Published in final edited form as:

Exp Neurol. 2014 July ; 0: 10–18. doi:10.1016/j.expneurol.2014.04.005.

Glial cell line-derived neurotrophic factor promotes increased phenotypic marker expression in femoral sensory and motor-derived Schwann cell cultures

Nithya J. Jesuraj¹, Laura M. Marquardt¹, Jasmine A. Kwasa¹, and Shelly E. Sakiyama-Elbert^{1,2,*}

¹Department of Biomedical Engineering, Washington University, St. Louis, MO 63130, USA

²Division of Plastic and Reconstructive Surgery, Department of Surgery, Washington University School of Medicine, St. Louis, MO 63110, USA

Abstract

Schwann cells (SCs) secrete growth factors and extracellular matrix molecules that promote neuronal survival and help guide axons during regeneration. Transplantation of SCs is a promising strategy for enhancing peripheral nerve regeneration. However, we and others have shown that after long-term *in vitro* expansion, SCs revert to a de-differentiated state similar to the phenotype observed after injury. *In vivo*, glial cell-line derived neurotrophic factor (GDNF) may guide the differentiation of SCs to remyelinate regenerating axons. Therefore, we hypothesized that exogenous GDNF may guide the differentiation of SCs into their native phenotypes *in vitro* through stimulation of GDNF family receptor (GFR) α -1. When activated in SCs, GFR α -1 promotes phosphorylation of Fyn, a Src family tyrosine kinase responsible for mediating downstream signaling for differentiation and proliferation. In this study, SCs harvested from the sensory and motor branches of rat femoral nerve were expanded *in vitro* and then cultured with 50 or 100 ng/mL of GDNF. The exogenous GDNF promoted differentiation of sensory and motor-derived SCs back to their native phenotypes, as demonstrated by decreased proliferation after 7 days and increased expression of S100 β and phenotype-specific markers. Furthermore, inhibiting Fyn with Src family kinase inhibitors, PP2 and SU6656, and siRNA-mediated knockdown of Fyn reduced GDNF-stimulated differentiation of sensory and motor-derived SCs. These results demonstrate that activating Fyn is necessary for GDNF-stimulated differentiation of femoral nerve-derived SCs into their native phenotypes *in vitro*. Therefore GDNF could be incorporated into SC-based therapies to promote differentiation of SCs into their native phenotype to improve functional nerve regeneration.

© 2014 Elsevier Inc. All rights reserved.

*To whom correspondence should be addressed: Shelly Sakiyama-Elbert, PhD, Department of Biomedical Engineering, Washington University, Campus Box 1097, One Brookings Drive, St. Louis, MO 63130, Telephone: +1 (314) 935-7556, Fax: +1 (314) 935-7448, sakiyama@wustl.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Keywords

nerve regeneration; peripheral nerve; cell transplantation; differentiation; Fyn

Introduction

In the peripheral nervous system, nerve injury is followed by Wallerian degeneration, in which the axons undergo demyelination. Typically after injury, SCs also de-differentiate into an immature phenotype, proliferate, and align in the remaining basal lamina to guide regenerating axons from the proximal to the distal end of the nerve (Burnett and Zager, 2004; Waller, 1850). The de-differentiated SCs revert to an immature state and secrete supporting molecules, such as growth factors and extracellular matrix molecules, to further support the regenerating axons (Brenner et al., 2005; Bunge, 1993; Frostick et al., 1998; Nagarajan et al., 2002; Reynolds and Woolf, 1993). As axons grow and reestablish contact with SCs, SCs receive cues from the environment to re-differentiate into mature phenotypes to myelinate and support axonal function (Jessen and Mirsky, 2005; Mirsky and Jessen, 1996). Determining the identity of cues from the environment that promote differentiation will allow for improved SC-based therapies.

Growth factors represent one potential group of environmental cues that could influence SC differentiation. GDNF is a growth factor present in the environment during nerve regeneration, and it has been shown to affect the behavior of SCs (Frostick et al., 1998). In SC-neuron co-cultures, the presence of GDNF enhances the myelination of axons (Hoke et al., 2003; Iwase et al., 2005). GDNF exerts its effects on SCs by binding to the glycosylphosphatidyl-inositol-anchored GFR α -1 (Naveilhan et al., 1997). The GFR α -1 receptor does not have an intracellular domain and thus needs to couple with other proteins to induce downstream signaling. In motor neurons, GFR α -1 couples with the proto-oncogene Ret to signal downstream (Soler et al., 1999). However, Ret is not expressed in SCs, therefore GFR α -1 couples with neural cell adhesion molecule (NCAM), which can act as a co-receptor to facilitate GDNF-mediated signaling in SCs (Iwase et al., 2005; Paratcha et al., 2003b). GDNF stimulation of GFR α -1/NCAM triggers phosphorylation of Fyn, a Src family tyrosine kinase. Previous studies have shown that exogenous GDNF affected SC proliferation and differentiation through signaling pathways downstream of Fyn (Iwase et al., 2005; Paratcha et al., 2003b). Therefore, GDNF may be a cue that promotes re-differentiation of SCs *in vivo* to myelinate axons.

The goal of the present study was to determine if GDNF affects SC differentiation *in vitro*. Previously, our lab and others have shown that SCs derived from the motor and sensory branches of rat femoral nerve have different phenotypic profiles, which are dysregulated after SC expansion in culture (Hoke et al., 2006; Jesuraj et al., 2012). We hypothesized that exogenous GDNF would promote differentiation of SCs after expansion culture and restore expression of SC phenotypic markers. Differentiation was assessed using a proliferation assay and quantitative real time polymerase chain reaction (qPCR) was used to measure gene expression of differentiation and phenotypic markers in SCs after 3 and 7 days of GDNF exposure.

To confirm that GDNF-mediated differentiation required activation of Fyn, inhibitors of Src family kinases, PP2 (Hanke et al., 1996) and SU6656 (Blake et al., 2000), were used to block Fyn phosphorylation. To verify the specificity of this pathway, LY294002, a phosphoinositide 3 (PI3) kinase inhibitor that blocks Ret-dependent GDNF signaling, was used as a control. Since Ret is not expressed in SCs, LY294002 should not affect GDNF-mediated differentiation in SCs. siRNA knockdown of Fyn was also performed to validate the role of Fyn activation in GDNF-mediated differentiation of SCs. The combined results of this study demonstrate that exogenous GDNF promotes differentiation and increased phenotypic marker expression in both sensory and motor-derived SCs *in vitro* in a dose-dependent manner that requires the activation of Fyn.

Materials and Methods

SC Isolation and Culture

SC cultures were prepared as previously described (Pruss, 1982; Raff et al., 1978). Briefly, sensory and motor branches of the rat femoral nerve were harvested and placed in Leibovitz's 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 x g for 5 min, the pellet was washed with Dulbecco's modified Eagle medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich, St. Louis, MO) and 1% antibiotic antimycotic (ABAM, Invitrogen). The cells were then seeded on 10 cm dishes coated with poly-L-lysine (pLL) (Sigma-Aldrich). Tissue culture plates 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, 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). Complement killing of fibroblasts was done as necessary, and the purity of the culture was assessed visually. After two passages, SCs were used for experiments.

