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Mechanisms of olfactory ensheathing cell-enhanced neurite outgrowth and axon regeneration after spinal cord injury

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Mechanisms of olfactory ensheathing cell-enhanced neurite outgrowth and axon regeneration after spinal cord injury

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Molecular, Cellular, and Integrative Physiology

by

Rana R. Khankan

2015
ABSTRACT OF THE DISSERTATION

Mechanisms of olfactory ensheathing cell-enhanced neurite outgrowth and axon regeneration after spinal cord injury

by

Rana R. Khankan

Doctor of Philosophy in Molecular, Cellular, and Integrative Physiology

University of California, Los Angeles, 2015

Professor Patricia Emory Phelps, Chair

Olfactory ensheathing cells (OECs) provide a pro-regenerative environment for the axons of olfactory receptor neurons and therefore are a promising candidate for cell transplantation therapy following spinal cord injury. We previously showed that OEC transplantation supports axon regeneration and functional re-connectivity following complete spinal cord injury, yet lack of an OEC-specific marker limited our ability to determine how they promoted these beneficial effects. Using both in vitro and in vivo models, we investigated the mechanisms by which OECs mediate axon regeneration. OECs enhance neurite outgrowth of postnatal cortical neurons in a scar-like culture model. We provide strong evidence that direct OEC-neurite alignment is critical to enhance neurite outgrowth in scar-like astrocyte and meningeal fibroblast inhibitory environments. We also tested eGFP-OECs from transgenic rats and showed that they facilitate neurite outgrowth in vitro. Then in a short-term study, we analyzed OEC survival, migration, and distribution within the lesion site of complete spinal cord transected rats. We found that rats transplanted with OECs preserve and associate with axons and neurons in the lesion core, reduce
the presence of inhibitory CSPGs and myelin debris, and reduce secondary tissue damage due to microglial and macrophage activation and infiltration post-injury. Collectively, these data support a neuroprotective and proregenerative role of OECs through the modulation of glial scar formation following a complete spinal cord transection.
The dissertation of Rana R. Khankan is approved.

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University of California, Los Angeles

2015
DEDICATION

I dedicate my dissertation work to my family for their unwavering support and encouragement over the years. A special thanks to my parents, Rachad and Wahida, for providing me with the opportunity to achieve my highest dreams. My sister, Dr. Rima Khankan, for encouraging me to find my own path and my nieces, Roya and Ruba for their endless inquiries about science!
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Curriculum Vitae

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My PhD dissertation evaluates the effects of olfactory ensheathing cells (OECs) on axon regeneration in vivo using a complete spinal cord transection model and neurite outgrowth in vitro using an inhibitory scar-like culture model. I investigated the mechanisms of OEC-mediated axon regeneration following spinal cord injury and provide evidence that OECs are neuroprotective, immunomodulatory, and facilitate axon regeneration by modifying the inhospitable environment that forms after SCI. I identified a spatially restricted and contact-dependent growth-promoting effect of OECs on neurites that is independent of the scar-like environment using glial and neuronal cultures. In search for novel OEC markers, in my MS thesis, I identified that OECs express and secrete Reelin and investigated Reelin’s function in the olfactory system. I also found that OECs express integrin alpha-7.

Publications


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Chapter 1. Introduction

*Glial scar formation after central nervous system (CNS) trauma*

Adult central nervous system (CNS) neurons have a reduced capacity to regrow functional axons after injury due to the inhibitory milieu of the lesion site. Both CNS and peripheral cells respond to the trauma in addition to environmental factors that together contribute to the non-permissive environment found at the injury site (Cregg et al., 2013; Fitch and Silver, 2008). Among the CNS cells that respond to injury are the astrocytes that become reactive, secrete pro-inflammatory cytokines, produce inhibitory extracellular matrix proteins, and form a barrier to reduce secondary tissue damage (Faulkner et al., 2004; Sofroniew and Vinters, 2010). Microglia, the resident phagocytic cells of the CNS, are activated by injury, secrete cytotoxic factors and recruit blood-derived immune cells to the lesion site to remove debris (Nakajima and Kohsaka, 2004). Macrophages, of microglial or hematopoietic origin, release of cytotoxic mediators such as free radicals, cytokines, and metalloproteases that cause axonal dieback and secondary tissue damage (Horn et al., 2008). Apoptotic oligodendrocyte cell death contributes to axonal damage further away from the lesion epicenter and Wallerian degeneration results in inhibitory myelin debris (Chew et al., 2012; Shuman et al., 1997). The oligodendrocyte precursor response to injury involves the production of NG2, an inhibitory chondroitin sulfate proteoglycan (CSPG), which is also produced by macrophages (Jones et al., 2002).

Peripheral cells such as meningeal fibroblasts then invade the lesion site, form a fibrotic scar, and secrete molecules like semaphorin 3A that inhibits axonal outgrowth (Bundesen et al., 2003; Pasterkamp et al., 2001). In addition, the breakdown of the blood brain barrier induces perivascular pericytes and fibroblasts to proliferate and contribute to fibrotic scar formation and
extracellular matrix deposition (Göritz et al., 2011; Soderblom et al., 2013). Together these
multicellular responses to injury generate the inhibitory milieu which prevents CNS axon
regeneration following injury.

Olfactory ensheathing cells (OECs) are a promising therapy for spinal cord injury

