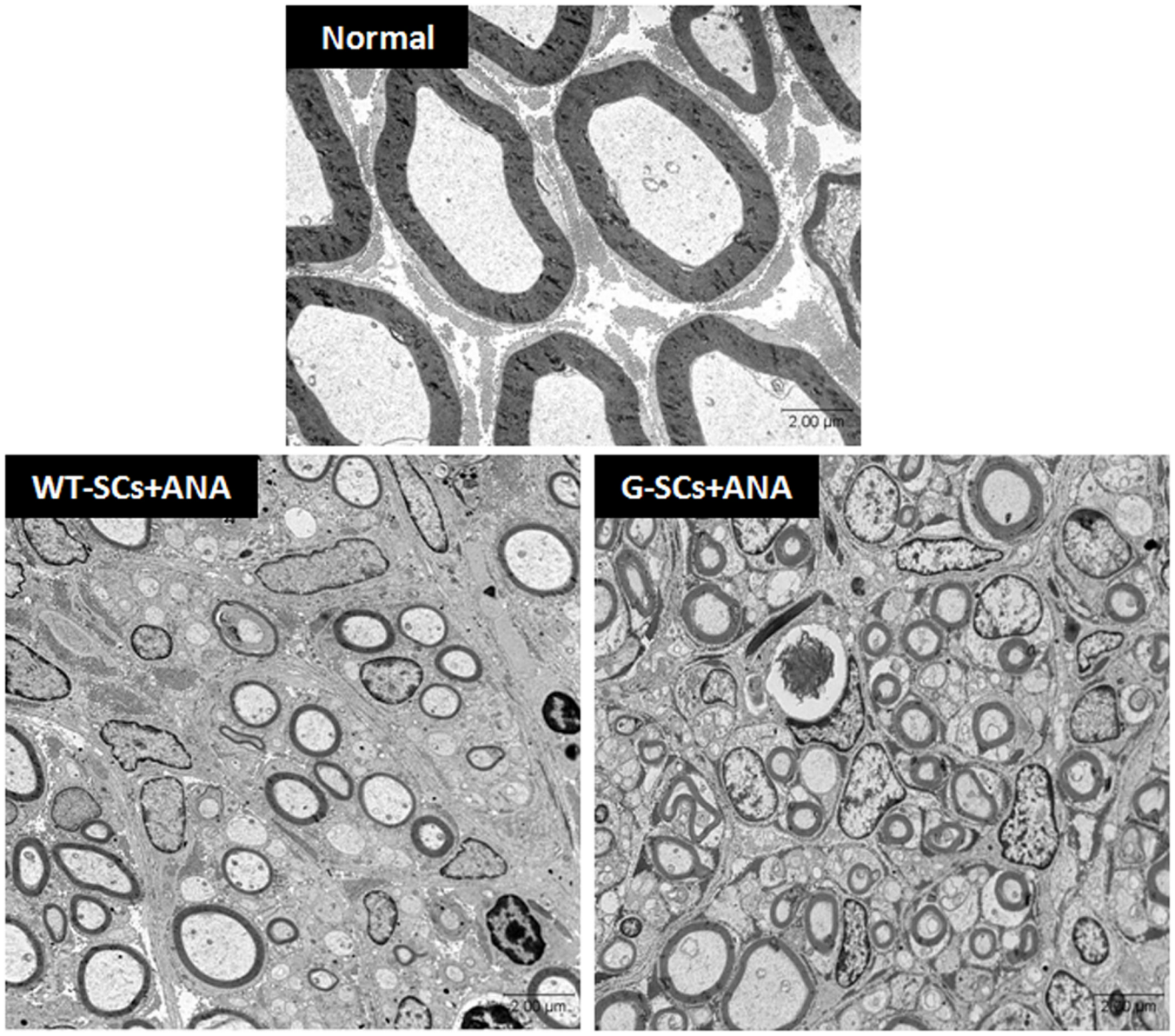

Figure 6. Electron micrographs of normal nerve, nerve allografts with SCs and G-SCs at mid-graft 6 weeks post-transplantation

The G-SCs+ANA group demonstrated an increased amount of axon bundling and myelination at the midpoint of the graft compared to the WT-SCs+ANA group.