Experimental design for dosage studies with GDNF

Motor or sensory-derived SCs were seeded separately onto 48-well plates at 3000 cells/cm² and serum-starved for 4 hours prior to growth factor addition. Previously, it has also been shown that nerve growth factor (NGF) promotes the differentiation of sciatic nerve-derived SCs through the p75 receptor (Hirata et al., 2001). Thus, NGF was used as a positive control for differentiation, and unsupplemented media (no NGF or GDNF, but including FBS) was used as a negative control. Cells were cultured with various growth factor concentrations in the media (0 ng/mL growth factor, 50 ng/mL GDNF, 100 ng/mL GDNF, 50 ng/mL NGF, and 100 ng/mL NGF) for 7 days in media containing DMEM, FBS and ABAM, changed every 2 days. SC pellets were collected on days 3 and 7 for proliferation analysis or RNA was collected from SCs on days 3 and 7. RNA was extracted and purified using an RNeasy Mini Kit (Qiagen). Samples were stored at -80°C until they were analyzed.

Inhibition of GDNF pathway

To determine the effects of inhibiting GDNF signaling via NCAM or Ret on SCs, sensory or motor-derived SCs were treated with different inhibitors (Table 1) of GDNF signaling. PP2 and SU6656 inhibit NCAM-mediated Fyn signaling (Blake et al., 2000; Hanke et al., 1996) and LY294002 inhibits Ret-mediated PI3 kinase signaling (Soler et al., 1999). SCs were seeded onto 48-well plates and incubated in serum-free media for 4 hours prior to treatment with PP2, SU6656 and LY294002 for 30 minutes as previously described (Iwase et al., 2005). The inhibitors were removed, GDNF (100 ng/ml) was then added to the media containing DMEM, FBS, ABAM, and the SCs were cultured for 7 days with media changed every 2 days. GDNF without inhibitor was used as a positive control and unsupplemented media (no NGF or GDNF, with FBS) was used as the negative control after 4 hours of serum starvation. SC pellets were collected on days 3 and 7 for proliferation analysis or RNA was collected from SCs on days 3 and 7 as described above.

Proliferation Assay

A CyQUANT® cell proliferation assay was used to assess the number of cells present in culture after the different treatments, according to the manufacturer's protocol. Cells were lysed, and the number of cells was determined from a standard curve of known cell number and intensity, using fluorescence with CyQUANT® GR dye, which strongly binds to nucleic acids.

A BrdU incorporation assay (Millipore, Billerica, MA) was also used to test the effect of GDNF treatment on motor and sensory-derived SC proliferation. SCs were seeded onto PLL-coated 96 well plates at 3,000 cells/well and cultured in SC media with 0, 50, or 100 ng/mL of GDNF for 3 and 7 days. BrdU measurements were performed according to manufacturer's instructions. Briefly, 24 hours prior to reading, BrdU reagent was added to culture media. Cells were fixed and subsequently stained with BrdU detection antibody and then an IgG- peroxidase conjugate secondary antibody. Absorbance measurements were read using a Multiskan RC plate reader at 450 and 590 nm.

Quantitative Real Time Polymerase Chain Reaction (qPCR)

cDNA was synthesized from isolated RNA using the QuantiTect® Reverse Transcription Kit (Qiagen). Using the QuantiTect® SYBR® Green PCR Mastermix (Qiagen) in combination with gene-specific QuantiTect® primers, qPCR was performed with an Applied Biosystems 7000 Real-Time PCR thermocycler. The genes studied included S100β (differentiated SC marker), nestin (undifferentiated SC marker) (Brookes et al., 1979), motor-specific markers, vascular endothelial cell growth factor (VEGF) and protein kinase C α (PRKCi), and sensory-specific markers, brain derived neurotrophic factor (BDNF) and myelin basic protein (MBP) (Hoke et al., 2006; Jesuraj et al., 2012). Primers (Table 2) were added to the cDNA for the motor and sensory-derived SCs. The qPCR was conducted using the following conditions: (1) 50°C for 2 min (2) 95°C for 15 min, and (3) forty cycles of 95°C for 15 seconds, 55°C for 30 seconds, and 72°C for 30 seconds (Gaumond et al., 2006). Target genes were normalized to an internal control (β -actin) to account for the variation in cDNA concentration between samples, and appropriate negative control samples were present (no template control). To estimate the mRNA concentrations, the differences in

gene expression levels between two samples were calculated using the comparative delta crossover threshold (C_t) method (Livak and Schmittgen, 2001; Pfaffl, 2001; Schmittgen and Livak, 2008).

Fyn siRNA knockdown

To verify the role of Fyn in GDNF signaling, knockdown of Fyn was performed using Fyn specific ON-Target plus SMARTpool siRNA (Dharmacon, Thermo Scientific, Lafayette, CO). Targeted RNA sequences included UCGAACGCAUGAAUUAUUAU, GUAGUCCCCUGUCACAAAG, AGAGGUACCUUUCUUAUCC, and UGACCUCCAUCCCCGAACUA. Briefly, sensory and motor-derived SCs were seeded at 3×10^3 cells/well into 48 well tissue culture plates in ABAM-free media. After 24 hours, media was removed and SCs were treated with 400 nM Fyn siRNA with oligofectamine reagent (Invitrogen) in Opti-MEM and ABAM-free, serum-free DMEM media for 4 hours. Control SCs were not treated with Fyn siRNA. After 4 hours, cells were given DMEM media supplemented with 3x normal FBS alone or with 100 ng/mL GDNF. Media was left alone for 72 hours, and then changed every 2 days until cell lysates were collected at 7 days. mRNA was extracted and qPCR was performed to probe for Fyn and S100 β expression levels.

GDNF Protein Release from SCs

GDNF released from motor and sensory-derived SCs was measured to determine baseline levels of GDNF production in de-differentiated SCs. SC media from sensory and motor-derived SC cultures was collected at days 2 and 7, and then stored at -20°C until analysis. GDNF levels in media were quantified using enzyme-linked immunosorption assay (ELISA) GDNF DuoSet (R&D Systems, Minneapolis, MN). Using a Multiskan RC multiwell plate spectrophotometer, the absorbance was read at 450 nm, with a 590 nm baseline subtraction, according to manufacturer's instructions. Standard curves using known GDNF concentrations in SC media allowed for calculation of GDNF concentration in samples.

Statistical Analysis

Statistical analyses were performed using SigmaStat 3.0 (Systat Software, San Jose, CA), and all data were evaluated with one-way analysis of variance (ANOVA), followed by a Scheffe's F test for comparisons between groups when significance ($p < 0.05$) was present. All results are reported as mean $\pm$ standard deviation.

Results

GDNF addition to the SC cultures reduces the proliferation of SCs in a dose-dependent manner

Previous studies have shown that SCs expanded in culture revert to a proliferative, de-differentiated state in response to mitogenic media (Monje et al., 2010; Morgan et al., 1991; Salzer et al., 1980). However, when SCs differentiate into mature cells, they transition into a post-mitotic state (Mirsky and Jessen, 1996). To determine whether exogenous GDNF would slow the proliferation of sensory and motor-derived SCs, the number of cells present in culture was determined after 3 and 7 days of GDNF exposure. Other studies have

suggested that NGF induces differentiation of SCs through p75 signaling (Hirata et al., 2001), which would also slow the proliferation of SCs. Therefore NGF was used as a positive control for promoting differentiation and unsupplemented media (without GDNF or NGF) was used as the negative control.

