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GDNF reverses the inhibitory properties of reactive astrocytes allowing robust axonal regeneration through Schwann cell-seeded guidance channels after spinal cord injury

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Abstract

Reactive astrogliosis has been considered as a major impediment for axonal regeneration after injuries in the mammalian central nervous system (CNS). Here we report that glial cell line-derived neurotrophic factor (GDNF), combined with transplanted Schwann cells (SCs), effectively reversed the inhibitory properties of astrocytes at graft-host interfaces allowing robust axonal regeneration, concomitant with vigorous migration of host astrocytes, into SC-seeded semi-permeable guidance channels implanted into a right-sided spinal cord hemisection at the 10th thoracic (T10) level. Within the graft, migrated host astrocytes were in close association with regenerated axons with their processes extended parallel to the axons, implying that the migrated astrocytes were not inhibitory and might have promoted directional growth of regenerated axons. *In vitro*, GDNF induced migration of SCs and astrocytes toward each other in an astrocyte-SC confrontation assay. GDNF also enhanced migration of astrocytes on a SC monolayer in an inverted coverslip migration assay, suggesting that this effect is mediated by a direct cell-cell contact between the two cell types. Morphologically, GDNF administration reduced astrocyte hypertrophy and induced elongated process extension of these cells, similar to what was observed *in vivo*. Notably, GDNF treatment significantly reduced production of glial fibrillary acidic protein (GFAP) and chondroitin sulfate proteoglycans (CSPGs), two hallmarks of astrogliosis, in both the *in vivo* and *in vitro* models. Thus, our study has demonstrated, for the first time, a novel role and mechanism of GDNF on modification of spinal cord injury (SCI)-induced astrogliosis resulting in robust axonal regeneration in adult rats.

Keywords

axonal regeneration; GDNF; reactive astrocytes; Schwann cells; Schwann cell; spinal cord injury; transplantation; astrogliosis; axon; myelination; regeneration

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Introduction

Glial cell line-derived neurotrophic factor (GDNF) and its receptors are widely expressed in the developing (Oppenheim et al., 1995) and adult central nervous system (CNS) (Arenas et al., 1995; Buj-Bello et al., 1995). Two receptors for GDNF, i.e. GFR α 1 and/or c-Ret, are expressed in not only neurons, but also Schwann cells (SCs) and astrocytes (Widenfalk et al., 2001). In addition to its effect on neuron survival (Kordower et al., 2000; Perrelet et al., 2002) and axonal regeneration (Iannotti et al., 2003; Mills et al., 2007), growing evidence suggests that the GDNF effect on axon regeneration may be mediated through affecting the behavior of glial cells (Paratcha et al., 2003; Iwase et al., 2005). Whether GDNF plays a role in modification of astrogliosis and subsequently promotion of axonal regeneration remain unclear.

Reactive astrogliosis, developed in response to injuries of the CNS, is a major impediment for axonal regeneration. Following spinal cord injury (SCI), astrocytes at and near the injury border adopt a reactive phenotype in characters of hypertrophic morphology, expressing elevated levels of glial fibrillary acidic protein (GFAP), and releasing inhibitory extracellular matrix molecules chondroitin sulphate proteoglycans (CSPGs) (Predy and Malhotra, 1989; Chau et al., 2004). It is the physical and chemical barriers formed by reactive astrogliosis inhibited axonal regeneration through and beyond injuries in the CNS (Reier et al., 1983; Fitch and Silver, 2008). However, in the developing and mature CNS, astrocytes play multifaceted roles. Radial glial cells, precursors to astrocytes, are generated alongside neurons and are important for supporting neuronal migration and axon guidance (Vaccarino et al., 2007). In the mature CNS, astrocytes regulate synaptic activity, modulate the extracellular ionic environment and maintain the blood-brain barrier (Walz, 2000; Abbott et al., 2006; Tanaka, 2007). Even after injury, reactive astrocytes may show adaptive plasticity by secreting many cytokines and neurotrophic factors (Aubert et al., 1995; Levison et al., 1996), restoring extracellular ionic environment (Sykova et al., 1992), and upregulating various cellular surface molecules and extracellular matrix molecules such as L1, lamina, and fibronectin (Le Gal La Salle et al., 1992; Alonso and Privat, 1993; Frisen et al., 1993). Indeed, reactive astrocytes were shown to protect tissue and preserve function after SCI (Faulkner et al., 2004). Thus, a repair strategy aimed at minimizing the inhibitory properties of astrocytes and yet at the same time maximizing their growth-promoting properties would be extremely attractive.

Previously, we co-administered recombinant human GDNF (rhGDNF or GDNF) and SCs in semi-permeable guidance channels grafted into hemisectioned spinal cords and found that GDNF alleviated astroglial reaction and modify morphological properties of reactive astrocytes (Iannotti et al., 2003). However, the role and mechanism by which GDNF mediates such an action remains unclear. The goal of this study was to determine whether GDNF, over-expressed by SCs, would intensify this modification which, in turn, improves graft-host interfaces leading to enhanced axonal regeneration following SCI.

Materials and Methods

Generation of purified Schwann cells (SCs) and astrocytes

SCs were purified as described previously (Morrissey et al., 1991; Xu et al., 1995). Briefly, SCs were harvested from the sciatic nerves of adult female Sprague-Dawley (SD) rats (Harlan, Indianapolis, IN) under aseptic conditions, then purified and expanded in culture. Purified SCs (purity >98%) at the third or fourth passage were collected for either *in vitro* experiments or seeding into mini-guidance channels for transplantation. Astrocytes were purified from the cortex of neonatal rat brains (Muir et al., 2002). Briefly Cortices from

postnatal day (P) 0–1 rats were minced in Hank's Buffered Salt Solution (HBSS) after the removal of meninges, digested in 0.25% trypsin (Sigma, St. Louis, MO), triturated in DMEM with 10% fetal bovine serum (FBS, Sigma), and centrifuged for 5 min at 1000 g. The cells were plated in Dulbecco's Modified Eagle's Medium (DMEM) with 10% FBS in tissue culture flasks. Once the cells had reached confluence, they were shaken for 170 rpm for 18 hours in an incubator shaker to remove microglia and oligodendrocyte progenitor cells, followed by 4 days culture in 20 mM anti-mitogen AraC to eliminate the fibroblasts, and then passaged and grown to confluence. Astrocyte cultures were >98% GFAP positive and maintained in DMEM containing 10% FBS (D10).

Transduction of SC in vitro

SCs were seeded into 6-well plates at a density of 5×10^5 cells/well for *in vitro* enzyme-linked immuno-sorbent assay (ELISA) or into a 25 ml flask at a density of 1×10^6 cells/flask for transplantation. When cells were grown to over 90% confluences, they were pre-treated with 4–6 $\mu\text{g/ml}$ polybrene (Sigma) for 30–60 min, and then infected by lentiviruses expressing either green fluorescence protein (lenti-GFP) or GDNF (lenti-GDNF) for 12 hours at a multiplicity of infection (MOI) of 4, resulting in about 50% infection of cells (Abdellatif et al., 2006). Infection media was then replaced with fresh media and, 3 days later, conditioned media in 6 well plates was collected for ELISA. Cells in 25 ml flasks were prepared for transplantation.

ELISA

The GDNF levels secreted by SCs after infection *in vitro* were measured by ELISA (Abdellatif et al., 2006). 3 days after infection, the supernatant of SC was collected and centrifuged at 20,000 g for 10 min at 4°C. The procedure for ELISA followed the supplier's recommendations (G1620, Promega, Madison, WI).