A major goal of spinal cord injury (SCI) research is to facilitate axonal regeneration
across the injury site and functional synaptic re-connectivity. Many therapies are targeted to
reduce these extracellular post-traumatic growth-inhibitory factors to enhance the intrinsic
capacity of neurons to regenerate. One CNS glial cell type that is able to enhance axonal
outgrowth is associated with the pro-regenerative olfactory system and is considered a promising
treatment following SCI (Granger et al., 2012; Lu et al., 2002; Lopez-Vales et al., 2006; Kubasak
et al., 2008; Ramón-Cueto et al., 1998, 2000; Tabakow et al., 2014; Takeoka et al., 2011; Ziegler
et al., 2011). Olfactory receptor neurons are continually generated throughout life and their axons
then project from the nasal epithelium to their CNS targets in the olfactory bulb (Graziadei and
Monti Graziadei, 1985). Their remarkable growth abilities are linked to olfactory ensheathing
cells (OECs), a distinctive glial cell with features that resemble both Schwann cells and
astrocytes (Doucette, 1991; Ramón-Cueto and Valverde, 1995). After an olfactory nerve lesion,
the growth-promoting OECs survive and maintain a conduit for olfactory receptor axons to grow
along as they project to their olfactory bulb targets (Li et al., 2005). However, olfactory receptor
axons fail to regenerate following an olfactory bulbectomy which removes OECs (Pasterkamp et
al., 1998). OECs secrete trophic factors that have neurite outgrowth promoting properties such as
brain derived neurotrophic factor (BDNF) and ciliary neurotrophic factor (CNTF; Lipson et al.,
2003; Woodhall et al., 2001). OECs also express adhesion molecules such as L1-NCAM and
neuropilin-1 that are involved in axonal outgrowth and allow OEC to ensheath growing axons to protect them from inhibitory molecules (Witheford et al., 2013; Roet et al., 2013). These characteristics make them excellent candidates for use as therapeutic cellular grafts after SCI.

**OECs support axon regeneration after a complete SCI**

Over the past decade, several studies have reported that OECs support axon regeneration even after a complete spinal cord transection in rodents. Anatomical evidence of the regeneration of corticospinal tract axons across the lesion site correlated with improved grid climbing ability 8 months after injury and OEC transplantation (Ramón-Cueto et al., 2000). Kubasak et al. (2008) showed that when OEC transplantation was combined with extensive manual step-training on a treadmill, the spinal rats significantly improved their stepping ability, exhibited smaller injury sites and had more noradrenergic axons around the lesion site than seen in media-injected spinal rats. Takeoka et al. (2011) reported that 8 months after a complete spinal cord transection, 70% of OEC-grafted rats displayed motor-evoked potentials in their hindlimb muscles after transcranial electrical stimulation versus 0% of media-injected controls. Furthermore, when the OEC-grafted rats had their spinal cords retransected, these evoked potentials disappeared (Takeoka et al., 2011). Additionally, following spinal cord transection and OEC-treatment, rats showed a direct correlation between improved grid climbing ability and the recovery of motor-evoked potentials in hindlimb muscles (Ziegler et al., 2011). These motor-evoked potentials also could be altered by pharmacological interventions such as quipazine administration, a 5-HT agonist (Ziegler et al., 2011).
**Proposed research project**

Despite these functional improvements, these studies could not identify OECs at 6-8 months post-transection and implantation due to the lack of an OEC-specific marker. The irrefutable identification of transplanted OECs after a complete SCI has hampered our investigation of their proregenerative mechanisms. We propose the use of enhanced green fluorescent protein (eGFP)-labeled OECs to eliminate the difficulty of identifying transplanted OECs following a complete spinal cord transection. In the current studies, we first evaluate how OECs promote neurite outgrowth using an *in vitro* scar-like model, composed of astrocyte and meningeal fibroblast co-cultures that are mechanically stretched to induce reactive gliosis and mimic the inhibitory glial barrier formed after a complete spinal cord transection (Wanner et al., 2008; Khankan et al., 2015).

**Specific Aim 1:** Determine if OECs and cell tracker green-labeled OECs stimulate neurite outgrowth in an inhibitory glial scar-like environment.

*Hypothesis 1.1:* OECs promote neurite outgrowth in a growth inhibitory astrocyte and meningeal fibroblast scar-like environment by a direct contact-mediated mechanism.

*Hypothesis 1.2:* Cell tracker green-labeled OECs and eGFP-labeled OECs enhance neurite outgrowth to a similar degree and thus can be used interchangeably.

Next we use enhanced eGFP-labeled OECs to investigate the mechanisms by which they mediate axon regeneration following a complete spinal cord transection.

**Specific Aim 2:** Determine the time course of survival and extent of cell migration into the injury site following transplantation of eGFP-labeled OECs or eGFP-labeled FB (cellular control) after complete spinal cord transection. Determine whether or
not OECs bridge the lesion site, ensheath regenerating axons, and enhance neuronal survival around the injury site.

*Hypothesis 2.1: OECs but not FBs, survive and migrate past the glial scar and into the lesion site, and thus provide a cellular bridge across the injury.*

*Hypothesis 2.2: OECs associate with and protect regenerating axons, enhance neuronal survival, and reduce inhibitory factors within the injury site.*

Lastly, we administered cyclosporine-A (CSA) to enhance the survival of eGFP-labeled cells following transplantation.

**Specific Aim 3:** Determine if the survival of FBs and OECs was reduced due to the host immune response following complete SCI and whether or not graft survival can be enhanced with CSA administration.

*Hypothesis 3.1: The reduced survival of FBs and OECs is due to the innate immune response and concomitant secondary tissue damage.*

*Hypothesis 3.2: CSA administration enhances the survival of transplanted OECs that will fill the lesion core to provide a regenerative cellular bridge following complete spinal cord transection.*

*Hypothesis 3.3: Enhanced OEC survival as a result of CSA-treatment protects regenerating axons, preserves neurons, and reduces inhibitory factors within the injury site.*
References


Ziegler, M.D., Hsu, D., Takeoka, A., Zhong, H., Ramón-Cueto, A., Phelps, P.E., Roy, R.R.,
Edgerton, V.R., 2011. Further evidence of olfactory ensheathing glia facilitating axonal
Chapter 2. Olfactory ensheathing cell–neurite alignment enhances neurite outgrowth in scar-like cultures

Abstract

The regenerative capacity of the adult CNS neurons after injury is strongly inhibited by the spinal cord lesion site environment that is composed primarily of the reactive astroglial scar and invading meningeal fibroblasts. Olfactory ensheathing cell (OEC) transplantation facilitates neuronal survival and functional recovery after a complete spinal cord transection, yet the mechanisms by which this recovery occurs remain unclear. We used a unique multicellular scar-like culture model to test if OECs promote neurite outgrowth in growth inhibitory areas. Astrocytes were mechanically injured and challenged by meningeal fibroblasts to produce key inhibitory elements of a spinal cord lesion. Neurite outgrowth of postnatal cerebral cortical neurons was assessed on three substrates: quiescent astrocyte control cultures, reactive astrocyte scar-like cultures, and scar-like cultures with OECs. Initial results showed that OECs enhanced total neurite outgrowth of cortical neurons in a scar-like environment by 60%. We then asked if the neurite growth-promoting properties of OECs depended on direct alignment between neuronal and OEC processes. Neurites that aligned with OECs were nearly three times longer when they grew on inhibitory meningeal fibroblast areas and twice as long on reactive astrocyte zones compared to neurites not associated with OECs. Our results show that OECs can independently enhance neurite elongation and that direct OEC-neurite cell contact can provide a permissive substrate that overcomes the inhibitory nature of the reactive astrocyte scar border and the fibroblast-rich spinal cord lesion core.
Introduction