The number of cells present after addition of GDNF to the media was determined at 3 and 7 days using a proliferation assay. Supplementation of both sensory and motor-derived SCs with 50 or 100 ng/mL of GDNF resulted in a lower number of cells than the unsupplemented media control at both 3 and 7 days (Figure 1). The number of cells present after GDNF supplementation was similar to NGF for both types of SCs at each time point. To determine whether this effect was due to cell death or decreased proliferation, BrdU incorporation was measured for both sensory and motor-derived SCs with 50 or 100 ng/mL of GDNF in the media. Decreased BrdU incorporation was observed for both sensory and motor-derived SCs with 100 ng/mL of GDNF in the media (data not shown), suggesting a decrease in proliferation. The decrease in SC proliferation compared to unsupplemented media suggests that GDNF may be driving the SCs into a post-mitotic state, similar to NGF.

Inhibiting the GDNF signaling pathway reduces the proliferation of SCs in vitro

To further isolate the effects of GDNF on SC proliferation, GDNF signaling was inhibited by PP2, SU6656, and LY294002 (Table 1). PP2 and SU6656 are Src family kinase inhibitors that block GDNF-mediated ERK 1/2 and CREB phosphorylation from NCAM activation via inhibition of Fyn (Blake et al., 2000; Hanke et al., 1996; Iwase et al., 2005). LY294002 is an inhibitor that blocks GDNF-mediated PI3 kinase phosphorylation from Ret-dependent GDNF signaling in neurons. LY294002 was used as a control inhibitor because Ret-dependent signaling is not found in SCs (Iwase et al., 2005; Soler et al., 1999). After treatment with the different inhibitors, the numbers of both types of SCs were determined at 3 and 7 days. GDNF without inhibitors (100 ng/mL) was used as a positive control and unsupplemented media was used as a negative control. Groups treated with PP2 and SU6656 prior to GDNF addition resulted in lower numbers of cells than the unsupplemented group, but the cell number was higher than the groups treated with GDNF (no inhibitor) and LY294002 (Figure 2). These results suggest that inhibition of Fyn by PP2 and SU6656 prevented GDNF-dependent decreases in proliferation for both sensory and motor-derived SCs. Furthermore, addition of LY294002 to SCs has no effect on GDNF-dependent decreases in proliferation, suggesting that GDNF signaling in SCs is not PI3 kinase dependent.

GDNF promotes the differentiation of sensory and motor-derived SCs in a dose-dependent manner

To determine whether the reduction in SC proliferation was due to GDNF-mediated differentiation, qPCR was performed on RNA extracts collected after 3 and 7 days of GDNF exposure. The expression levels of S100 β and nestin were evaluated and compared to the expression levels in unsupplemented media at the same time point. For both sensory and motor-derived SCs, addition of 100 ng/mL GDNF increased the expression of S100 β after 7 days in a dose dependent manner compared to the 50 ng/mL and unsupplemented groups (Figure 3). The nestin expression levels were lower than the unsupplemented group at days 3

and 7 for the 100 ng/mL dose of GDNF in both types of cells. Although, the expression trend of increased S100 β and decreased nestin for the GDNF groups were similar to groups treated with NGF, the magnitude of the increased S100 β expression was greater with GDNF at day 7 compared to the NGF-treated groups (Figure 3). These expression patterns suggest GDNF may trigger downstream signaling in SCs to promote differentiation into a more mature phenotype *in vitro*, similar to NGF.

The increased expression of S100 β suggests that GDNF may also promote phenotype-specific marker expression in sensory and motor-derived SCs. In previous studies, phenotypic markers for both sensory and motor-derived SCs were identified (Table 2), and it was found that these markers are dysregulated when SCs are expanded in culture over 30 days (Jesuraj et al., 2012). Therefore, phenotypic marker expression was also evaluated using qPCR and compared to expression in unsupplemented cultures at the same time point. Exogenous GDNF promoted increased expression of phenotypic markers in sensory and motor-derived SC cultures in a dose-dependent manner. Sensory markers, MBP and BDNF, were upregulated in sensory-derived SCs after 7 days of 100 ng/mL GDNF treatment compared to the unsupplemented group (Figure 4A). In motor-derived SCs, the motor markers, VEGF and PRKCi, were upregulated with 100 ng/mL of GDNF after 7 days compared to the unsupplemented group (Figure 4B). These results together suggest that exogenous GDNF promotes differentiation of both types of SCs and increased expression of native sensory and motor phenotypic markers *in vitro*.

Inhibition of the GDNF signaling pathway prevents differentiation of SCs

To verify that SC differentiation was due to GDNF signaling through Fyn, GDNF signaling was blocked with kinase inhibitors PP2 and SU6656. SCs were treated with inhibitor for 4 hours prior to addition of GDNF (100 ng/mL) in the absence of serum. The expression levels of differentiation markers and phenotypic markers were evaluated and compared to the unsupplemented group. PP2 and SU6656-treated groups had S100 β and nestin expression levels similar to the unsupplemented group for both types of SCs (Figure 5). Furthermore, inhibition of Fyn signaling pathway with PP2 and SU6656 also prevented upregulation of native phenotypic marker expression. In sensory-derived SCs, MBP and BDNF expression levels were similar to that of the unsupplemented group (Figure 6A). Similarly, in motor-derived SCs, motor markers PRKCi and VEGF were expressed at similar levels compared to the unsupplemented group (Figures 6B). The effect of PP2 and SU6656 on native phenotypic marker expression suggests that these inhibitors blocked the downstream cascade necessary to promote differentiation of the SCs. However, groups treated with LY294002 prior to GDNF addition had similar expression profiles to GDNF-treated groups implying that LY294002, a PI3 kinase inhibitor, does not affect GDNF-stimulated differentiation in SCs. These results suggest that Fyn activation is necessary for GDNF-mediated differentiation of SCs into their native phenotypes.

Knockdown of Fyn prevents the differentiation of SCs

To further validate that Fyn activation is necessary for GDNF-mediated differentiation in SCs, target specific siRNA was used to knockdown Fyn in both sensory and motor-derived SCs. Knockdown of Fyn was observed in both sets of SCs 7 days after treatment, and

mRNA levels of Fyn in siRNA-treated SCs were approximately 50% that of SCs collected prior to treatment (Figure 7). Untreated and GDNF-treated SCs showed no change in Fyn mRNA levels, as well as SCs treated with both GDNF and Fyn siRNA, perhaps indicating recovery of Fyn expression with exogenous GDNF treatment. Furthermore, knockdown of Fyn had significant effects on the maturation of SCs using GDNF (Figure 8). In GDNF-treated SCs, S100 β levels were significantly upregulated compared to SCs at day 0 as previously mentioned. However, S100 β was downregulated in Fyn siRNA-treated SCs, and S100 β was not upregulated in SCs treated with both GDNF and siRNA-treated SCs, which further confirms the need for Fyn activation for the differentiation of SCs in response to GDNF.

Supraphysiological levels of GDNF are necessary to induce differentiation of SCs

To understand the levels of GDNF stimulation necessary to induce differentiation of SCs, the amounts of GDNF secreted by de-differentiated SCs were measured using ELISAs. The amounts of GDNF secreted by both sensory and motor-derived SCs were 30 – 60 pg/mL, which were more than three orders of magnitude lower than the amounts necessary to induce differentiation of the SCs into their mature native phenotypes (Table 3). This result indicates that de-differentiated SCs do not product enough endogenous GDNF to stimulate re-differentiation, and therefore require supraphysiological levels of GDNF to induce differentiation of SCs.