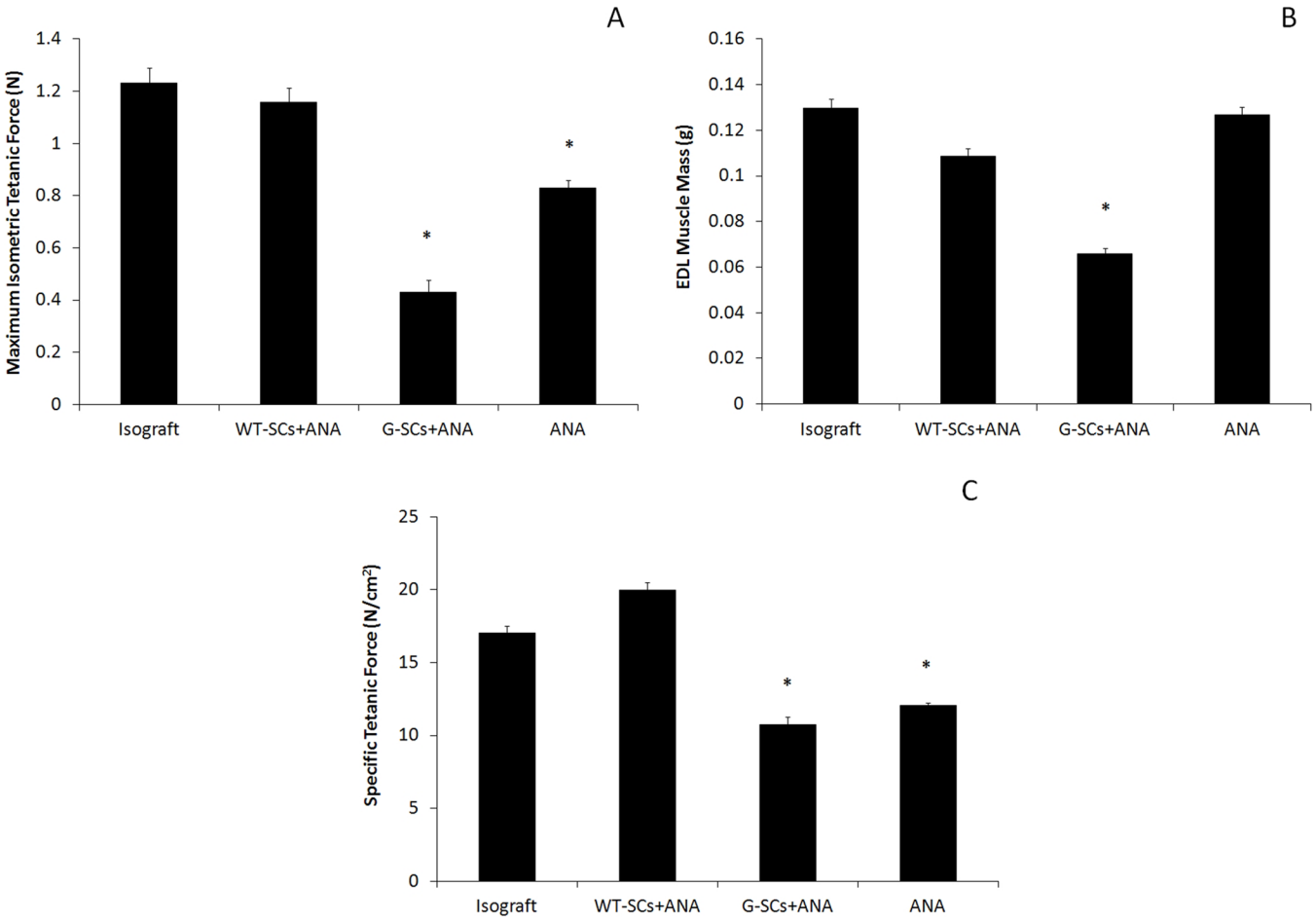

Figure 7. Evoked muscle force measurements reveal decreased motor recovery in distal musculature 12 weeks after implantation of processed nerve allografts seeded with Schwann cells overexpressing GDNF

(A) Measurements of maximum isometric tetanic force production in EDL muscle innervated by repaired sciatic nerve demonstrate that the ANA and G-SCs+ANA groups support significantly lower degrees of motor recovery compared to fresh nerve isografts. In comparison, the WT-SCs+ANA group supported an equivalent degree of motor recovery compared to fresh nerve isografts. (B) Assessment of EDL muscle mass shows that muscles innervated by sciatic nerves repaired with G-SCs+ANA exhibited significant atrophy unlike muscles innervated by sciatic nerves repaired with WT-SCs+ANA and ANA groups. (C) Calculation of specific tetanic force production in reinnervated EDL muscle reveals that, upon correction for differences in muscle atrophy, the ANA and G-SCs+ANA groups supported significantly lower degrees of motor recovery compared to fresh nerve isografts. Error bars represent the standard error of the mean (n = 6). * - *denotes $p<0.05$ versus isograft.*