Olfactory receptor neurons are generated and then project their axons from the peripheral into the central nervous system throughout life (CNS; Graziadei and Monti Graziadei, 1985). The regenerative ability of the olfactory receptor neurons is enhanced by olfactory ensheathing cells (OECs), a distinct glia with features of both Schwann cells and astrocytes (Doucette, 1991; Ramón-Cueto and Valverde, 1995). After an olfactory nerve injury, OECs survive and maintain a conduit so that newly generated axons can grow into the damaged inhibitory areas of the adult olfactory system, cross the glia limitans, and contact their olfactory bulb targets (Doucette, 1991; Li et al., 2005). Due to these attributes, OECs are considered a promising treatment following spinal cord injury (SCI; Lu et al., 2002; Lopez-Vales et al., 2006; Kubasak et al., 2008; Ramón-Cueto et al., 1998, 2000; Tabakow et al., 2014; Takeoka et al., 2011; Ziegler et al., 2011).

While adult CNS neurons have a capacity to regenerate, they usually fail to regrow functional axons due to a non-permissive or inhibitory environment. After injury, astrocytic cell bodies hypertrophy, and their processes widen, cluster together, elongate, and display increased glial fibrillary acidic protein (GFAP) immunoreactivity, a response defined as reactive astrogliosis (Barrett et al., 1981; Reier and Houle, 1988; Silver and Miller, 2004; Sofroniew, 2009). A GFAP-positive scar border of reactive astrocytes forms due to both the injury and the invasion of meningeal fibroblasts (Silver and Miller, 2004; Wanner et al., 2013). The processes of newly generated reactive astrocytes become oriented transversely to isolate the intact spinal cord from the lesion core, a response that limits axon regeneration (Barnabé-Heider et al., 2010; Li et al., 2012; Wanner et al., 2013). Additionally, increased chondroitin sulfate proteoglycan (CSPG) and class 3 semaphorin (Sema3) expression contribute to the inhibitory environment formed at the lesion site (Buss et al., 2009; Fitch and Silver, 2008; Hu et al., 2010; Pasterkamp et
A scar-like culture model (Wanner et al., 2008) replicates the wide-spread reduction of neurite outgrowth as a result of reactive astrogliosis and elevation of inhibitory CSPGs phosphacan, neurocan, and tenascin. The scar-like environment in this model is generated by the addition of two injury-inducing factors to quiescent astrocytes: 1) confrontation with meningeal fibroblasts and 2) mechanical stretch (Wanner et al., 2008).

OECs may overcome the injury site inhibition and promote neurite sprouting and outgrowth by providing both an adhesive cellular substrate and permissive soluble factors (Chung et al., 2004; Kafitz and Greer 1999; Pellitteri et al., 2009; Sonigra et al., 1999). Indeed OECs express multiple adhesion molecules involved in axon outgrowth, secrete trophic factors, and ensheath growing axons to protect them from inhibitory molecules (Doucette, 1990; Lipson et al., 2003; Ramón-Cueto and Valverde, 1995; Woodhall et al., 2001). Trophic factors, such as brain-derived neurotrophic factor (BDNF), contribute to the ability of OECs to enhance axon regeneration on an inhibitory substrate (Ruitenber et al., 2003; Runyan and Phelps, 2009), but the contact-mediated OEC-neuron interactions are not well studied.

The growth-promoting characteristics of OECs lead to their use as therapeutic cellular grafts following SCI. Over the past decade a number of studies reported that OECs support axon regeneration in vivo, even after a complete transection (Kubasaki et al, 2008; Lopez-Vales et al., 2006; Lu et al., 2002; Ramón-Cueto et al., 1998, 2000; Tabakow et al., 2014; Takeoka et al., 2011; Ziegler et al., 2011). Despite the reported functional improvements, most of these studies could not identify OECs post-implantation and consequently the OEC interactions in a SCI environment remain unclear. In the present study, we used an established model of SCI that recapitulates the inhibitory environment of the astroglial scar and its fibroblast border (Wanner et al., 2008) to test how OEC transplantation facilitates neurite regeneration. We identified OEC-
neurite alignment as a critical regulator of neurite outgrowth on the growth-inhibitory substrates in scar-like cultures.

**Materials and Methods**

*Astrocyte-meningeal fibroblast co-culture*

Methods to prepare co-cultures of astrocytes and meningeal cells (predominantly fibroblasts, but also microglia and blood vessels) were similar to those reported in Wanner et al. (2008) and (2012). Astrocytes were cultured from neonatal rat cerebral cortices in 5% fetal bovine serum (FBS, Hyclone, Logan, UT) and upon reaching confluence, cultures were switched to a mixture of 1:1 DMEM and Ham’s F12 (D/F medium, Gibco) supplemented with 5% horse serum. Approximately 200,000 astrocytes were seeded onto deformable membranes (962 mm² Bioflex 6 well plates, Flexcell Int. Corp., Hillsborough, NC) previously coated with collagen (Fig. 1; control culture). Astrocyte cultures were slowly withdrawn from serum and kept serum-free until the addition of meningeal cells.