Discussion

A great deal of research has been done to study the differentiation of SCs in response to environmental cues during injury and development (Jaegle et al., 1996; Jessen and Mirsky, 2005; Mirsky and Jessen, 1996; Zorick and Lemke, 1996; Zorick et al., 1996). Understanding the environmental cues that influence the differentiation of SCs is important in designing of SC-based therapies for peripheral nerve injury. Environmental cues, such as growth factors, are present after injury and have been shown to affect the behavior of SCs. One such growth factor, GDNF, has been shown to promote not only the regeneration of injured axons but also the migration, proliferation, and differentiation of SCs *in vivo* (Airaksinen and Saarma, 2002; Iwase et al., 2005). Therefore, GDNF may be an important factor to include in SC-based therapies to promote differentiation of SCs for faster and robust myelination of regenerating axons. Prior to incorporating SCs and GDNF into therapies, it is important to assess the effects GDNF on de-differentiated SCs. In this study, we sought to evaluate *in vitro* the effects of GDNF on the differentiation of SCs derived from the sensory and motor branches of rat femoral nerve. The effects were studied using a proliferation assay to determine the number of cells present after GDNF addition and qPCR to evaluate the gene expression patterns of differentiation markers (S100 β – mature myelinating SCs, nestin – undifferentiated) and phenotypic markers (sensory SC – BDNF and MBP and motor SC – VEGF and PRKCi). Additionally, to isolate and to validate that SC differentiation was due to GDNF-mediated Fyn activation, multiple inhibitor (Table 1) and knockdown studies were conducted *in vitro*.

To transplant a sufficient number of SCs after injury, SCs must first be expanded *in vitro*. However, long term expansion of SCs in culture promotes their de-differentiation and dysregulation of phenotypic markers (Jesuraj et al., 2012). Although it has been shown that transplanting de-differentiated SCs at the injury site is beneficial and promotes regeneration similar to the isograft (Jesuraj et al., 2013), this approach could be improved with additional cues to promote better regeneration and functional recovery than isografts. More importantly, SCs may provide the trophic support necessary to guide regenerating sensory or motor axons to their correct end organ target. Without signaling from the environment, SCs de-differentiate into an immature state *in vitro* (Jessen and Mirsky, 2005; Mirsky and Jessen, 1996). Thus it is very important to assess environmental cues that promote the differentiation of sensory or motor-derived SCs into their appropriate native phenotype.

We found that exogenous GDNF promoted the differentiation of both sensory and motor-derived SCs, as demonstrated by increased expression of S100 β . Exogenous GDNF also increased the expression of phenotype-specific markers in both types of SCs: GDNF stimulated increased expression of sensory markers in sensory-derived SCs and motor markers in motor-derived SCs. This outcome is critical for restoring mature SC functions and we have not found any other factor that promotes this result *in vitro*.

Mechanistically, GDNF has been shown to activate pathways in SCs, through the GFR α -1 surface receptor (Naveilhan et al., 1997), implicated in cell migration, proliferation, differentiation and growth factor production (Ellerbroek et al., 2003; Grimm et al., 1998; Iwase et al., 2005; Kim et al., 1997; Kinameri and Matsuoka, 2003; Klemke et al., 1997; Lang et al., 1996; Meintanis et al., 2001; Morgan et al., 1991; Verity et al., 1998). Inhibitor studies to block the phosphorylation of Fyn and knockdown studies to reduce Fyn expression in SCs, demonstrate the need for Fyn activation for GDNF-mediated differentiation in SCs. However, the knockdown studies have also shown that after siRNA-mediated knockdown, addition of GDNF to the cultures restores the Fyn expression to baseline levels. This phenomenon may be due to the fact that NCAM levels increase in SCs in response to GDNF (Iwase et al., 2005). Greater NCAM levels present within a cell can lead to homophilic NCAM binding, which has also been shown to stimulate the activation of Fyn, thus increasing its expression level in SCs (Paratcha et al., 2003a; Sjostrand and Ibanez, 2008). Therefore, GDNF stimulation of GFR α -1/NCAM may not be the only mechanism by which Fyn is activated, but the increases in NCAM homophilic binding can also contribute to Fyn phosphorylation. Although the direct interaction of GDNF with the GFR α -1/NCAM co-receptors may not be the only method to activate Fyn, this study does demonstrate that GDNF promotes SC differentiation via a Fyn dependent mechanism, either through GDNF binding with GFR α -1/NCAM or homophilic binding of NCAM.

SC differentiation will slow down the proliferation of SCs as they transition into a post-mitotic state. In this study, addition of GDNF to the cultures slowed the proliferation of SCs and promoted expression of differentiation markers and native phenotypic markers in both sensory and motor-derived SCs. Furthermore, blocking the GDNF signaling pathway with PP2 and SU6656 prevented the differentiation of SCs, suggesting that Fyn activation is required for GDNF-mediated SC differentiation. These results suggest that GDNF can provide a cue to restore SC phenotypic (motor or sensory-specific) memory *in vitro*.

The delivery of growth factors to the site of injury is a common therapy used to guide the regeneration of axons across a nerve defect (Airaksinen and Saarma, 2002; Barras et al., 2002; Boyd and Gordon, 2003; Fine et al., 2002; Wood et al., 2009). Inclusion of SCs in therapies has also been shown to promote functional regeneration of peripheral nerves (Aszmann et al., 2008; Guenard et al., 1992; Hu et al., 2007; Kim et al., 1994; Levi et al., 1994). However, both types of treatments fail to surpass the regenerative capacity of isografts, which mimics the autografts used for clinical nerve repair. Therefore, incorporation of SCs and growth factors into therapeutic devices or grafts may improve beyond that of isografts. NGF and GDNF are two growth factors that have been used to promote regeneration of axons, and both are found in regenerating nerves (Barras et al., 2002; Costigan et al., 2002; Fu and Gordon, 1997; Lee et al., 2003; Nguyen et al., 1998; Otto et al., 1987; Santos et al., 1998; Wang et al., 2002; Wood et al., 2009). However, only GDNF has been shown to support the regeneration of both sensory and motor axons (Hoke et al., 2000; Naveilhan et al., 1997). Other studies have successfully seeded SCs overexpressing GDNF into guidance channels to guide regenerating axons in spinal cord injury (Deng et al., 2013; Deng et al., 2011). However, when SCs overexpressing GDNF were transplanted in acellular nerve grafts to treat peripheral nerve injury, the axons failed to regenerate beyond the graft into the distal stump (Santosa et al., 2013). Therefore, for greater chances of functional recovery, it is an advantage to use a combinatorial strategy incorporating SC transplantation and GDNF into a delivery system within a scaffold, such as fibrin (Wood et al., 2010; Wood et al., 2009). This controlled delivery of GDNF could promote differentiation into specific SC phenotypes, as well as guide the regeneration of both sensory and motor axons. Prior to transplanting SCs with GDNF, additional studies need to be done *in vitro* to verify that SCs can still differentiate into sensory or motor phenotypes while exposed to GDNF within a scaffold.

Another area of interest in designing peripheral nerve therapies is the inclusion of sufficient guidance cues to regenerate sensory or motor-specific axons. During regeneration, when axons are given equal access to sensory and motor pathways, motor axons tend to regenerate down the motor pathway. This phenomenon, preferential motor reinnervation, may be influenced by trophic support from the end organs (e.g. muscle and skin) or the SCs present within terminal nerve pathways (Brushart, 1988, 1993; Madison et al., 1996; Madison et al., 1999; Madison et al., 2007; Madison et al., 2009). Another study has shown that sensory axons may also regenerate preferentially down sensory pathways (Hoke et al., 2006). In a recent study, it was shown that motor axons regenerate towards the muscle even in the absence of SCs at the injury site. With no end organ contact, motor axon projection into the muscle branch was limited. In this study, it was concluded that the influence of the end organ was greater than the influence of SCs during regeneration (Madison et al. 2009). While this may be true for small defects in which direct suturing techniques are used to bridge the gap, SCs may play a greater role in guiding the regenerating axons towards their correct target end-organs in larger nerve gaps. Moreover, the phenotype of SCs may influence the regeneration accuracy of sensory or motor-specific axons. Therefore, designing therapies that could incorporate cues such as GDNF to differentiate SCs into their native phenotypes following transplantation could enable sensory or motor-specific

functional nerve regeneration that would surpass the functional regeneration capability of an isograft.