Seeding SCs into mini-guidance channels

Semi-permeable 60:40 poly-acrylonitrile/poly-vinylchloride (PAN/PVC) copolymer guidance channels with an outer diameter of 1.25 mm (Provided by Dr. Xuejun Wen, Clemson University, Charleston, SC) were cleaned and sterilized according to the established methods (Xu et al., 1999; Bamber et al., 2001). SCs were suspended in a 60:40 (v:v) of DMEM and Matrigel (MG, Collaborative Research, Bedford, MA) at a final density of 120×10^6 cells/ml and seeded into guidance channels as described previously (Xu et al., 1999). The channel contents include 1) SCs alone (SCs), 2) SCs infected with lenti-GFP (lenti-GFP SCs), 3) SCs co-administered with GDNF protein (GDNF protein + SCs), and 4) SCs infected with lenti-GDNF (lenti-GDNF SCs). In channels when GDNF was co-administered, an amount of DMEM was replaced with an equal volume of concentrated GDNF to achieve a final concentration of GDNF at 5 $\mu\text{g}/\mu\text{l}$ (Iannotti et al., 2003). After seeding, the channel was closed at both ends with PAN/PVC glue and kept in DMEM for 2–3 hours at 37°C to allow polymerization of the MG.

Spinal cord hemisection and transplantation of SC-seeded guidance channels

Adult female SD rats (180–200 grams, Harlan) were randomly divided into four groups that received grafts of: 1) SCs alone (n=10), 2) lenti-GFP SCs (n=10), 3) GDNF protein + SCs (n=10), and 4) lenti-GDNF SCs (n=10). The procedures for spinal cord hemisection and mini-guidance channel implantation, as well as for pre- and post-operative animal care, were described in detail in previous publications (Xu et al., 1999; Bamber et al., 2001). Briefly, a right-sided spinal cord hemisection was performed at the 9th and 10th thoracic (T) levels to create a 2.8 mm gap longitudinally followed by implantation of a 3 mm-long piece of SC-seeded guidance channel into the lesion site. In all groups, rats were sacrificed at 6 weeks

post-implantation. All animal handling, surgical procedures, and post-operative care were performed in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996) and the Guidelines and Policies for Rodent Survival Surgery provided by the Animal Care Committees of Indiana University.

Collection of Schwann cell conditioned medium (SCM)

When cultures of purified SCs in T25 flasks were confluent, they were rinsed twice with DMEM and kept in D10 without or with GDNF (100 ng/ml) for 24 hours. Then cultures were replaced with GDNF-free medium and maintained for additional 4 days before medium collection. The medium was centrifuged and filtrated through a 0.2 μ m filter and stored (Millipore, Hertfordshire, UK).

Scratch wound healing migration Assay

The scratch migration assay was used to measure two-dimensional cell movement (Boran and Garcia, 2007). After astrocytes were grown to confluence in 24-well plates, a scratch was made on the monolayer using a sterile 200 μ l pipette tip. Then the astrocyte cultures were exposed to the following five treatment groups: 1) medium only (D10), 2) GDNF (100 ng/ml in D10), 3) SC conditioned medium (SCM; at 1:1 ratio to D10), 4) SCM + GDNF (100ng/ml), and 5) SCM pretreated with GDNF (GDNF-pretreated SCM, at 1:1 ratio to D10). At the beginning of experiment ($t = 0$ h), a digital image of the scratch was taken at a magnification of 10 x. At 24 hours later ($t = 24$ h), the same region was imaged again. The images were quantified using an NIH Image J program to determine the two-dimensional movements of the astrocytes by measuring the surface area of migrated cells at $t = 0$ h and comparing it with that at $t = 24$ h. Experiments were performed for at least three times and measurements were made in triplicate.

Inverted coverslip migration assay

This assay has been used to detect the migratory ability of one type of cells above another type (Fok-Seang et al., 1995). In this experiment, astrocytes were pre-labeled with Di-I (20 mM), a carbocyanine fluorescent tracer, for 5 min at 37°C (Molecular Probes, Leiden, The Netherlands). Di-I-labeled astrocytes were plated onto coverslip fragments (≈ 2 mm²) pre-coated with poly-lysine. After 16–18 hours, the coverslips were washed to remove loose cells and placed invertedly (with cells facing downward) onto a SC monolayer, and incubated in serum-free DMEM medium with or without the administration of GDNF (100 ng/ml) for 3 days to allow cell migration. Cultures were then fixed and the maximum distance of cells migrated away from the edge of the fragment was measured. The number of cells that migrated out from the edge of the fragment was also counted at every 100 μ m distance. Experiments were carried out in the presence of anti-mitogen Ara-C (5 μ g/ml) to make sure that the movement of cells away from the coverslip was solely caused by migration instead of proliferation.

Astrocyte and Schwann cell confrontation assay

The confrontation assay was performed according to a previously described method (Lakatos et al., 2003). Briefly, 10 μ l of two opposing parallel strips of SCs and astrocytes (1×10^4 /cell type), respectively, were seeded on a PLL-coated coverslip. The width of gap between two cell types was about 1 mm. Cells were allowed to attach for 1 hour before washing in DMEM to remove nonattached cells. Cultures were then maintained in D10 and allowed to grow towards each other over a period of 12–14 days, giving time for cells to make contact and interact. In the GDNF treated group, GDNF (100 ng/ml) were added to the cultures after the cells had contacted each other for two days. Using the NIH Image J, a 300 μ m line was drawn along the interface between astrocytes and SCs. The numbers of SCs and

astrocytes crossing this line were counted and averaged over five randomly chosen fields. Experiments were performed for at least three times and measurements were made in triplicate.

Astrocytes and Schwann cell coculture assay

Co-cultures of astrocytes and SCs were performed as described previously (Lakatos et al., 2000). Briefly, astrocytes and SCs were mixed at a ratio of 1:3, respectively, with a total cell number of 4×10^4 per well for 24 well plates. For creating cell lysates, a total cell number of 1.6×10^5 was added to each well of six well plates, each containing 2 ml of D10. GDNF (100ng/ml) were added from the second day and fresh medium with or without GDNF was replaced everyday. The cultures were maintained for 14 days by which time astrocyte responses occurred. To assess whether GDNF affected the hypertrophic changes of astrocytes, we prepared astrocyte culture in the presence of vehicle (DMEM), GDNF (100ng/ml), SCM (1:1 with DMEM), SCM+GDNF, SCM (Pre-GDNF), SCs, and SCs +GDNF. The ratio of astrocytes and SCs was 1:3. All culture media at various conditions were kept for 7 days before immunostaining for GFAP (astrocytes) and/or p75NTR (SCs). Astrocyte hypertrophy was assessed by measuring changes in individual astrocyte area using the NIH Image J software.

Western blotting

Western blotting followed procedures described previously (Liu and Xu, 2006). Briefly, protein samples (20 μ g) were electrophoresed on SDS-polyacrylamide gels, and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). The blots were incubated with primary antibodies against GFAP (1:1000) or CS-56 (1:1000; an antibody recognize CSPGs) overnight followed by incubation with HRP-conjugated secondary antibody for 1 hour at room temperature (1:5000). Blots were visualized using the enhanced chemiluminescence (ECL) plus detection system (GE Healthcare, Little Chalfont, UK).

Light and electron microscopy

The preparation for light and electron microscopy was described previously (Xu et al., 1999). Transverse 1 μ m-thick semi-thin plastic sections through the mid-point of the guidance channel were stained in 1% toluidine blue-1% sodium borate in order to quantify the mean number of myelinated axons (N), and tissue cable size (S) according to a previously published method (Xu et al., 1999). The density of axons (D) was calculated as $D = N/S$. For electron microscopy (EM), Ultra-thin sections were stained with uranyl acetate and lead citrate and examined with an electron microscope (FEI Tecnai G2 F20, Hillsboro, OR).

Immunohistochemistry

Immunohistochemistry was performed as described previously (Iannotti et al., 2003; Liu et al., 2006). Briefly, a 10 mm spinal segment containing the transplanted channel was removed, cryoprotected, sectioned on a cryostat at 20-25 μ m, and mounted on microscope slides. The sections were incubated in primary antibodies overnight at 4°C. Polyclonal rabbit anti-GFAP antibody (GFAP; 1:100, Chemicon, Temecula, CA) was used to identify astrocytes, anti-NGFR^{P75} antibody (1:100; Sigma) was used to identify Schwann cells, and anti-P0 antibody (1:100, a gift from Dr J. J. Archelos) was used to identify myelin formed by transplanted SCs. Monoclonal mouse anti-SMI-31 antibody (1:1000; Chemicon) was used to identify axons, and anti-chondroitin sulfate proteoglycans (CSPG) antibody (1:500, Chemicon) was used to identify the glial scar. On the following day, cultures were incubated with either rhodamine-conjugated goat anti-rabbit (1:100, ICN-Cappel, Aurora, OH) or AMCA-conjugated affinipure donkey anti-mouse IgG(H+L) (1:100, Jackson

ImmunoResearch Lab., West Grove, PA), and Hoechst 33342 (10 µg/ml, Sigma), a fluorescent nuclear dye. Slides were washed, mounted, examined and photographed using an Olympus DX60 fluorescent microscope. Primary antibody omission and mouse and rabbit isotype controls (Zymed Lab Inc., San Francisco, CA) were used to confirm the specificity of the antibodies.