Meningeal fibroblasts were isolated from newborn rat cortical meninges, dissociated and resuspended with 3% trypsin, collagenase, and DNase I. They were grown for 5 days on poly-L-lysine-coated dishes (PLL; Sigma, St. Louis, MO). Meningeal fibroblasts were added to the astrocytes on deformable membranes after 4 weeks *in vitro* with 130,000 cells per culture in 10% FBS/DF medium (Fig. 1). Astrocyte-fibroblast co-cultures grown on deformable membranes were given two short pressure pulses (3.5-3.8 psi) with a pressure controller (Ellis et al., 1995) that induced abrupt membrane deformation and mechanical trauma to the cells (Fig. 1; scar-like culture).
**OEC primary culture**

Olfactory bulbs were collected from 8-10 weeks old Sprague Dawley rats and the leptomeninges were removed to reduce fibroblast contamination. Methods to prepare OEC primary cultures were adopted from Ramón-Cueto et al. (2000). OECs were dissected from the first two layers of the olfactory bulb and washed in Hank’s Balanced Salt Solution (HBSS, Gibco, Rockville, MD) prior to tissue centrifugation at 365 g for 5 min. The tissue pellet was first resuspended in 0.1% trypsin and HBSS without Ca\(^{2+}/Mg^{2+}\) (Gibco), then placed in a 37°C water bath, and mixed intermittently for 10 min. D/F medium supplemented with 10% FBS and 1% Penicillin/Streptomycin (P/S, Gibco) was used to inactivate trypsin prior to centrifugation. Dissociated cells were rinsed and centrifuged 3 times, and then plated into 25 cm\(^2\) culture flasks pre-coated with 0.05 mg/ml PLL. Cells were maintained at 37°C for 7 days and D/F medium was changed every 2 days.

**OEC immunopurification**

Hydrophobic petri dishes were coated overnight with Biotin-SP-conjugated AffiniPure goat anti-mouse IgG (1:1000; Jackson Immunoresearch Laboratories, West Grove, PA) in 50 mM Tris buffer. Dishes were washed 4 times with 25mM PBS (Gibco) and then incubated overnight with antibody against p75-nerve growth factor receptor (anti-p75-NGFR, 1:5; clone 192, Chandler et al., 1984) at 4°C. Dishes were rinsed 3 times with 25mM PBS and treated with a mixture of PBS and 0.5% BSA for 1 h at room temperature. Before immunopanning cells, antibody-treated dishes were washed with PBS and DMEM.

OEC primary cultures were treated with 0.25% trypsin in HBSS without Ca\(^{2+}/Mg^{2+}\) for 3 min at 37°C before trypsin inactivation with D/F medium. Cells were centrifuged at 216 g for 10
min and resuspended in D/F medium. Cells were added to pre-treated anti-p75-NGFR dishes and incubated at 37°C for 10 min. Unbound cells were washed off and a cell scraper was used to recover bound cells which were then subjected to a second immunopanning. Purified p75-NGFR-positive OECs were resuspended, plated on PLL-coated culture flasks, and incubated at 37°C for 7 days with medium changed every 2 days. Purified OECs were stimulated with pituitary extract (20 μg/ml, Gibco) and forskolin (2 μM, Sigma). Mitogens were removed and cells were pretreated with CellTracker™ Green CMFDA (7 μM, Molecular Probes, Eugene, OR) for 1 h at 37°C. After rinsing, OECs were incubated with D/F medium for 1 hr, washed with PBS, and trypsinized. 100,000 OECs were added to the scar-like cultures 5-6 hours after the mechanical stretch (Fig. 1; scar-like + OEC culture).

Cerebral cortical neuron cultures

Neurons were obtained from postnatal day 6-8 rat cerebral cortices after the removal of the leptomeninges. Cerebral cortices were harvested in Hibernate-A medium (BrainBits, Inc., Springfield, IL) with P/S, L-glutamine (L-glu, Gibco), and B27 supplement (Gibco), then chopped and digested with warm papain (2 mg/ml, Worthington, Lakewood, NJ) in Hibernate-A medium with P/S and L-glu. Neurons were enriched with an OptiPrep step gradient (Axis-Shield, Norton, MA). One day after stretch, all cultures received 100,000 neurons pipetted into the center of the culture (Fig. 1). After 24 h, cultures were fixed using 4% paraformaldehyde in Tris-buffered saline (TBS) for 1 h and washed. Additional 24-hour co-cultures were tested for neuron viability and most neurons without processes co-localized with live uptake of propidium iodide, indicating the loss of cell integrity. Four independent culture experiments were conducted and 1-3 wells per experiment were analyzed for each experimental variable.
**Immunocytochemical procedures**

Cultures were permeabilized with 0.3% Triton in TBS for 30 min, blocked with 5% donkey and goat serum (Sigma) overnight, and incubated with anti-GFAP (1:500, mouse or rabbit, BD Biosciences Pharmingen, San Jose, CA; Dako, Carpinteria, CA) and rabbit anti-β3 tubulin (1:1500, Covance, Berkeley, CA) or mouse anti-fibronectin (1:500, BD Biosciences Pharmingen) overnight at 4°C. To visualize immunostaining, Cy3- and Cy5-conjugated fluorescent secondaries (1:250, 1:80, respectively; Jackson ImmunoResearch, West Grove, PA) were used. Nuclei were stained with Hoechst dye (Sigma) for 5 min. Cultures were washed with TBS, dried, and then cover slipped onto glass slides with Fluorogel (Electron Microscopy Sciences, Hatfield, PA).

**Imaging, neuron tracing, and statistical analysis**

Images were obtained using a Zeiss LSM 510 confocal microscope. Approximately 16 fields from the central-most area of the stretch wells that contained GFAP-negative zones and multiple neurons were analyzed for neurite outgrowth. Peripheral areas were excluded for consistency. Neurons and their neurites were quantified and traced using the Neurolucida 7.50.4 neuron reconstruction program (MicroBrightField, Inc., Williston, VT). Individual neurite lengths and cell body details were exported to Microsoft Excel (Redmond, WA) using Neurolucida Explorer 4.50.4 (MicroBrightField, Inc., Williston, VT). Neurite processes were measured and the extent of their glial association was scored as aligned, crossing, or not associated. Additionally, neurons and neurite processes were binned based on their location in either a GFAP-positive or negative area. Neurons were counted in the GFAP-negative fibroblast zone only if neurites remained entirely within the GFAP-negative area. Neuron counts were
normalized per image and reported as the sum of neurons analyzed in all 16 fields of each culture. Culture experiments were conducted in triplicate or quadruplicate and data sets presented as a mean ± SEM. Statistical analyses of differences between means was calculated using a two-way ANOVA, performed with JMP Software (version 10.0.0 for Microsoft Windows, SAS Institute Inc., Cary, NC). Statistical significance was determined by \( p < 0.05. \)