In conclusion, we have shown that exogenous GDNF in the environment of de-differentiated SCs derived from both sensory and motor branches of the rat femoral nerve promotes the differentiation into mature SCs expressing native sensory or motor markers *in vitro*. Additionally, activation of Fyn is necessary for GDNF-stimulated differentiation of SCs into their native phenotypes. Understanding how SC gene expression is affected by environmental cues will aid the design of more efficient SC-based therapies to promote faster and more robust regeneration of the peripheral nerves.

Acknowledgments

The authors were funded and supported by the NIH RO1 grant NS051706. The authors of this paper would also like to thank the Hope Center for Neurological Disorders at Washington University for use of the qPCR thermocycler. We would like to thank Dr. Susan Mackinnon's lab for providing us with the nerves from which we derived our SCs.

References

- Airaksinen MS, Saarna M. The GDNF family: signalling, biological functions and therapeutic value. *Nat Rev Neurosci.* 2002; 3:383–394. [PubMed: 11988777]
- Aszmann OC, Korak KJ, Luegmair M, Frey M. Bridging critical nerve defects through an acellular homograft seeded with autologous schwann cells obtained from a regeneration neuroma of the proximal stump. *J Reconstr Microsurg.* 2008; 24:151–158. [PubMed: 18438750]
- Barras FM, Pasche P, Bouche N, Aebischer P, Zurn AD. Glial cell line-derived neurotrophic factor released by synthetic guidance channels promotes facial nerve regeneration in the rat. *J Neurosci Res.* 2002; 70:746–755. [PubMed: 12444596]
- Blake RA, Broome MA, Liu X, Wu J, Gishizky M, Sun L, Courtneidge SA. SU6656, a selective src family kinase inhibitor, used to probe growth factor signaling. *Mol Cell Biol.* 2000; 20:9018–9027. [PubMed: 11074000]
- Boyd JG, Gordon T. Glial cell line-derived neurotrophic factor and brain-derived neurotrophic factor sustain the axonal regeneration of chronically axotomized motoneurons in vivo. *Experimental neurology.* 2003; 183:610–619. [PubMed: 14552902]
- Brenner MJ, Lowe JB 3rd, Fox IK, Mackinnon SE, Hunter DA, Darcy MD, Duncan JR, Wood P, Mohanakumar T. Effects of Schwann cells and donor antigen on long-nerve allograft regeneration. *Microsurgery.* 2005; 25:61–70. [PubMed: 15481042]
- Brockes JP, Fields KL, Raff MC. Studies on cultured rat Schwann cells. I. Establishment of purified populations from cultures of peripheral nerve. *Brain Res.* 1979; 165:105–118. [PubMed: 371755]
- Brushart TM. Preferential reinnervation of motor nerves by regenerating motor axons. *J Neurosci.* 1988; 8:1026–1031. [PubMed: 3346713]
- Brushart TM. Motor axons preferentially reinnervate motor pathways. *J Neurosci.* 1993; 13:2730–2738. [PubMed: 8501535]
- Bunge RP. Expanding roles for the Schwann cell: ensheathment, myelination, trophism and regeneration. *Current opinion in neurobiology.* 1993; 3:805–809. [PubMed: 8260833]
- Burnett MG, Zager EL. Pathophysiology of peripheral nerve injury: a brief review. *Neurosurgical focus.* 2004; 16:E1. [PubMed: 15174821]
- Costigan M, Belfort K, Karchewski L, Griffin RS, D'Urso D, Allchorne A, Sitariski J, Mannion JW, Pratt RE, Woolf CJ. Replicate high-density rat genome oligonucleotide microarrays reveal hundreds of regulated genes in the dorsal root ganglion after peripheral nerve injury. *BMC Neurosci.* 2002; 3:16. [PubMed: 12401135]
- Deng LX, Deng P, Ruan Y, Xu ZC, Liu NK, Wen X, Smith GM, Xu XM. A novel growth-promoting pathway formed by GDNF-overexpressing Schwann cells promotes propriospinal axonal

- regeneration, synapse formation, and partial recovery of function after spinal cord injury. *J Neurosci.* 2013; 33:5655–5667. [PubMed: 23536080]
- Deng LX, Hu J, Liu N, Wang X, Smith GM, Wen X, Xu XM. GDNF modifies reactive astrogliosis allowing robust axonal regeneration through Schwann cell-seeded guidance channels after spinal cord injury. *Experimental neurology.* 2011; 229:238–250. [PubMed: 21316362]
- Ellerbroek SM, Wennerberg K, Burridge K. Serine phosphorylation negatively regulates RhoA in vivo. *J Biol Chem.* 2003; 278:19023–19031. [PubMed: 12654918]
- Fine EG, Decosterd I, Papaloizos M, Zurn AD, Aebischer P. GDNF and NGF released by synthetic guidance channels support sciatic nerve regeneration across a long gap. *Eur J Neurosci.* 2002; 15:589–601. [PubMed: 11886440]
- Frostick SP, Yin Q, Kemp GJ. Schwann cells, neurotrophic factors, and peripheral nerve regeneration. *Microsurgery.* 1998; 18:397–405. [PubMed: 9880154]
- Fu SY, Gordon T. The cellular and molecular basis of peripheral nerve regeneration. *Mol Neurobiol.* 1997; 14:67–116. [PubMed: 9170101]
- Gaumont G, Tyropolis A, Grodzicki S, Bushmich S. Comparison of direct fluorescent antibody staining and real-time polymerase chain reaction for the detection of *Borrelia burgdorferi* in Ixodes scapularis ticks. *J Vet Diagn Invest.* 2006; 18:583–586. [PubMed: 17121087]
- Grimm L, Holinski-Feder E, Teodoridis J, Scheffer B, Schindelhauer D, Meitinger T, Ueffing M. Analysis of the human GDNF gene reveals an inducible promoter, three exons, a triplet repeat within the 3'-UTR and alternative splice products. *Hum Mol Genet.* 1998; 7:1873–1886. [PubMed: 9811930]
- Guenard V, Kleitman N, Morrissey TK, Bunge RP, Aebischer P. Syngeneic Schwann cells derived from adult nerves seeded in semipermeable guidance channels enhance peripheral nerve regeneration. *J Neurosci.* 1992; 12:3310–3320. [PubMed: 1527582]
- Hanke JH, Gardner JP, Dow RL, Changelian PS, Brissette WH, Weringer EJ, Pollok BA, Connelly PA. Discovery of a novel, potent, and Src family-selective tyrosine kinase inhibitor. Study of Lck- and FynT-dependent T cell activation. *J Biol Chem.* 1996; 271:695–701. [PubMed: 8557675]
- Hirata H, Hibasami H, Yoshida T, Ogawa M, Matsumoto M, Morita A, Uchida A. Nerve growth factor signaling of p75 induces differentiation and ceramide-mediated apoptosis in Schwann cells cultured from degenerating nerves. *Glia.* 2001; 36:245–258. [PubMed: 11746763]
- Hoke A, Cheng C, Zochodne DW. Expression of glial cell line-derived neurotrophic factor family of growth factors in peripheral nerve injury in rats. *Neuroreport.* 2000; 11:1651–1654. [PubMed: 10852218]
- Hoke A, Ho T, Crawford TO, LeBel C, Hilt D, Griffin JW. Glial cell line-derived neurotrophic factor alters axon schwann cell units and promotes myelination in unmyelinated nerve fibers. *J Neurosci.* 2003; 23:561–567. [PubMed: 12533616]
- Hoke A, Redett R, Hameed H, Jari R, Zhou C, Li ZB, Griffin JW, Brushart TM. Schwann cells express motor and sensory phenotypes that regulate axon regeneration. *J Neurosci.* 2006; 26:9646–9655. [PubMed: 16988035]
- Hu J, Zhu QT, Liu XL, Xu YB, Zhu JK. Repair of extended peripheral nerve lesions in rhesus monkeys using acellular allogenic nerve grafts implanted with autologous mesenchymal stem cells. *Experimental neurology.* 2007; 204:658–666. [PubMed: 17316613]
- Iwase T, Jung CG, Bae H, Zhang M, Soliven B. Glial cell line-derived neurotrophic factor-induced signaling in Schwann cells. *J Neurochem.* 2005; 94:1488–1499. [PubMed: 16086701]
- Jaegle M, Mandemakers W, Broos L, Zwart R, Karis A, Visser P, Grosveld F, Meijer D. The POU factor Oct-6 and Schwann cell differentiation. *Science.* 1996; 273:507–510. [PubMed: 8662541]
- Jessen KR, Mirsky R. The origin and development of glial cells in peripheral nerves. *Nat Rev Neurosci.* 2005; 6:671–682. [PubMed: 16136171]
- Jesuraj NJ, Nguyen PK, Wood MD, Moore AM, Borschel GH, Mackinnon SE, Sakiyama SE. Differential Gene Expression in Motor and Sensory Schwann Cells in the Rat Femoral Nerve. *J Neurosci Res.* 2012; 90:96–104. [PubMed: 21932366]
- Jesuraj NJ, Santosa KB, Macewan MR, Moore AM, Kasukurthi R, Ray WZ, Flagg ER, Hunter DA, Borschel GH, Johnson PJ, Mackinnon SE, Sakiyama-Elbert SE. Schwann cells seeded in acellular nerve grafts improve functional recovery. *Muscle & nerve.* 2013