BrdU incorporation

Cell proliferation was assayed by measuring the incorporation of BrdU according to methods described previously (Hu et al., 2008). Briefly, astrocytes were seeded at low density onto PLL-coated coverslips and left to adhere overnight. Cells were treated with DMEM for 12 hours and then exposed to the following five treatment conditions: 1) medium only (D10), 2) GDNF (100 ng/ml in D10), 3) SCM (at 1:1 ratio to D10), 4) SCM + GDNF (100ng/ml), and 5) SCM pretreated with GDNF (GDNF-pretreated SCM, at 1:1 ratio to D10). For the astrocyte and SC coculture, control groups were kept in D10 and GDNF groups were treated with GDNF (100 ng/ml) for 16 hours. In all groups, 10 µM BrdU (Sigma) was added to label dividing cells and cultures were maintained for an additional 16 hours. Then, cells were fixed, immuno-labeled with mouse anti-BrdU antibody (1:80; Dako, Santa Barbara, CA) for 40 min, followed by the secondary IgG1-FITC antibody (1:100, ICN) for 30 min. The percentage of BrdU-positive cells was calculated by counting 200 DAPI-labeled nuclei at three random sites and averaged, and the experiments were performed in triplicate.

Assessments of GFAP and CSPG immunoreactivity in vivo

Fluorescence intensity of GFAP and CSPG immunoreactivity (IR) was measured to estimate the fold increase in GFAP and CSPG levels at the lesion border over baseline levels of uninjured spinal cord, as described previously (Iannotti et al., 2003). The GFAP-IR was used as a marker to outline the boundary between the grafted and host tissues. After outlining of the astrogliotic region at the graft-host interfaces, the intensity of GFAP-IR and CSPG-IR was determined using an Olympix digital camera and NIH Image J. For each animal, 20µm serial sections at equal medio-lateral distances were used for analysis. The intensity of GFAP-IR and CSPG-IR at the rostral and caudal graft-host interfaces were measured from three longitudinal sections through the guidance channel and tissue cable (one section through the middle of the channel and the other two located 100 µm medially and laterally away from the central section). Sections from each group were processed simultaneously for GFAP-IR and CSPG-IR. The total intensity values were then averaged for each group.

Quantification of astrocyte migration and process directionality

In all cases quantification was performed with the experimenter blind to the treatment group. For quantification of GFAP-labeled astrocytes, three GFAP-stained longitudinal sections at equivalent distances through the guidance channel and tissue cable were used for analysis. We defined the host-graft boundary as a starting point “0” to measure the distances of host astrocytes migrated into the SC-seeded guidance channel at every 200 µm intervals. The number of GFAP-positive astrocyte is presented as astrocyte index at 0.2, 0.4, 0.6, 0.8, 1.0, 1.2 mm position, relative to the graft-host interface which is indicated as “0” position. Astrocyte index is a ratio of GFAP astrocyte number at a specific position over the astrocyte number at “0” position, similar to a recent study (Liu et al., 2008). For the orientation of astrocyte processes, GFAP-IR processes were randomly selected within the interface area and ‘best fit’ lines were traced over them using the Image J software. Angles between the lines and longitudinal axis of the channel were calculated with the Image J with a 0° to be parallel and 90° perpendicular to the channel axis. 30 randomly chosen astrocytic processes in each section were analyzed and the mean and median angles were determined.

Statistical analysis

Data were expressed as mean $\pm$ standard deviation (S.D.) of the mean. One-way ANOVA with Tukey's post-hoc test was used to determine statistical significance. A *p* value of < 0.05 was considered statistically significant.

Results

GDNF induced migration of host astrocytes into the SC grafts

We first evaluate the effects of GDNF on the migratory ability of astrocyte, stained with GFAP, in longitudinal sections through the channel (Fig. 1). A total of 4 transplantation groups that contained 1) SCs alone, 2) lenti-GFP SCs, 3) GDNF protein + SCs, and 4) lenti-GDNF SCs ($n=10/\text{group}$) were analyzed. In the groups that received transplantation of SCs alone or lenti-GFP SCs, a dense meshwork of reactive astrocytes (GFAP-IR) was found on the host side of the spinal cord demarcating a clear interface between the host and grafted tissues (Fig. 1C, E, F & H). Few astrocytes, if any, migrated into the graft region (Fig. 1D & G). In contrast, in grafts when GDNF is co-administered with SCs (GDNF protein + SCs) or overexpressed in SCs by lentiviral-mediated gene transfer (lenti-GDNF SCs), robust migration of astrocytes into the transplants was observed (Fig. 1I-N). The migrated astrocytes contained elongated processes which extended in parallel to the longitudinal axis of the graft (Fig. 1J & M). Accordingly, the astrogliotic response at the graft-host interface in the GDNF-treated groups was reduced (Fig. 1K & N) as compared to the control groups lacking GDNF (Fig. 1E & H). In the GDNF-treated groups, the astrocytes migrated throughout the entire length of the graft as they were seen in the rostral and caudal graft segments by immunohistochemistry (IH) as well as in the middle segment by EM. For example, in the caudal segment, astrocytes migrated for up to 1.2 mm rostrally (the longest distance that was examined) within the channels from the caudal graft-host interface (Fig. 1O). Moreover, the amount of migrated GFAP-IR cells, indicated as astrocyte index, over distances within the channels was shown and significant differences between the GDNF treated and non-treated groups are clearly seen (Fig. 1O). Finally, GDNF treatments also altered astrocyte morphology in that these cells became less reactive and gave rise to elongated processes in parallel to the graft axis which was in sharp contrast to the hypertrophic morphology of the reactive astrocytes with thicker and randomly extended processes in the non-GDNF treatment groups (Fig. 1E & H vs. K & N). The astrocyte processes were more parallel to the graft axis in the GDNF-treated groups (lenti-GDNF SCs, $18.85 \pm 18.80^\circ$; GDNF protein + SCs, $24.46 \pm 21.34^\circ$) as compared to the non-GDNF-treated groups (lenti-GFP SCs, $60.25 \pm 20.33^\circ$; SCs alone, $68.94 \pm 28.64^\circ$) with 0° designated as complete parallel and 90° perpendicular to the graft axis (Fig. 1P).

GDNF reduced GFAP and CSPG expression at the graft-host interface

Since GFAP is a hallmark of reactive astrogliosis and CSPGs are a major class of axon growth inhibitors associated with reactive astrocytes (Davies et al., 1999; Bradbury et al., 2002; Chau et al., 2004; Hsu and Xu, 2005), we investigated the expression of GFAP and CSPG at the graft-host interfaces in the four groups that received either GDNF or non-GDNF treatments, as described above (Fig. 2). In grafts that contained SCs alone or lenti-GFP SCs, increased expression of GFAP (Fig. 2A & D) and CSPG (Fig. 2B & E) was clearly seen. In contrast, in grafts that contained GDNF protein + SCs or lenti-GDNF SCs, both GFAP and CSPG expressions were significantly reduced (Fig. 2G-L) and the differences between the GDNF treated and non-treated groups were statistically significant (Fig. 2M & N).