**Results**

**Scar-like cultures mimic key aspects of spinal cord injury**

To better understand how OECs interact with injured neurons *in vivo*, we tested OECs in an *in vitro* assay that recapitulates the features of a traumatic SCI. We used a scar-like culture model composed of astrocytes challenged by meningeal fibroblasts that is then mechanically stretched to induce molecular and morphological features of reactive astrogliosis and fibroblast clustering (Fig. 1; Wanner et al., 2008). Scar-like cultures reproduce features of the inhibitory environment found near the glial scar border and the lesion core after complete spinal cord transection (Fig. 2). At 1 month post-SCI, stellate GFAP-positive astrocytic processes bundle together to encircle the GFAP-negative, non-neural tissue in the spinal cord injury site (Fig. 2A, B). Similarly in scar-like cultures, elongated processes of GFAP-positive reactive astrocytes surround clusters of fibronectin-labeled meningeal fibroblasts (Fig. 2E). Thus, borders are established separating the astrocyte and fibroblast areas similar to those found after SCI (Fig. 2C-E; Göritz et al., 2011; Reier and Houle, 1988; Wanner et al., 2008, 2013). Neurites typically avoided growing into the non-permissive GFAP-negative fibroblast areas (Fig. 2C). Therefore, our scar-like cultures provide multiple small-scale zones of astrocytic scar borders reminiscent of the inhibitory scar border formed following SCI.
Features of reactive astrogliosis and GFAP immunoreactivity remained following the addition of OECs to the scar-like cultures (Fig. 2C, D). OECs intermingled with astrocytes, and interestingly, they crossed the reactive astrocyte borders, and often entered into the fibroblast-filled, GFAP-negative areas (Fig. 2D, E). Neurons grown in scar-like + OEC cultures adhered and extended neurites into the non-permissive fibroblast territory primarily when they associated with OECs (Fig. 2D). These cultures, therefore, are suitable to assess if OECs can stimulate neurite outgrowth in a scar-like environment.

**OECs promote neurite outgrowth in scar-like cultures**

To evaluate the extent to which OECs promoted neurite outgrowth in growth-inhibitory scar-like cultures, we compared neurite outgrowth on quiescent astrocytes (positive control), and on injured astrocyte-meningeal fibroblast co-cultures in the absence or presence of OECs (scar-like or scar-like + OEC). Approximately 5300 neurons were traced and reconstructed, and the lengths of 12,700 neurites were quantified. The same percentage of neurons initiated neurite outgrowth in the three culture conditions (control 85±0.01% of neurons; scar-like 85±0.02%; scar-like + OEC 85±0.02%; mean ± SEM; n=4). Additionally, no differences were found in the mean number of neurons with neurites sampled per culture (control 117±16 neurons; scar-like 135±20; scar-like + OEC 135±17; n=4). Likewise, the number of neurons that failed to grow any neurites did not differ between cultures. These neurons were excluded from further analysis as more than 90% co-localized with propidium iodide.

Postnatal cerebral cortical neurons grew long, branching neurites over control quiescent astrocyte lawns, while neurons on reactive astrocytes in scar-like cultures had short unbranched processes (Fig. 3A, B). When OECs were added to scar-like cultures, neurite outgrowth was
restored to levels closer to control cultures (compare panel A to panel C in Fig. 3), and these long neurites commonly were associated with OECs (Fig. 3D). Total neurite tree lengths of neurons grown on control astrocyte cultures were longer than those grown on scar-like cultures without or with OECs (Fig. 3E; control 304±26 µm; scar-like 136±17 µm; p< 0.0001; scar-like + OECs 217±17 µm; p< 0.05; n=4). The addition of OECs to the scar-like cultures increased total neurite outgrowth 60% above that measured in scar-like cultures alone (Fig. 3B-E; p< 0.01).

**OECs enhance neurite outgrowth by neurite-OEC association**

Having established the ability of OECs to promote neurite outgrowth in scar-like cultures, we next asked how OECs enhance neurite outgrowth in an inhibitory environment. To determine if there was a contact-mediated effect between OEC processes and neurites, scar-like + OEC cultures were examined in confocal images. Each neurite-OEC interaction was scored into one of three categories: 1) neurites aligned with and extensively contacting an OEC process (Fig. 4A1; Align), 2) neurites that crossed an OEC process (Fig. 4A2; Cross), and 3) neurites that did not interact with OECs (Fig. 4A3; None). When single neurites were separated by association category, 52±8% of neurites aligned with OEC processes, whereas only 23±4% (p<0.01) crossed, and 26±5% (p<0.01) never contacted OECs (Fig. 4B). Measurements of neurites aligned with OEC processes were significantly longer (Fig. 4A1, C; 135±15 µm) than those that crossed (Fig. 4A2, C; 53±9 µm; p< 0.001) or never associated with OEC processes (Fig. 4A3, C; 30±3 µm; p< 0.0001). The length of neurites that crossed OECs did not differ from those with no interaction and are combined in future analyses (p= 0.14). These data show that OECs enhanced neurite elongation through direct alignment of neuronal and OEC processes in scar-like cultures.
**Fibroblast zones are more growth inhibitory than astrocyte zones**

To better understand OEC-enhanced neurite outgrowth, we then characterized the effect of the two distinct cellular substrates of scar-like cultures, i.e., reactive astrocytes (Fig. 5A, B) and meningeal fibroblasts (Fig. 5C, D), on neuronal adhesion and neurite outgrowth. In scar-like cultures without OECs, the percent of neurons with regenerating neurites was much greater in astrocyte than in fibroblast areas (Fig. 5E; 79±5% versus 21±5%; \(p<0.0001; n=3\)). In addition, the majority of neurites grew on astrocytes rather than on fibroblasts (Fig. 5F; 90±1% of neurites versus 10±1% of neurites; \(p<0.0001\)), and the average neurite length was nearly three times longer on astrocytes than fibroblasts (60±12 µm versus 23±5 µm; \(p<0.01; n=3\)). The addition of OECs to the scar-like cultures increased the distribution of neurons adhering to fibroblast areas by 50% and doubled the number of neurites within those areas (Fig. 3E; 32±2% of neurons; 20±7% of neurites). Neurites also were significantly longer in astrocyte zones with, than without OECs (Fig. 5F; 92±5 µm versus 60±12 µm; \(p<0.05\)). In fibroblast zones, however, neurite lengths in the presence or absence of OECs did not significantly differ (Fig. 5F; 41±6 µm versus 23±5 µm; \(p=0.14\)). This suggests that fibroblasts inhibited neurite outgrowth more than reactive astrocytes and that OECs acted independent of astrocytic inhibitory factors to enhance neurite elongation.