- Kim DH, Connolly SE, Kline DG, Voorhies RM, Smith A, Powell M, Yoes T, Daniloff JK. Labeled Schwann cell transplants versus sural nerve grafts in nerve repair. *J Neurosurg.* 1994; 80:254–260. [PubMed: 8283264]
- Kim HA, DeClue JE, Ratner N. cAMP-dependent protein kinase A is required for Schwann cell growth: interactions between the cAMP and neuregulin/tyrosine kinase pathways. *J Neurosci Res.* 1997; 49:236–247. [PubMed: 9272646]
- Kinameri E, Matsuoka I. Autocrine action of BMP2 regulates expression of GDNF-mRNA in sciatic Schwann cells. *Brain Res Mol Brain Res.* 2003; 117:221–227. [PubMed: 14559157]
- Klemke RL, Cai S, Giannini AL, Gallagher PJ, de Lanerolle P, Cheresch DA. Regulation of cell motility by mitogen-activated protein kinase. *J Cell Biol.* 1997; 137:481–492. [PubMed: 9128257]
- Lang P, Gesbert F, Delespine-Carmagnat M, Stancou R, Pouchelet M, Bertoglio J. Protein kinase A phosphorylation of RhoA mediates the morphological and functional effects of cyclic AMP in cytotoxic lymphocytes. *Embo J.* 1996; 15:510–519. [PubMed: 8599934]
- Lee AC, Yu VM, Lowe JB 3rd, Brenner MJ, Hunter DA, Mackinnon SE, Sakiyama-Elbert SE. Controlled release of nerve growth factor enhances sciatic nerve regeneration. *Experimental neurology.* 2003; 184:295–303. [PubMed: 14637100]
- Levi AD, Guenard V, Aebischer P, Bunge RP. The functional characteristics of Schwann cells cultured from human peripheral nerve after transplantation into a gap within the rat sciatic nerve. *J Neurosci.* 1994; 14:1309–1319. [PubMed: 8120626]
- Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods.* 2001; 25:402–408. [PubMed: 11846609]
- Madison RD, Archibald SJ, Brushart TM. Reinnervation accuracy of the rat femoral nerve by motor and sensory neurons. *J Neurosci.* 1996; 16:5698–5703. [PubMed: 8795625]
- Madison RD, Archibald SJ, Lacin R, Krarup C. Factors contributing to preferential motor reinnervation in the primate peripheral nervous system. *J Neurosci.* 1999; 19:11007–11016. [PubMed: 10594081]
- Madison RD, Robinson GA, Chadaram SR. The specificity of motor neurone regeneration (preferential reinnervation). *Acta physiologica (Oxford, England).* 2007; 189:201–206.
- Madison RD, Sofroniew MV, Robinson GA. Schwann cell influence on motor neuron regeneration accuracy. *Neuroscience.* 2009; 163:213–221. [PubMed: 19505536]
- Meintanis S, Thomaidou D, Jessen KR, Mirsky R, Matsas R. The neuron-glia signal beta-neuregulin promotes Schwann cell motility via the MAPK pathway. *Glia.* 2001; 34:39–51. [PubMed: 11284018]
- Mirsky R, Jessen KR. Schwann cell development, differentiation and myelination. *Current opinion in neurobiology.* 1996; 6:89–96. [PubMed: 8794046]
- Monje PV, Soto J, Bacallao K, Wood PM. Schwann cell dedifferentiation is independent of mitogenic signaling and uncoupled to proliferation: role of cAMP and JNK in the maintenance of the differentiated state. *J Biol Chem.* 2010; 285:31024–31036. [PubMed: 20634285]
- Morgan L, Jessen KR, Mirsky R. The effects of cAMP on differentiation of cultured Schwann cells: progression from an early phenotype (04+) to a myelin phenotype (P0+, GFAP-, N-CAM-, NGF-receptor-) depends on growth inhibition. *J Cell Biol.* 1991; 112:457–467. [PubMed: 1704008]
- Nagarajan R, Le N, Mahoney H, Araki T, Milbrandt J. Deciphering peripheral nerve myelination by using Schwann cell expression profiling. *Proceedings of the National Academy of Sciences of the United States of America.* 2002; 99:8998–9003. [PubMed: 12084938]
- Naveilhan P, ElShamy WM, Ernfors P. Differential regulation of mRNAs for GDNF and its receptors Ret and GDNFR alpha after sciatic nerve lesion in the mouse. *Eur J Neurosci.* 1997; 9:1450–1460. [PubMed: 9240402]
- Nguyen QT, Parsadanian AS, Snider WD, Lichtman JW. Hyperinnervation of neuromuscular junctions caused by GDNF overexpression in muscle. *Science.* 1998; 279:1725–1729. [PubMed: 9497292]
- Otto D, Unsicker K, Grothe C. Pharmacological effects of nerve growth factor and fibroblast growth factor applied to the transected sciatic nerve on neuron death in adult rat dorsal root ganglia. *Neurosci Lett.* 1987; 83:156–160. [PubMed: 3441295]
- Paratcha G, Ledda F, Ibanez CF. The neural cell adhesion molecule NCAM is an alternative signaling receptor for GDNF family ligands. *Cell.* 2003a; 113:867–879. [PubMed: 12837245]