GDNF induced parallel alignment between migrated astrocytes, regenerated axons and new myelin formed by grafted Schwann cells

We next examined the association of migrated astrocytes with regenerated axons and new myelin formed by grafted Schwann cells under the influence of GDNF. In both GDNF-treated groups (Fig. 3G & H), greater axonal growth into the guidance channels was found as compared to the non-GDNF-treated groups (Fig. 3E & F). Toluidine blue stained cross sections taken from the graft mid-point showed that the total number and density of myelinated axons were all significantly increased in the GDNF-treated groups as compared to the non-GDNF-treated groups (Suppl. Fig. 2), which was consistent with our previous results (Iannotti et al., 2003; Zhang et al., 2009). We further examined whether migrated astrocytes, after GDNF treatment, were permissive to axon growth and subsequent myelination by grafted SCs using immunofluorescence double labeling of GFAP with SMI-31, an axon marker, or P0, a marker for SC myelin. In non-GDNF-treated groups (SCs alone or lenti-GFP SCs), astrocytes did not migrate into the graft environment and, therefore, no association of astrocytes with axons (Fig. 3A, B, E & F) or myelin (Fig. 3I, J, M & N) was found within the grafts. Interestingly, the lack of migrated astrocytes in the control groups correlated well with reduced amount of SMI-31-IR axons within the channel (Fig. 3. E & F). At the graft-host interface, the astrocyte processes were misaligned with regenerated axons in the control groups (Fig. 3E & F). In contrast, in the GDNF-treated groups, astrocytes vigorously migrated into the channel with their processes extended longitudinally and in close alignment with regenerated axons (Fig. 3C, G, D & H). Furthermore, migrated astrocytes were also in close association with new myelin (P0-IR) formed by grafted SCs (Fig. 3K, O, L & P). Note that GDNF treatments induced greater myelin formation than the non-GDNF treatments (Fig. 3O & P vs. M & N). In contrast to the clear dissociation between myelinated SCs and host astrocytes in the non-GDNF-treated groups (Fig. 3I & J), migrated astrocytes in the GDNF-treated groups showed parallel alignment of their processes with new myelin formed by grafted SCs (Fig. 3O & P). At the EM level, the association of astrocytes or their processes with regenerated axons, both myelinated and unmyelinated, were clearly seen (Fig. 3Q & R).

GDNF promoted interdigitative migration between astrocytes and SCs

To determine the effect of GDNF on astrocyte-SC interaction, a confrontation assay was employed. Such an assay was used previously to demonstrate that SCs did not intermingle with astrocytes (Wilby et al., 1999; Lakatos et al., 2000; Grimpe et al., 2005). In the absence of GDNF, a sharp and straight boundary (Fig. 4A, red line) was formed between two seeded cell populations. In contrast, GDNF administration induced migration of both cell populations towards each other, resulting in an interdigitated boundary between the two (Fig. 4B, red line). Such an interaction between astrocytes and SCs could be further appreciated when these cells were immunostained with their phenotypic markers GFAP and p75, respectively (Fig. 4C & D). Quantitative analysis showed that the number of cells that crossed a 300 μ m line (Fig. 4A & B, yellow line) in both SCs and astrocytes were significantly increased in the GDNF treated group as compared to the control group (Fig. 4E; $p < 0.01$). Thus, GDNF induced interdigitative migration of both astrocytes and SCs towards their counterparts.

GDNF had no direct effect on astrocytes migration but promoted their migration on the monolayer of SCs

To determine the effect of GDNF and/or SC-conditioned medium (SCM) on astrocyte migration, we used a scratch wound-healing model (Boran and Garcia, 2007). Administration of GDNF failed to reduce the scratch gap at 24 hours, compared to the vehicle control (Fig. 5A & B), suggesting that GDNF had no direct effect on astrocyte migration. In contrast, the size of the wound was significantly reduced in astrocytes treated

with SCM (Fig. 5A & B; $p<0.01$) indicating that factors secreted by SCs accelerated astrocyte migration. Interestingly, SCM from GDNF-pretreated SCs [SCM (pre-GDNF)] reduced the scratch gap and enhanced astrocyte migration (Fig. 5A & B), suggesting that GDNF may affect astrocyte migration indirectly through modification of SCs or their production of SCM. To determine whether SCM or GDNF had any effect on astrocytes proliferation which may affect their migration, a BrdU incorporation assay was performed. Results showed that SCM but not GDNF stimulated astrocyte proliferation (Suppl. Fig. 3), similar to a previous report (Santos-Silva et al., 2007). Our experiment was done in the presence of an anti-mitogen Ara-C (5ug/ml) to eliminate the compound effect of cell proliferation on the cell migration assay.

To test whether cell-cell contact mediated GDNF-induced astrocyte migration, we used an inverted coverslip migration assay (Fig. 5C). In this experiment, the anti-mitogen Ara-C was also used for the reason mentioned above. At 3 days after the GDNF treatment, both migratory distances and the number of astrocytes per migratory distances on the SC monolayer increased significantly, as compared to the control group (Fig. 5C-E; $p<0.01$).

GDNF reversed SC-induced hypertrophy of astrocytes and promoted their processes extension

To assess whether GDNF affected the hypertrophic changes of astrocytes, we prepared astrocyte culture at various culture conditions (Fig. 6). Results showed that the GDNF did not have a direct effect on astrocyte area as compared to the vehicle group (Fig. 6B & H). SCM alone (Fig. 6C) or with the addition of (Fig. 6D) or pretreated (Fig. 6E) with GDNF, increased the astrocyte area or hypertrophy as compared to the vehicle control (Fig. 6H, $p<0.05$). The same effect was observed when astrocytes were co-cultured with SCs and compared with when astrocytes were cultured alone (Fig. 6F & H; $p<0.01$), indicating that the average size of individual astrocytes was significantly increased in the presence of SCs. Interestingly, when GDNF was added to the astrocyte-SC co-culture system, it reduced astrocyte size and promoted elongation of astrocyte processes (Fig. 6 G-G' & H; $p<0.01$) a phenomenon which was also observed *in vivo* (Fig. 1). To rule out the possibility that GDNF reverted the hypertrophy of astrocytes by inducing SCs' secretion of other factors, we pretreated SCs with GDNF, and collected such GDNF-pretreated SCM [SCM (Pre-GDNF)] as an additional control. The SCM (Pre-GDNF) did not reverse the hypertrophic change of astrocytes in the SCM group indicating that GDNF did not have an effect on the production of secondary trophic factors as examined previously (Zhang et al., 2009). Thus, GDNF may reverse SC-induced astrocyte hypertrophy through the interaction between the two cell populations.

GDNF reduced expressions of GFAP and CSPG in astrocyte-SC co-cultures

Lastly, we examined the effect of GDNF on expressions of GFAP and CSPG, two hallmarks of astrogliosis, in astrocyte single cultures or astrocyte-SC co-cultures. Western blot analysis showed that neither GDNF nor SCM alone had an effect on the expression of GFAP or CS-56 in astrocyte single cultures as compared to the vehicle group (Fig. 7A & B). Administration of a combination of GDNF and SCM in astrocyte single cultures had no effect on GFAP expression but significantly reduced that of CS-56. Only when GDNF was administered to the astrocyte-SC co-cultures did it significantly reduce GFAP and CS-56 expression (Fig. 7A & B; $p<0.01$). These results correlate well with the observation that GDNF reduced astrocytic atrophy *in vitro* (as shown in Figure 5) and gliosis *in vivo* (as shown in Fig. 2) in the presence of both astrocytes and SCs. Together, these results suggest that the effects of GDNF on reducing astrogliosis must be mediated through the interaction between astrocytes and SCs.

Discussion

To our knowledge, this is the first study demonstrating an important role of GDNF on modifying astrogliotic responses at graft-host interfaces allowing robust axonal regeneration into SC grafts and subsequently remyelination following SCI. A key finding was that GDNF, administered with or over-expressed by grafted SCs, induced active migration of host astrocytes into SC grafts concomitant with enhanced axonal regeneration and remyelination. GDNF administration or overexpression also significantly reduced GFAP and CSPG induction at the graft-host interfaces. Such a GDNF effect on astrogliotic responses *in vivo* was further confirmed *in vitro* and was considered to be mediated through interactions between astrocytes and SCs. Importantly, these studies indicate that the inhibitory properties of reactive astrocytes, induced by a CNS injury, can be readily modified by a trophic factor GDNF which leads to enhanced axonal regeneration and remyelination. They also indicate that modifying the inhibitory properties of reactive astrocytes may represent a novel and attractive strategy to promote greater axonal regeneration and recovery of function following SCI.