**Neurite growth aligned with OECs is enhanced on both substrates**

Because OECs could enhance neurite outgrowth better in astrocyte than in fibroblast areas, we asked if this enhanced neurite outgrowth depended upon cell surface interactions between neurites and OECs. We quantified this cell surface-mediated effect by separating individual neurites into groups that aligned or did not interact with OECs and remained entirely
within reactive astrocyte or fibroblast zones. On both substrates, the mean neurite length was greatest when neurites aligned with OECs compared to neurites that did not contact OECs (Fig. 5B-D, G; aligned 136±17 µm versus non-aligned 37±5 µm on astrocytes, p< 0.0001; aligned 64±12 µm versus non-aligned 20±2 µm on fibroblasts, p< 0.001). This implies that the OEC-enhanced outgrowth in fibroblast zones was previously masked when aligned and non-aligned neurite lengths were combined (Fig. 5F). We next asked if the stunted neurite outgrowth on fibroblasts in scar-like cultures (Fig. 5C, inset) could be overcome when neurites aligned to OEC surfaces (Fig. 5B, D). Neurites were nearly three times longer in fibroblast zones when they aligned with OECs compared to when they did not interact (Fig. 5G; 64±12 µm versus 23±5 µm; p< 0.01). Confocal images of neurites aligned with OECs show evidence of direct cell-to-cell contact (Fig. 5H). This shows that the inhibitory factors from fibroblasts can be overcome if the neurites are directly aligned with OECs.

Finally, to determine if neurite-OEC alignment was sufficient to enhance neurite elongation, we compared neurites that were aligned with OECs to those in quiescent astrocyte cultures and reactive astrocyte zones in scar-like cultures. When neurites aligned with OECs in reactive astrocyte zones, they were longer than neurites in quiescent or reactive astrocyte zones without OECs (Fig 5G; 136±17 µm versus quiescent astrocytes 109±7 µm, p< 0.05; or reactive astrocytes 60±12 µm, p< 0.0001). Moreover, the direct association between neurites and OEC processes in the fibroblast zones attenuated the stunted neurite outgrowth to the extent that neurite lengths matched those in the astrocyte zones of scar-like cultures (Fig. 5A, D, G; 64±12 µm versus 60±12 µm, p< 0.78). Together our findings support that direct cell surface alignment with OECs is required to enhance neurite elongation in both scar-like astrocyte and fibroblast environments.
Discussion

We used an established glial scar model that replicates key features of the inhibitory environment following SCI to assess if OECs enhance neurite outgrowth of postnatal cerebral cortical neurons. Analyses of neuron reconstructions showed that the presence of OECs increased the total neurite tree length of neurons grown in scar-like cultures. We identified OEC-neurite alignment as a novel method by which OECs enhanced neurite elongation. In fact, alignment with OECs more than doubled the mean neurite lengths that grew in both the astrocyte and fibroblast zones. Additionally, neurites that aligned with OECs in the scar-like astrocyte zones were significantly longer than those in permissive quiescent astrocyte areas. In combination, our data suggest that OECs provide a spatially restricted and contact-dependent growth-promoting effect on neurites that is independent of the scar-like environment.

OECs can interact with inhibitory components of the glial scar

After SCI both meningeal fibroblasts and astrocytes along the scar border act as physical and chemical barriers that disrupt regeneration (Davies et al., 1997; Pasterkamp et al., 2001; Wanner et al., 2008, 2013). Similarly, neurite outgrowth was stunted in scar-like cultures due to the scar border generated by the presence of fibroblasts and mechanical stretch (Wanner et al., 2008). These fibroblast and reactive astrocyte zones reportedly contain two major molecules implicated in neurite outgrowth inhibition, Sema3A and CSPGs (Pasterkamp et al., 1998, 2001; Wanner et al., 2008, 2013). Sema3A is the best known substrate-bound, growth-inhibitory factor expressed by meningeal fibroblasts in vitro (Niclou et al., 2003; Shearer et al., 2003) and inhibits regeneration of axons that express the Sema3A receptor neuropilin-1 (NRP-1; Pasterkamp et al., 1998, 2001). Based on studies of the developing olfactory bulb, NRP-1-positive olfactory
receptor neuron axons are repelled by Sema3A, and this inhibition contributes to pre-target axon sorting between the olfactory epithelium and the olfactory bulb (Imai et al., 2009). A subset of OECs in the olfactory nerve layer express Sema3A during development and likely contribute to axon orientation during their growth into the olfactory bulb (Crandall et al., 2000; Imai et al., 2009; Schwarting et al., 2000). OECs also express NRP-1 and the down-regulation of NRP-1 expression on OECs reduces axonal outgrowth of dorsal root ganglia neurons (Roet et al., 2013). Thus, because OECs express both Sema 3A and its receptor NRP-1, they are not repelled by meningeal fibroblasts (Fig. 2D, 5D) and therefore can influence axon orientation (Fig. 5B, D).

After SCI, astrocytes and fibroblasts up-regulate and secrete CSPGs (Bradbury et al., 2002; Burda et al., 2014; McKeon et al. 1995; Morgenstern et al. 2002). Treatment with chondroitinase ABC following injury enhances axon growth and blocks interactions between CSPGs and Sema3A to reverse the repulsive properties on neurite outgrowth (Bradbury et al., 2002; de Wit et al., 2005; Grimpe et al. 2005; McKeon et al. 1995; Zuo et al. 1998). Reportedly OECs express proteoglycanases, such as A disintegrin and metalloproteinase with thrombospondin motifs-1 and -4 (Guerout et al., 2010; Roet et al., 2013), and appear to reduce CSPG immunoreactivity in the injured spinal cord (Lakatos et al., 2003). Together with these reports our results suggest that OECs may modulate extracellular matrix components by favorably interacting with both reactive astrocytes and meningeal fibroblasts to provide a more permissive substrate and stimulate neurite regeneration.