- Paratcha G, Ledda F, Ibáñez CF. The Neural Cell Adhesion Molecule NCAM Is an Alternative Signaling Receptor for GDNF Family Ligands. *Cell*. 2003b; 113:867–879. [PubMed: 12837245]
- Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic acids research*. 2001; 29:e45. [PubMed: 11328886]
- Pruss RM. The potential use of cultured cells to detect non-neuronal targets of neuropeptide action. *Peptides*. 1982; 3:231–233. [PubMed: 6750565]
- Raff MC, Abney E, Brockes JP, Hornby-Smith A. Schwann cell growth factors. *Cell*. 1978; 15:813–822. [PubMed: 215315]
- Reynolds ML, Woolf CJ. Reciprocal Schwann cell-axon interactions. *Current opinion in neurobiology*. 1993; 3:683–693. [PubMed: 8260817]
- Salzer JL, Williams AK, Glaser L, Bunge RP. Studies of Schwann cell proliferation. II. Characterization of the stimulation and specificity of the response to a neurite membrane fraction. *J Cell Biol*. 1980; 84:753–766. [PubMed: 7358797]
- Santos X, Rodrigo J, Hontanilla B, Bilbao G. Evaluation of peripheral nerve regeneration by nerve growth factor locally administered with a novel system. *J Neurosci Methods*. 1998; 85:119–127. [PubMed: 9874148]
- Santosa KB, Jesuraj NJ, Viader A, MacEwan M, Newton P, Hunter DA, Mackinnon SE, Johnson PJ. Nerve allografts supplemented with schwann cells overexpressing glial-cell-line-derived neurotrophic factor. *Muscle & nerve*. 2013; 47:213–223. [PubMed: 23169341]
- Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. *Nat Protoc*. 2008; 3:1101–1108. [PubMed: 18546601]
- Sjostrand D, Ibanez CF. Insights into GFRalpha1 regulation of neural cell adhesion molecule (NCAM) function from structure-function analysis of the NCAM/GFRalpha1 receptor complex. *J Biol Chem*. 2008; 283:13792–13798. [PubMed: 18353777]
- Soler RM, Dolcet X, Encinas M, Egea J, Bayascas JR, Comella JX. Receptors of the glial cell line-derived neurotrophic factor family of neurotrophic factors signal cell survival through the phosphatidylinositol 3-kinase pathway in spinal cord motoneurons. *J Neurosci*. 1999; 19:9160–9169. [PubMed: 10531419]
- Verity AN, Wyatt TL, Hajos B, Eglén RM, Baecker PA, Johnson RM. Regulation of glial cell line-derived neurotrophic factor release from rat C6 glioblastoma cells. *J Neurochem*. 1998; 70:531–539. [PubMed: 9453547]
- Waller AV. Experiments on the glossopharyngeal and hypoglossal nerves of the frog and observations produced thereby in the structure of their primitive fibres. *Phil Trans R Soc Lond*. 1850; 140
- Wang CY, Yang F, He XP, Je HS, Zhou JZ, Eckermann K, Kawamura D, Feng L, Shen L, Lu B. Regulation of neuromuscular synapse development by glial cell line-derived neurotrophic factor and neurturin. *J Biol Chem*. 2002; 277:10614–10625. [PubMed: 11790765]
- Wood MD, MacEwan MR, French AR, Moore AM, Hunter DA, Mackinnon SE, Moran DW, Borschel GH, Sakiyama-Elbert SE. Fibrin matrices with affinity-based delivery systems and neurotrophic factors promote functional nerve regeneration. *Biotechnol Bioeng*. 2010; 106:970–979. [PubMed: 20589674]
- Wood MD, Moore AM, Hunter DA, Tuffaha S, Borschel GH, Mackinnon SE, Sakiyama-Elbert SE. Affinity-based release of glial-derived neurotrophic factor from fibrin matrices enhances sciatic nerve regeneration. *Acta Biomater*. 2009; 5:959–968. [PubMed: 19103514]
- Zorick TS, Lemke G. Schwann cell differentiation. *Curr Opin Cell Biol*. 1996; 8:870–876. [PubMed: 8939676]
- Zorick TS, Syroid DE, Arroyo E, Scherer SS, Lemke G. The transcription factors SCIP and Krox-20 mark distinct stages and cell fates in Schwann cell differentiation. *Mol Cell Neurosci*. 1996; 8:129–145.

Highlights

- Schwann cell (SC) proliferation slows with GDNF and NGF treatment
- GDNF increases expression of S100 and decreases expression of nestin
- GDNF increase appropriate expression of phenotype-specific SC markers
- Fyn activity is required for GDNF induced changes in gene expression

Figure 1. Addition of GDNF to the SC media promotes decreased proliferation of SCs

The proliferation of sensory or motor-derived SCs in response to addition of GDNF to the culture was monitored over 7 days. SCs were supplemented with GDNF had a similar decrease in proliferation compared to NGF at both 50 and 100 ng/mL. The number of cells present after 7 days was lower than the unsupplemented group. Error bars represent standard deviation (n=6). Dotted line represents initial seeding density. * denotes $p < 0.05$ compared to the same time point in the NGF and GDNF treated groups.

Figure 2. Treatment of cultures with Src family kinase inhibitors of the GDNF signaling pathway prior to GDNF addition promotes proliferation

50μM PP2, 10μM SU6656 and 50μM LY294002 were added to sensory and motor-derived SC cultures for 30 minutes prior to treatment with 100 ng/mL of GDNF. The number of cells present after 3 and 7 days was then determined using a proliferation assay. The groups treated with PP2 and SU6656 had lower number of cells after 3 and 7 days compared to the unsupplemented group, but had a higher number than the groups treated with LY294002 and GDNF. The groups treated with LY294002 had a similar number of cells present after 3 and 7 days as the 100 ng/mL GDNF group. Error bars represent standard deviation (n=6). Dotted line represents initial seeding density. * denotes $p < 0.05$ compared to the same time point in all other groups, # denotes $p < 0.05$ compared to the same time point in for the LY294002 and GDNF groups.

Figure 3. Addition of GDNF to the media promotes increased expression of S100 β and decreased expression of nestin in a dose-dependent manner

The expression of S100 β and nestin was monitored over 7 days in the cultures treated with GDNF and NGF (positive control). qPCR was used to determine the gene expression of each marker and the values were normalized to β -actin. The fold difference in gene expression for sensory and motor-derived SCs were compared the unsupplemented control group. The expression levels of S100 β increased in the sensory and motor-derived SCs in a dose-dependent manner. The changes in S100 β levels for the GDNF-treated groups showed a similar trend to the NGF groups. However, increases in S100 β levels were higher for the 100 ng/mL GDNF group compared to NGF groups. Error bars represent the standard deviation (n=3). **dotted line at 2 is the threshold value for upregulation. The dotted line at 1 represents similar expression to unsupplemented. * denotes $p < 0.05$ vs. 50 ng/mL GDNF at the same time point, # denotes $p < 0.05$ vs. unsupplemented, \$ denotes $p < 0.05$ vs. 50 ng/mL GDNF group at same time point, ^ denotes $p < 0.05$ vs. 100 ng/mL NGF groups at same time point

Figure 4. SC cultures treated with GDNF promotes increased native phenotypic marker expression in a dose-dependent manner

The expression patterns of BDNF and MBP (sensory markers) (A) and VEGF and PRKCi (motor markers) (B) was monitored over 7 days after addition of GDNF to the cell culture. The fold difference of gene expression for SCs grown with and without GDNF was compared to the unsupplemented control. qPCR was used to determine the gene expression of each marker and the values were normalized to β -actin. Addition of GDNF to SC cultures promoted increased sensory marker expression in sensory-derived SCs and motor marker expression in motor-derived SCs over 7 days. Error bars represent the standard deviation (n=3). **dotted line at 2 is the threshold value for upregulation. The dotted line at 1 represents similar expression to unsupplemented. * denotes $p < 0.05$ vs. 50 ng/mL at the same time point, # denotes $p < 0.05$ vs. unsupplemented.