Astrocytes and SCs normally reside separately in the PNS and CNS, respectively, and do not interact with each other. At the peripheral nerve entry zone, astrocytes contribute to the formation of glial limitans that prevents SCs to migrate into the CNS and generates barriers to axonal regeneration after injury (Fraher, 1997; Golding et al., 1997). Such a glial limitans or barrier is similar to the one that we observed between grafted SCs and reactive astrocytes in this study. At this interface, astrocytes prevented the migration of SCs into the host spinal cord as they normally do at the glial limitans. In fact, very few grafted SCs can break through the astrocytic barrier to migrate into the host tissue (Baron-Van Evercooren et al., 1992; Sims et al., 1999) and only when the astrocytes were depleted can SCs migrate into and intermingle with the host tissue (Sims et al., 1999). The presence of GFP-SCs in the graft region of the present study clearly indicates that these cells survived SC bridge transplantation and that they did not migrate into the host spinal cord in the absence of GDNF. Our *in vitro* data also showed that, in the presence of GDNF, migration of SCs into the astrocyte monolayer occurred, supporting the possibility that GDNF may facilitate SC migration into the host tissue if appropriate environment is provided.

Similarly, astrocytes do not migrate into the SCs environment and, in fact, they form a strong inhibitory barrier at the host side of the spinal cord, as reported previously (Xu et al., 1995; Xu et al., 1997; Xu et al., 1999). *In vitro*, astrocytes and SCs form separate territories with sharp boundaries between them (Wilby et al., 1999; Lakatos et al., 2000). Previously, we demonstrated that co-administration of rhGDNF in the SC-seeded mini-channel transplantation model reduced the extent of reactive astrogliosis and cavity formation as well as induced mild migration of astrocytes into the SC grafted (Iannotti et al., 2003). In the present study, the remarkable effect of GDNF on the migration of astrocytes into the lenti-GDNF SC grafts may result from long-lasting overexpression of GDNF from lentiviral-transfected SCs. Contrarily, strong effect of GDNF on astrocyte migration was not found in a recent study (Tom et al., 2009). The difference between these two studies may be related, in part, to the difference in GDNF delivery, concentration or expression. In our case, GDNF was either slowly released from the Matrigel or by lentiviral infected SCs so that a focal and lasting releasing effect of GDNF may be achieved. Migrating astrocytes were observed dispersed amongst and in close association with grafted SCs indicating that SCs and astrocytes are less repellent to each other under the influence of GDNF. Such an optimal interaction between the two cell types, that used to repel each other, was also confirmed in our *in vitro* assays.

To interpret the effect of GDNF on astrocyte migration, two possibilities exist: a direct effect of GDNF on astrocytes or an indirect effect of GDNF through its action on SCs. Since GDNF did not have a direct effect on astrocyte migration in our *in vitro* assays, we favor the second possibility. For example, in the scratch wound healing assay, SC conditioned medium (SCM) induced astrocyte migration in astrocyte cultures indicating that SCM promotes astrocytes migration. However, such migration was not enhanced when GDNF was co-administered with SCM, indicating that GDNF has no additional effect on astrocyte migration. Interestingly, when SCs were pre-stimulated with GDNF, their conditioned medium, i.e. SCM-(Pre-GDNF), enhanced astrocyte migration. The strongest migration of astrocytes, however, were found only when astrocytes were in direct contact with SCs, as were shown in both the inverted coverslip assay *in vitro* and SC-seeded bridge transplantation experiment *in vivo*. These results collectively indicate that the GDNF effect on astrocyte migration requires the presence of and interaction with SCs. Although SCM alone can induce astrocyte migration to a certain extent, when astrocytes and SCs encounter each other physically this effect is not sufficient to induce astrocyte migration into the SC territory.

It is well accepted that reactive astrogliosis, developed in response to injuries of the CNS, is a major impediment for axonal regeneration (Predy and Malhotra, 1989; Silver and Miller, 2004; Fitch and Silver, 2008). One effective strategy to remove this impediment is to enzymatically remove inhibitory CSPGs produced by reactive astrocytes at the site of injury (Bradbury et al., 2002; Chau et al., 2004; Houle et al., 2006). An alternative approach, as presented in this study, is to modify the properties of reactive astrocytes making them less inhibitory and more permissive to axonal growth, as during development. If reactive astrocytes can be modified to create a directionally-oriented permissive environment, then this typically growth-inhibitory cell population can be transformed into a growth-promotive cell population to enhance axonal regeneration. Indeed, GDNF treatment in the present study reversed the inhibitory properties of reactive astrocytes, induced migration and aligned extension of their processes from the host into the SC graft, and reduced both GFAP and CSPG reactivities at the graft-host interfaces. Enzymatic removal of CSPG with ChABC was associated with migration of astrocytes into the caudal graft environment (Chau et al., 2004) which may provide a mechanism for GDNF-mediated astrocyte migration observed in the current study. These effects may collectively contribute to the conversion of reactive astrocytes from growth-inhibitory to growth-promotive to axonal regeneration. Within the SC graft, migrated astrocytes aligned along regenerated axons implying that these astrocytes facilitated directional growth of regenerated axons. Moreover, the astrocytic processes were in close contact with regenerated axons which provides a morphological basis for astrocyte-axon interaction that could be important for exchanging metabolites and supplying nutrients to regenerated axons (Nieto-Sampedro et al., 1988; Logan et al., 1994; Clatterbuck et al., 1996). The evidence that migrated astrocytes were widely dispersed amongst grafted SCs and that they were in close association with SC myelin indicates that the astrocyte-SC interaction, induced by GDNF, may enhance SC-mediated axonal regeneration and remyelination.

One interesting finding is that, in astrocyte-SC co-cultures, GDNF treatment significantly reduced astrocyte hypertrophy and induced their process extension. Such a GDNF effect was also observed *in vivo* at the graft-host interface concomitant with reduced production of GFAP and CSPG and enhanced axonal regeneration into the SC graft. Thus, GDNF not only affect the biochemical changes of reactive astrocytes but also their morphological changes in response to the injury.

Although the present study has identified a novel role of GDNF on modifying astrocyte responses to injury and regeneration, the role of GDNF on CNS injury and regeneration may

be more complicated than we thought. Previously, we demonstrated that GDNF reduced lesion volume and increased white matter sparing in a contusive SCI model (Iannotti et al., 2004). We also demonstrated that co-administration of GDNF protein with SCs increased axonal regeneration and reduced astrogliosis, cavity formation and infiltration of inflammatory cells at graft-host interfaces in a SC-seeded mini-channel implantation model (Iannotti et al., 2003). We further demonstrated that, in the same SC transplantation model, GDNF enhanced both the number and caliber of regenerated axons (Zhang et al., 2009). Moreover, GDNF significantly increased the number of myelin sheaths produced by SCs but had no effect on the proliferation of isolated SCs or their secretion of neurotrophins nerve growth factor (NGF), neurotrophin-3 (NT3), or brain-derived trophic factor (BDNF) *in vitro* (Zhang et al., 2009). These results collectively suggest that GDNF-enhanced axonal regeneration and SC myelination is mediated through multiple factors. Taken together, the previous and current results indicate that GDNF may orchestrate a multi-faceted response of both neurons and glial cells, including astrocytes and SCs, that eventually results in synergistic and beneficial effects on axonal regeneration and remyelination following SCI.