**OEC-neurite alignment stimulates outgrowth**

Our quantitative findings that OEC-neuron alignment enhances neurite outgrowth on meningeal fibroblast and reactive astrocyte territories is novel, but the mechanism of their
growth enhancement via cell-to-cell contact could not be addressed in this study due to the multi-
cellular nature of the scar-like cultures. Adhesion-type molecules expressed by OECs are likely
to be involved: integrins, N-cadherin, or other cell adhesion molecules (CAMs; Akins et al.,
2007; Fairless et al., 2005; Miragall et al., 1988; Roet et al., 2013). OECs express L1-CAM
(Runyan and Phelps, 2009; Shields et al., 2010) and are reported to support neurite outgrowth of
corticospinal tract neurons (Witthford et al., 2013). L1 also modulates the response to Sema3A
and associates with NRP-1 to change the Sema3A-induced chemorepulsion to chemoattraction
(Castellani et al., 2000). In the olfactory bulb and nerve, OECs and olfactory receptor axons
express NCAM during development and adulthood both at axon-axon and axons-OEC contacts
(Miragall et al., 1988; Yoshida et al., 1999). Additionally, siRNA knockdown of OEC-expressed
NCAM significantly reduced neurite outgrowth (Roet et al., 2013).

N-Cadherin regulates direct cell-to-cell adhesion and interacts with downstream signaling
cascades that promote neurite outgrowth (Bixby and Zhang, 1990; Kiryushko et al., 2004). OECs
express high levels of N-Cadherin yet do not appear to use it to adhere to other OECs or
astrocytes (Fairless et al., 2005). One possibility is that the neurites use N-Cadherin to align with
OECs, in a similar manner to that demonstrated for immature Schwann cells in vitro (Wanner
and Wood, 2002; Wanner et al., 2006). Alternatively, OECs may modulate the distribution and
function of N-Cadherin via the expression of the Scavenger Receptor Class B member 2, an
enhancer of regenerative sprouting (Roet et al., 2013).

OECs in this study appear to enhance neurite outgrowth in an inhibitory environment by
their unique ability to enter the fibroblast zones and promote neurite alignment along their
surfaces. In addition, OECs extend neurite outgrowth beyond that which growth-permissive
astrocytes and reactive astrocytes are able to provide. Thus, our results provide strong evidence
that direct OEC-neurite alignment is critical to enhance neurite outgrowth in these two different inhibitory environments.

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Conflict of interest statement:

None.
Figure 1. Schematic of experimental design and timeline.

Experiments were conducted on either quiescent astrocytes (control) or co-cultures of stretched astrocytes and meningeal fibroblasts (scar-like). Mature astrocytes (light blue background) were cultured on deformable membranes and first confronted with fibroblasts to induce astrogliosis (dark blue background). Then astrocyte-fibroblast co-cultures were mechanically stretched 5-6 hours prior to the addition of OECs labeled with Cell Tracker Green (scar-like + OEC). One day later, cerebral cortical neurons (P6-P8) were added to all experimental conditions. Neurons were grown for 24 hours and fixed.
Fig. 1

- Control
- Scar-like
- Scar-like + OEC

Seed astrocytes

Add fibroblasts

Day 1
- Stretch co-cultures
- 5-6 hours post-stretch
  - Add OECs

Day 2
- Add cortical neurons

Day 3
- Fix cultures
Figure 2. Scar-like cultures mimic the glial scar borders formed after spinal cord transection.

(A) Lesion site from an adult rat 1 month after a complete spinal cord transection. Reactive astrocytes express high levels of GFAP at the glial scar border (black, arrows) that divides the GFAP-positive and negative zones. (B) A GFAP-negative lesion area (*) is surrounded by elongated GFAP-expressing astrocytic processes (black arrowheads). (C) Scar-like culture has GFAP-negative lesion areas (*, black) that are surrounded by GFAP-positive astrocytes (blue). Neurites (yellow arrows) from a cortical neuron (white) typically do not enter inhibitory GFAP-negative zones (*). (D) OECs (green) in a scar-like culture intermingle with astrocytes (blue) and span the GFAP-negative zone (*, black). Neurons (white) extend processes along the OECs (yellow arrows) in both the GFAP-positive and GFAP-negative zones. (E) Astrocytic processes (blue) in a scar-like culture encircle fibronectin-labeled meningeal fibroblasts (red) that are concentrated in the GFAP-negative area (*). GFAP, Glial fibrillary acidic protein; β3-tub, β3-tubulin; OEC, olfactory ensheathing cell; FN, fibronectin. Scale A= 100 μm; B = 50 μm, C-E = 20 μm.
Fig. 2
Figure 3. OECs facilitate neurite outgrowth in scar-like cultures.

(A) Postnatal day 8 neurons extend long neurites (yellow arrows) on a quiescent astrocyte control culture. (B) A growth inhibitory scar-like culture reduces neurite extension (arrows). (C, D) The identical scar-like co-culture is illustrated without (C) or with OECs (D; green) to best visualize alignment of neurite outgrowth with OECs. Neurites marked by arrows in C align with OECs identified by arrowheads in D. (E) Average total neurite tree length per neuron in control, scar-like, and scar-like + OEC cultures. Each experiment (n=4) is represented by a black dot, while means ±SEM values for each variable are represented in red. *p < 0.05, **p < 0.01, and ***p < 0.001 for this and subsequent figures. GFAP, Glial fibrillary acidic protein; β3-tub, β3-tubulin; OEC, olfactory ensheathing cell. Scale A-D = 50 μm.
Fig. 3
**Figure 4. OEC-enhanced neurite outgrowth is mediated by neuron-OEC association.**

(A) In scar-like + OEC cultures associations between neurons and OECs were classified as: 1) aligned (single arrowheads), 2) crossing (double arrowhead), or 3) no interaction (double arrowhead). (B) An average of 52% of measured neurites aligned with OECs, while 23% of neurites crossed and 26% did not associate with OECs. (C) Neurites aligned with OECs were longer than those that crossed or did not associate with OECs. Individual experiments (n=4) in B and C are represented by black dots, with the means ±SEM values marked in red. GFAP, Glial fibrillary acidic protein; β3-tub, β3-tubulin; OEC, olfactory ensheathing cell. Scale A = 50 μm; A1-3 = 25 μm.
Fig. 4