Figure 5. Blocking the GDNF signaling pathway with Src family kinase inhibitors prevents increased expression of S100 β

The expression of S100 β and nestin was monitored over 7 days in the cultures treated with GDNF signaling pathway inhibitors and GDNF. qPCR was used to determine the gene expression of each marker and the values were normalized to β -actin. The fold difference in gene expression for sensory and motor-derived SCs was compared to the unsupplemented control. The expression levels of S100 β and nestin for groups treated with PP2 and SU6656 had similar levels of expression to the unsupplemented group. The group treated with LY294002 had similar levels of S100 β upregulation to the 100 ng/mL GDNF group. Error bars represent the standard deviation (n=3). **dotted line at 2 is the threshold value for upregulation. The dotted line at 1 represents similar expression to unsupplemented control. * denotes $p < 0.05$ vs. PP2 at the same time point, # denotes $p < 0.05$ vs. SU6656 at the same time point, \$ denotes $p < 0.05$ vs. the unsupplemented control.

Figure 6. Blocking the GDNF signaling pathway with Src family kinase inhibitors prevents expression of phenotypic markers in both sensory-derived SCs and motor-derived SCs
 The expression levels of MBP and BDNF (sensory markers) (A) and VEGF and PRKCI (motor markers) (B) was monitored over 7 days in the cultures treated with GDNF signaling pathway inhibitors (50 μ M PP2, 10 μ M SU6656 and 50 μ M LY294002) and 100 ng/mL GDNF. qPCR was used to determine the gene expression of each marker and the values were normalized to β -actin. The fold difference in gene expression for sensory and motor-derived SCs was compared to the unsupplemented control. The addition of PP2 and SU6656 prevented upregulation of sensory markers in sensory-derived SCs and motor markers in motor-derived SCs. The group treated with LY294002 had similar phenotypic marker expression levels compared to the 100 ng/mL GDNF group. Error bars represent the standard deviation (n=3). **dotted line at 2 is the threshold value for upregulation. The dotted line at 1 represents similar expression to unsupplemented control. * denotes $p < 0.05$ vs. PP2 at the same time point, # denotes $p < 0.05$ vs. SU6656 at the same time point, \$ denotes $p < 0.05$ vs. the unsupplemented control.

Figure 7. GDNF addition after siRNA-mediated Fyn knockdown raises Fyn mRNA expression to normal levels

The mRNA fold change of Fyn tyrosine kinase in untreated SCs, 100 ng/mL GDNF treated SCs, 400 nM Fyn siRNA treated SCs, and GDNF + Fyn siRNA treated SCs were evaluated at day 7 and compared to SCs at day 0. Fyn siRNA treatment leads ~50% decrease of mRNA levels in motor and sensory SCs at day 7. However, GDNF treatment trended to raise Fyn levels to normal after siRNA treatment. Data is shown as average fold change and dotted lines represent upregulation at 2 and downregulation at 0.5. * denotes $p < 0.05$ vs. untreated SCs and GDNF treated SCs.

Figure 8. Treatment of SCs with 400 nM of Fyn siRNA inhibits the effect GDNF treatment has on SC differentiation into mature SCs

Both sensory and motor-derived SCs treated with 100 ng/mL of GDNF show increased levels of S100B β compared to day 0. Treatment with Fyn siRNA alone, or in combination with GDNF, prevented any effect on S100B β levels. Data is shown as average fold change and dotted lines represent upregulation at 2 and downregulation at 0.5. * denotes $p < 0.05$ vs. all other conditions # denotes $p < 0.05$ vs. from Fyn treated SCs.

Table 1

Inhibitors used to block GDNF-stimulated downstream signalling

Inhibitor	Concentration	Characterization
PP2	50 μ M	Inhibitor of Src family kinases ¹
SU6656	10 μ M	Inhibitor of Src family kinases ²
LY294002	50 μ M	PI3 kinase inhibitor in Ret dependent signaling ³

¹ (Hanke et al. 1996)² (Blake et al. 2000)³ (Soler et al. 1999)

Blake RA, Broome MA, Liu X, Wu J, Gishizky M, Sun L, Courtneidge SA. 2000. SU6656, a selective src family kinase inhibitor, used to probe growth factor signaling. *Mol Cell Biol* 20:9018–27.

Hanke JH, Gardner JP, Dow RL, Changelian PS, Brissette WH, Weringer EJ, Pollok BA, Connelly PA. 1996. Discovery of a novel, potent, and Src family-selective tyrosine kinase inhibitor. Study of Lck- and FynT-dependent T cell activation. *J Biol Chem* 271:695–701.

Soler RM, Dolcet X, Encinas M, Egea J, Bayascas JR, Comella JX. 1999. Receptors of the glial cell line-derived neurotrophic factor family of neurotrophic factors signal cell survival through the phosphatidylinositol 3-kinase pathway in spinal cord motoneurons. *J Neurosci* 19:9160–9.

Table 2

List of genes used for qPCR

Gene	Marker of
S100	Differentiated SCs ^{1,2}
Nestin	Un-differentiated SCs ^{1,2}
Myelin basic protein (MBP)	Sensory-derived SCs ³
Brain-derived neurotrophic factor (BDNF)	Sensory-derived SCs ⁴
Protein kinase C iota (PRKCi)	Motor-derived SCs ³
Vascular endothelial growth factor (VEGF)	Motor-derived SCs ⁴
Fyn	Fyn, a Src tyrosine kinase

¹
(Brockes et al. 1979)²
(Raff et al. 1979)³
(Jesuraj et al. 2012)⁴
(Hoke et al. 2006)

Brockes JP, Fields KL, Raff MC. 1979. Studies on cultured rat Schwann cells. I. Establishment of purified populations from cultures of peripheral nerve. *Brain Res* 165:105–18.

Hoke A, Redett R, Hameed H, Jari R, Zhou C, Li ZB, Griffin JW, Brushart TM. 2006. Schwann cells express motor and sensory phenotypes that regulate axon regeneration. *J Neurosci* 26:9646–55.

Jesuraj NJ, Nguyen PK, Wood MD, Moore AM, Borschel GH, Mackinnon SE, Sakiyama SE. 2012. Differential Gene Expression in Motor and Sensory Schwann Cells in the Rat Femoral Nerve. *J Neurosci Res* 90:96–104.

Raff MC, Brockes JP, Fields KL, Mirsky R. 1979. Neural cell markers: the end of the beginning. *Prog Brain Res* 51:17–22.

Table 3

Endogenous GDNF levels in motor- and sensory-derived SCs

	GDNF Concentration (pg/mL)	
	Motor-derived SCs	Sensory-derived SCs
Day 2	44.28 ± 26.27	59.92 ± 5.22
Day 7	31.17 ± 21.86	45.58 ± 0.81