In summary, here we report a novel function of GDNF on modifying reactive astrogliosis, a classically considered inhibitory barrier to axonal regeneration, at the SC graft-host interface following SCI. Reversing the inhibitory properties of reactive astrocytes may thus open a new avenue to foster axonal regeneration, remyelination and recovery of function following SCI.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

- Abbott NJ, Ronnback L, Hansson E. Astrocyte-endothelial interactions at the blood-brain barrier. *Nat Rev Neurosci.* 2006; 7:41–53. [PubMed: 16371949]
- Abdellatif AA, Pelt JL, Benton RL, Howard RM, Tsoulfas P, Ping P, Xu XM, Whittemore SR. Gene delivery to the spinal cord: comparison between lentiviral, adenoviral, and retroviral vector delivery systems. *J Neurosci Res.* 2006; 84:553–567. [PubMed: 16786574]
- Alonso G, Privat A. Reactive astrocytes involved in the formation of lesional scars differ in the mediobasal hypothalamus and in other forebrain regions. *J Neurosci Res.* 1993; 34:523–538. [PubMed: 8478987]
- Arenas E, Trupp M, Akerud P, Ibanez CF. GDNF prevents degeneration and promotes the phenotype of brain noradrenergic neurons *in vivo*. *Neuron.* 1995; 15:1465–1473. [PubMed: 8845169]
- Aubert I, Ridet JL, Gage FH. Regeneration in the adult mammalian CNS: guided by development. *Curr Opin Neurobiol.* 1995; 5:625–635. [PubMed: 8580714]
- Bamber NI, Li H, Lu X, Oudega M, Aebischer P, Xu XM. Neurotrophins BDNF and NT-3 promote axonal re-entry into the distal host spinal cord through Schwann cell-seeded mini-channels. *Eur J Neurosci.* 2001; 13:257–268. [PubMed: 11168530]
- Baron-Van Evercooren A, Clerin-Duhamel E, Lapie P, Gansmuller A, Lachapelle F, Gumpel M. The fate of Schwann cells transplanted in the brain during development. *Dev Neurosci.* 1992; 14:73–84. [PubMed: 1396177]
- Boran MS, Garcia A. The cyclic GMP-protein kinase G pathway regulates cytoskeleton dynamics and motility in astrocytes. *J Neurochem.* 2007; 102:216–230. [PubMed: 17564679]

- Bradbury EJ, Moon LDF, Popat RJ, King VR, Bennett GS, Patel PN, Fawcett JW, McMahon SB. Chondroitinase ABC promotes functional recovery after spinal cord injury. *Nature*. 2002; 416:636–640. [PubMed: 11948352]
- Buj-Bello A, Buchman VL, Horton A, Rosenthal A, Davies AM. GDNF is an age-specific survival factor for sensory and autonomic neurons. *Neuron*. 1995; 15:821–828. [PubMed: 7576631]
- Chau CH, Shum DK, Li H, Pei J, Lui YY, Wirthlin L, Chan YS, Xu XM. Chondroitinase ABC enhances axonal regrowth through Schwann cell-seeded guidance channels after spinal cord injury. *Faseb J*. 2004; 18:194–196. [PubMed: 14630702]
- Clatterbuck RE, Price DL, Koliatsos VE. Ciliary neurotrophic factor stimulates the expression of glial fibrillary acidic protein by brain astrocytes in vivo. *J Comp Neurol*. 1996; 369:543–551. [PubMed: 8761927]
- Davies SJA, Goucher DR, Doller C, Silver J. Robust regeneration of adult sensory axons in degenerating white matter of the adult rat spinal cord. *J Neurosci*. 1999; 19:5810–5822. [PubMed: 10407022]
- Faulkner JR, Herrmann JE, Woo MJ, Tansey KE, Doan NB, Sofroniew MV. Reactive astrocytes protect tissue and preserve function after spinal cord injury. *J Neurosci*. 2004; 24:2143–2155. [PubMed: 14999065]
- Fitch MT, Silver J. CNS injury, glial scars, and inflammation: Inhibitory extracellular matrices and regeneration failure. *Exp Neurol*. 2008; 209:294–301. [PubMed: 17617407]
- Fok-Seang J, Mathews GA, French-Constant C, Trotter J, Fawcett JW. Migration of oligodendrocyte precursors on astrocytes and meningeal cells. *Dev Biol*. 1995; 171:1–15. [PubMed: 7556887]
- Fraher JP. Axon-glial relationships in early CNS-PNS transitional zone development: an ultrastructural study. *J Neurocytol*. 1997; 26:41–52. [PubMed: 9154528]
- Frisen J, Arvidsson U, Lindholm T, Fried K, Verge VM, Cullheim S, Hokfelt T, Risling M. *trkC* expression in the injured rat spinal cord. *Neuroreport*. 1993; 5:349–352. [PubMed: 8298102]
- Golding J, Shewan D, Cohen J. Maturation of the mammalian dorsal root entry zone--from entry to no entry. *Trends Neurosci*. 1997; 20:303–308. [PubMed: 9223223]
- Grimpe B, Pressman Y, Lupa MD, Horn KP, Bunge MB, Silver J. The role of proteoglycans in Schwann cell/astrocyte interactions and in regeneration failure at PNS/CNS interfaces. *Mol Cell Neurosci*. 2005; 28:18–29. [PubMed: 15607938]
- Houle JD, Tom VJ, Mayes D, Wagoner G, Phillips N, Silver J. Combining an autologous peripheral nervous system “bridge” and matrix modification by chondroitinase allows robust, functional regeneration beyond a hemisection lesion of the adult rat spinal cord. *J Neurosci*. 2006; 26:7405–7415. [PubMed: 16837588]
- Hsu JY, Xu XM. Early profiles of axonal growth and astroglial response after spinal cord hemisection and implantation of Schwann cell-seeded guidance channels in adult rats. *J Neurosci Res*. 2005; 82:472–483. [PubMed: 16240391]
- Hu JG, Fu SL, Wang YX, Li Y, Jiang XY, Wang XF, Qiu MS, Lu PH, Xu XM. Platelet-derived growth factor-AA mediates oligodendrocyte lineage differentiation through activation of extracellular signal-regulated kinase signaling pathway. *Neuroscience*. 2008; 151:138–147. [PubMed: 18093741]
- Iannotti C, Li H, Yan P, Lu X, Wirthlin L, Xu X-M. Glial cell line-derived neurotrophic factor-enriched bridging transplants promote propriospinal axonal regeneration and enhance myelination after spinal cord injury. *Exp Neurol*. 2003; 183:379–393. [PubMed: 14552879]
- Iannotti C, Zhang YP, Shields CB, Han Y, Burke DA, Xu X-M. A neuroprotective role of glial cell line-derived neurotrophic factor following moderate spinal cord contusion injury. *Exp Neurol*. 2004; 189:317–332. [PubMed: 15380482]
- 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]
- Kordower JH, Emborg ME, Bloch J, Ma SY, Chu Y, Leventhal L, McBride J, Chen E-Y, Palfi S, Roitberg Bz, Brown WD, Holden JE, Pyzalski R, Taylor MD, Carvey P, Ling Z, Trono D, Hantraye P, Deglon N, Aebischer P. Neurodegeneration prevented by lentiviral vector delivery of GDNF in primate models of Parkinson's disease. *Science*. 2000; 290. [PubMed: 10634780]

- Lakatos A, Franklin RJ, Barnett SC. Olfactory ensheathing cells and Schwann cells differ in their in vitro interactions with astrocytes. *Glia*. 2000; 32:214–225. [PubMed: 11102963]
- Lakatos A, Barnett SC, Franklin RJ. Olfactory ensheathing cells induce less host astrocyte response and chondroitin sulphate proteoglycan expression than Schwann cells following transplantation into adult CNS white matter. *Exp Neurol*. 2003; 184:237–246. [PubMed: 14637095]
- Le Gal La Salle G, Rougon G, Valin A. The embryonic form of neural cell surface molecule (E-NCAM) in the rat hippocampus and its reexpression on glial cells following kainic acid-induced status epilepticus. *J Neurosci*. 1992; 12:872–882. [PubMed: 1545243]
- Levison SW, Ducceschi MH, Young GM, Wood TL. Acute exposure to CNTF in vivo induces multiple components of reactive gliosis. *Exp Neurol*. 1996; 141:256–268. [PubMed: 8812159]
- Liu NK, Xu XM. beta-Tubulin Is a More Suitable Internal Control than beta-Actin in Western Blot Analysis of Spinal Cord Tissues after Traumatic Injury. *J Neurotrauma*. 2006; 23:1794–1801. [PubMed: 17184189]
- Liu NK, Zhang YP, Titsworth WL, Jiang X, Han S, Lu PH, Shields CB, Xu XM. A novel role of phospholipase A2 in mediating spinal cord secondary injury. *Ann Neurol*. 2006; 59:606–619. [PubMed: 16498630]
- Liu Y, Wang X, Lu CC, Kerman R, Steward O, Xu XM, Zou Y. Repulsive Wnt signaling inhibits axon regeneration after CNS injury. *J Neurosci*. 2008; 28:8376–8382. [PubMed: 18701700]
- Logan A, Berry M, Gonzalez AM, Frautschy SA, Sporn MB, Baird A. Effects of transforming growth factor beta 1 on scar production in the injured central nervous system of the rat. *Eur J Neurosci*. 1994; 6:355–363. [PubMed: 8019673]
- Mills CD, Allchorne AJ, Griffin RS, Woolf CJ, Costigan M. GDNF selectively promotes regeneration of injury-primed sensory neurons in the lesioned spinal cord. *Mol Cell Neurosci*. 2007; 36:185–194. [PubMed: 17702601]
- Morrissey TK, Kleitman N, Bunge RP. Isolation and functional characterization of Schwann cells derived from adult peripheral nerve. *J Neurosci*. 1991; 11:2433–2442. [PubMed: 1869923]
- Muir EM, Adcock KH, Morgenstern DA, Clayton R, von Stillfried N, Rhodes K, Ellis C, Fawcett JW, Rogers JH. Matrix metalloproteases and their inhibitors are produced by overlapping populations of activated astrocytes. *Brain Res Mol Brain Res*. 2002; 100:103–117. [PubMed: 12008026]
- Nieto-Sampedro M, Gomez-Pinilla F, Knauer DJ, Broderick JT. Epidermal growth factor receptor immunoreactivity in rat brain astrocytes. Response to injury. *Neurosci Lett*. 1988; 91:276–282. [PubMed: 3185965]
- Oppenheim RW, Houenou LJ, Johnson JE, Lin L-FH, Li L, Lo AC, Newsome AL, Prevette DM, Wang S. Developing motor neurons rescued from programmed and axotomy-induced cell death by GDNF. *Nature*. 1995; 373:344–346. [PubMed: 7830769]
- Paratcha G, Ledda F, Ibanez CF. The neural cell adhesion molecule NCAM is an alternative signaling receptor for GDNF family ligands. *Cell*. 2003; 113:867–879. [PubMed: 12837245]
- Perrelet D, Ferri A, Liston P, Muzzin P, Korneluk RG, Kato AC. IAPs are essential for GDNF-mediated neuroprotective effects in injured motor neurons in vivo. *Nat Cell Biol*. 2002; 4:175–179. [PubMed: 11813002]
- Predy R, Malhotra SK. Reactive astrocytes in lesioned rat spinal cord: effect of neural transplants. *Brain Res Bull*. 1989; 22:81–87. [PubMed: 2713718]
- Reier, PJ.; Stensaas, LJ.; Guth, L. The astrocytic scar as an impediment to regeneration in the central nervous system. In: Kao, CC.; Bunge, RP.; Reier, PJ., editors. *Spinal cord reconstruction*. Raven Press; New York: 1983. p. 163–196.
- Santos-Silva A, Fairless R, Frame MC, Montague P, Smith GM, Toft A, Riddell JS, Barnett SC. FGF/heparin differentially regulates Schwann cell and olfactory ensheathing cell interactions with astrocytes: a role in astrocytosis. *J Neurosci*. 2007; 27:7154–7167. [PubMed: 17611269]
- Silver J, Miller JH. Regeneration beyond the glial scar. *Nat Rev Neurosci*. 2004; 5:146–156. [PubMed: 14735117]
- Sims TJ, Durgun MB, Gilmore SA. Transplantation of sciatic nerve segments into normal and glia-depleted spinal cords. *Exp Brain Res*. 1999; 125:495–501. [PubMed: 10323296]
- Sykova E, Svoboda J, Simonova Z, Jendelova P. Role of astrocytes in ionic and volume homeostasis in spinal cord during development and injury. *Prog Brain Res*. 1992; 94:47–56. [PubMed: 1283792]

- Tanaka K. Role of glutamate transporters in astrocytes. *Brain Nerve*. 2007; 59:677–688. [PubMed: 17663138]
- Tom VJ, Sandrow-Feinberg HR, Miller K, Santi L, Connors T, Lemay MA, Houle JD. Combining peripheral nerve grafts and chondroitinase promotes functional axonal regeneration in the chronically injured spinal cord. *J Neurosci*. 2009; 29:14881–14890. [PubMed: 19940184]
- Vaccarino FM, Fagel DM, Ganat Y, Maragnoli ME, Ment LR, Ohkubo Y, Schwartz ML, Silbereis J, Smith KM. Astroglial cells in development, regeneration, and repair. *Neuroscientist*. 2007; 13:173–185. [PubMed: 17404377]
- Walz W. Role of astrocytes in the clearance of excess extracellular potassium. *Neurochem Int*. 2000; 36:291–300. [PubMed: 10732996]
- Widenfalk J, Lundstromer K, Jubran M, Brene S, Olson L. Neurotrophic factors and receptors in the immature and adult spinal cord after mechanical injury or kainic acid. *J Neurosci*. 2001; 21:3457–3475. [PubMed: 11331375]
- Wilby MJ, Muir EM, Fok-Seang J, Gour BJ, Blaschuk OW, Fawcett JW. N-Cadherin inhibits Schwann cell migration on astrocytes. *Mol Cell Neurosci*. 1999; 14:66–84. [PubMed: 10433818]
- Xu XM, Guenard V, Kleitman N, Bunge MB. Axonal regeneration into Schwann cell-seeded guidance channels grafted into transected adult rat spinal cord. *J Comp Neurol*. 1995; 351:145–160. [PubMed: 7896937]
- Xu XM, Chen A, Guenard V, Kleitman N, Bunge MB. Bridging Schwann cell transplants promote axonal regeneration from both the rostral and caudal stumps of transected adult rat spinal cord. *J Neurocytol*. 1997; 26:1–16. [PubMed: 9154524]
- Xu XM, Zhang SX, Li H, Aebischer P, Bunge MB. Regrowth of axons into the distal spinal cord through a Schwann-cell-seeded mini-channel implanted into hemisectioned adult rat spinal cord. *Eur J Neurosci*. 1999; 11:1723–1740. [PubMed: 10215926]
- Zhang L, Ma Z, Smith GM, Wen X, Pressman Y, Wood PM, Xu XM. GDNF-enhanced axonal regeneration and myelination following spinal cord injury is mediated by primary effects on neurons. *Glia*. 2009 In press.

Figure 1. GDNF induced migration of host astrocytes into the Schwann cell (SC) grafts
 (A) Dorsal view of the brain and spinal cord of an adult rat subjected to a SC-seeded guidance channel transplantation. (B) A high magnification image of transplanted area illustrated how tissues were processed. A 1 mm segment at the graft mid-point was cut transversely and processed for EM. The remaining proximal and distal segments of the graft were cut longitudinally for immunohistochemistry (IH). Images shown in (C-N) are representative photomicrographs of the caudal graft-host interface from grafts that contained 1) SCs alone, 2) lenti-GFP SCs, 3) GDNF protein + SCs, and 4) lenti-GDNF SCs. (C-E) In the SC alone graft, a dense meshwork of hypertrophic astrocytes, labeled with GFAP, was seen at the host side of the caudal graft-host interface (yellow dashed line). Note that host astrocytes did not migrate into the SC graft in the absence of GDNF. (F-H) In the lenti-GFP SC graft, a similar dense meshwork of hypertrophic astrocytes was found. The survival of grafted SCs, evidenced by GFP-staining, with elongated processes extending along the axis of the graft was clearly seen. In this group, host astrocytes did not migrate into the SC graft. (I-K) In the GDNF protein + SC graft, numerous host astrocytes migrated into the graft environment. (L-N) In the lenti-GDNF SC graft, remarkably more host astrocytes migrated into the graft environment for considerable distances. (D, G, J & M) and (E, H, K & N) are high magnifications of boxed areas of the graft proper and caudal graft-host interface, respectively, shown in (C, F, I & L). Yellow dashed lines indicate the graft-host interfaces. White dash lines in (C, F, I & L) depict the graft proper. (O) A comparison in migratory distances of astrocytes, represented as *astrocyte index*, into the SC graft among the four transplantation groups. (P) A comparison in orientation of astrocyte processes at the distal graft-host interfaces among the four transplantation groups. 0° is parallel and 90° is perpendicular to the channel. ***: $p < 0.001$ (lenti-GDNF SCs vs. GDNF protein + SCs); ++: $p < 0.001$ (lenti-GDNF SCs vs. SCs alone); \$\$\$: $p < 0.001$ (lenti-GDNF SCs vs. lenti-GFP SCs); ** or ***: $p < 0.01$ or $p < 0.001$ (GDNF protein + SCs vs. SC alone); ## or ###: $p < 0.01$

or $p < 0.001$ (GDNF protein + SCs vs. lenti-GFP SCs). Scale bars: C, F, I & L = 400 μ m; D, E, G, H, J, K, M & N = 100 μ m.

Figure 2. GDNF reduced GFAP and CSPG expression at graft-host interfaces *in vivo* (A-L) Representative photomicrographs show the caudal graft-host interface of channels that contained 1) SCs alone (A-C), 2) lenti-GFP SCs (D-F), 3) GDNF protein + SCs (G-I), and 4) lenti-GDNF SCs (J-L). In grafts containing either SCs alone (A-C) or lenti-GFP SCs (D-F), increased expression of GFAP (A & D) and CSPG (B & E) was found at the distal graft-host interface, which could be further appreciated in the merged images (C & F). In contrast, in grafts containing either GDNF protein + SCs (G-I) or lenti-GDNF SCs (J-L), the expression of both GFAP (G & J) and CSPG (H & K) was considerably reduced, as clearly seen in the merged images of (I & L). Such reduction was correlated with significant migration of host astrocytes into the graft environment in groups when GDNF was administered (I & L). White dash lines indicate the caudal graft-host interfaces whereas white asterisks in (A-F) indicate enhanced expression of GFAP and CSPG at the caudal graft-host interface. (M & N) Quantitative analyses show that the difference in GFAP (M) and CSPG (N) expressions in the four groups were statistically significant (*: $P<0.05$; **: $P<0.01$). Scale bar: 100 μ m.

Figure 3. GDNF induced parallel alignment between migrated astrocytes, regenerated axons and SC myelin

(A-P) Representative photomicrographs show astrocyte-axon relationship at the caudal graft-host interface (A-H) as well as astrocyte-myelin association within the graft proper (I-P) in channels that contained 1) SCs alone (1st row), 2) lenti-GFP SCs (2nd row), 3) GDNF protein + SCs (3rd row), and 4) lenti-GDNF SCs (4th row). (A-H) In both GDNF treated groups, significantly more axons (SMI-31-positive) grew into the graft environment (G & H) as compared to the grafts containing no GDNF (E & F). Such robust axonal regeneration in the GDNF treated groups was concomitant with vigorous migration of astrocytes (GFAP-positive) into the grafts within which astrocytic processes aligned in parallel to regenerated axons (G & H; arrows). In contrast, in the absence of GDNF, no astrocytic migration into the grafts was found (E & F). (I-P) Within the grafts of both GDNF treated groups, close association between migrated astrocytes (K & L; GFAP-positive) and SC myelin (O & P; P0-positive) was found, which was in high contrast to the lack of astrocyte migration (I & J) and limited SC myelination (M & N) in control grafts in the absence of GDNF. Dashed line in A-H indicates the caudal graft-host interface. Arrows in rows 3 and 4 indicate migrated astrocytes and their association with regenerated axons (G & H) or SC myelin (O & P). (Q & R) At the EM level, close association of an astrocytic process (Q; white asterisk) or astrocyte cell body (R; white asterisk) with regenerated myelinated (white arrows) or unmyelinated (black arrows) axons were clearly seen. Black asterisks indicate SC nuclei. Bars: A—P = 100 μ m; Q = 2 μ m; R = 0.5 μ m.

Figure 4. GDNF induced interdigitative migration between astrocytes and Schwann cells (SCs) (A & B) Phase contrast images show that, in a confrontational assay, a sharp boundary was formed between the SC and astrocyte (AC) monolayers in a control group receiving no GDNF (A; red line). In contrast, GDNF administration induced vigorous migration of both cell populations towards each other, resulting in an interdigitative boundary between the two (B; red line). (C & D) The migratory abilities of astrocytes and SCs in the absence (C) or presence (D) of GDNF could be further appreciated when the cultures were immunostained with their phenotypic markers GFAP and p75, respectively. (E) Quantitative analysis showed that the number of cells that crossed a 300 μm line (A and B; yellow lines) in both SCs and astrocytes were significantly increased in the GDNF treated group as compared to the non-treated group (**; $p < 0.01$). Bars: A-D = 100 μm.

Figure 5. Effects of GDNF on astrocytes migration in the absence or presence of Schwann cell (SC) monolayers

(A) Live cell time-lapse imagings of astrocyte (AC) monolayers under different treatment conditions after a scratch injury. Composite phase contrast photomicrographs were captured at 0 and 24 hours after the scratch. Movement of cells into the scarred region resulted in a decrease in the surface area of the scar. Bar = 100μm. (B) Quantification of the percentage of AC migration area to the total wound area showed that SC conditioned medium (SCM) induced astrocyte migration (**: $p < 0.01$). However, GDNF, used alone or in combination with SCM, did not induce or enhance SCM-mediated astrocyte migration. Interestingly, SCM from GDNF-pretreated SCs [SCM (pre-GDNF)] reduced the scratch gap and enhanced astrocyte migration. (C) DiI pre-labeled astrocytes were seeded on a SC monolayer using an inverted cover-slip assay. Merged phase contrast and immunofluorescent images showed that both migratory distances and the number of astrocytes per migratory distance on the SC monolayer increased significantly as compared to the control group. Bar = 100 μm. (D & E) Quantitative analysis showed that both the number of migrated astrocytes over distances away from the edge of inverted coverslip (D) and the maximum migratory distances (E) were significantly increased in the GDNF-treated group as compared to the non-treated group indicating that GDNF significantly promoted astrocyte migration on the SC monolayer. *: $p < 0.01$; **: $p < 0.01$.

Figure 6. GDNF reduced hypertrophy of astrocytes (AS) *in vitro* and promoted their processes extension

(A & B) GDNF alone did not have an effect on astrocyte morphology (H). (C-E) Schwann cell (SC)-conditioned medium (SCM) alone (C), SCM + GDNF (D), or SCM pretreated with GDNF [SCM(pre-GDNF)](E), increased the astrocyte area as compared to the vehicle control (H, $p < 0.05$). (F) A similar effect was observed when astrocytes were co-cultured with SCs (compared with astrocyte cultured alone; H, $p < 0.01$). (G-G') When GDNF was added to the astrocyte-SC co-cultures, it reduced astrocyte hypertrophy and promoted elongation of astrocyte processes (white arrows) as were shown in both the single staining of GFAP (G) and double staining of GFAP (red) and p75 (green), a SC marker (G'). All sections were counterstained with Hoechst 33342 (blue), a fluorescent nuclear dye. (H) Quantification of astrocyte area was performed using a NIH Image J software. *: $p < 0.05$; **: $p < 0.01$. Bar = 100μm.

Figure 7. GDNF reduced expressions of GFAP and CSPG in astrocyte-SC co-cultures (A and B) Western blot analysis showed that only when GDNF was administered to the astrocyte-SC co-cultures, it significantly reduced GFAP and CS-56 expression ($p < 0.01$). Additionally, administration of a combination of GDNF and SCM in astrocyte single cultures had no effect on GFAP expression (A) but significantly reduced that of CS-56 (B, $p < 0.01$). The top panels show representative GFAP and CS-56 expressions after different treatments. The bottom panels show compiled results in bar graphs after these treatments ($n = 6/\text{group}$). AC: astrocytes; SC: Schwann cells; SCM: Schwann cell-conditioned medium; SCM (pre-GDNF): SCM pretreated with GDNF.