A. Scar-like + OEC

Align
Cross
None

β3tub GFAP OEC

B. **Percent of Neurites**

Align Cross None

C. ***Neurite Length (μm)**

Align Cross None
**Figure 5. Neurite-OEC alignment enhances neurite outgrowth in both astrocyte and fibroblast zones**

(A, C) In scar-like cultures neurons within the GFAP-positive reactive astrocyte zones (A, blue) have longer neurites (arrows) than neurons in the GFAP-negative fibroblast zones (C, black area). Retraction bulbs (arrow, inset C) are found in fibroblast areas. (B, D) In scar-like + OEC cultures neurons extend long processes (yellow arrowheads) within both astrocyte (B) and fibroblast zones (D) if they associate with OECs. Neurites in scar-like + OEC cultures (D) can abruptly alter their direction to maintain alignment with OECs (white arrowheads). (E) Quantification of the percentage of neurites that grew into astrocyte (blue) versus fibroblast (black) zones. If OECs are present, however, the percentage of neurites within the fibroblast zones doubled. (F) Mean neurite lengths in astrocyte (blue) versus fibroblast (black) zones show that neurites are longer in scar-like + OEC cultures in both zones than in scar-like cultures alone. (G) Comparison of all neurites measured and sorted by their alignment status with OECs. In both the astrocyte and fibroblast zones, neurites are significantly longer if they align with OECs (align) versus no alignment (none). Neurites that align with OECs in reactive astrocyte zones are 25% longer than neurites from quiescent astrocyte cultures alone. Neurite-OEC alignment also enhances the average neurite length in the fibroblast zones to the same level of outgrowth without OECs in the astrocyte zones. Blue (astrocyte zone) or black (fibroblast zone) dots represent neurite length averages per culture experiment (n=3) and the mean ±SEM corresponds to large dots. (H) Orthogonal view of direct cell-to-cell contact between an OEC (green) and neurites (red) growing exclusively in a fibroblast zone (see D). Scale A-D = 50 μm.
Fig. 5

Astrocyte zone

Fibroblast zone

A. Scar-like

C. Scar-like

B. Scar + OEC

D. Scar + OEC

E. Percent of Neurites

F. Neurite Length (µm)

G. Neurite Length (µm)

H. 

β3tub GFAP OEC

- OEC  scar-like + OEC

- OEC  scar-like + OEC

Astrocyte zone

Fibroblast zone
References


Chapter 3. Olfactory ensheathing cell transplantation following spinal cord injury mediates neuroprotective and immunomodulatory mechanisms

Abstract

Following spinal cord injury, multiple neural and peripheral cell types rapidly respond to tissue damage to form a structurally and chemically inhibitory scar that limits axon regeneration. Astrocytes form the physical glial scar, produce inhibitory chemicals such as chondroitin sulfate proteoglycans (CSPGs), and activate microglia and recruit blood-derived immune cells to the lesion for debris removal. One beneficial therapy, olfactory ensheathing cell (OEC) transplantation, has demonstrated functional improvements and promoted axon regeneration following spinal cord injury. The lack of an OEC-specific marker, however, has limited the investigation of mechanisms underlying the pro-regenerative effects of OECs. We compared the effects of enhanced green fluorescent (GFP)-labeled FB and OEC transplants acutely following complete spinal cord transection in adult rats. We assessed the preservation of neurons and serotonergic axons, the levels of inhibitory CSPGs and myelin debris, and the extent of immune cell activation between 1 and 8 weeks post-injury. Our findings indicate that OECs survive longer than FBs post-transplantation, preserve axons and neurons in the lesion core, and reduce the presence of inhibitory molecules. Additionally, we show that OECs limit immune cell activation and infiltration whereas FBs altered the formation of the astroglial scar and subsequently had increased immune cell infiltration and concomitant secondary tissue damage. Administration of cyclosporine-A to enhance graft survival demonstrated that immune suppression can augment OEC-mediated immunosuppression, enhance axon regeneration, and protect neurons during the first 2 weeks after injury. Collectively, these data suggest that OECs
utilize neuroprotective and immunomodulatory mechanisms to create an environment that supports neuronal survival and axon regeneration.

**Introduction**

The regenerative capacity of adult mammalian neurons after injury is reduced by astroglial scar formation, inhibitory myelin-associated proteins, chondroitin sulfate proteoglycan (CSPG) deposition, and inflammatory cell activation that together contribute to a non-permissive environment and minimal functional recovery (Buss et al., 2009; Fitch et al., 1999; Fitch and Silver, 2008; Hu et al., 2010; Reier and Houle, 1988). To facilitate axonal regeneration and subsequent synaptic connectivity, therapies must overcome these post-traumaic growth-inhibitory factors and promote tissue preservation. Examples of such therapies include the reduction of glial scar inhibition, the removal of cellular debris, and the establishment of a cellular bridge across the lesion site. Additionally, treatments that provide neurotrophic factors to spared neurons and function in axonal guidance can enhance the intrinsic capacity of injured neurons to regenerate functional synaptic connections and initiate recovery.

After an olfactory nerve injury, olfactory ensheathing cells (OECs), facilitate the generation of new olfactory receptor neurons and maintain a conduit for their axons to grow along as they project to their olfactory bulb targets (Li et al., 2005; Lipson et al., 2003). OECs express adhesion molecules involved in axonal outgrowth, secrete trophic factors such as brain derived neurotrophic factor (BDNF) and ciliary neurotrophic factor (CNTF), and ensheathe growing axons to protect them from inhibitory molecules. Over the past decade, many studies have reported that OECs support axon regeneration even after a severe spinal cord transection in rodents and humans (Granger et al., 2012; Kubasak et al., 2008; Lopez-Vales et al., 2006; Lu et
al., 2002; Ramón-Cueto et al., 1998, 2000; Tabakow et al., 2014; Takeoka et al., 2011; Ziegler et al., 2011). Collectively these studies have generated anatomical, physiological, and behavioral evidence that OECs support the regeneration of corticospinal tract axons, preserve neurons and axons near the lesion site, increase the recovery of motor-evoked potentials in the hind limb muscles, and correlate with the improved stepping and grid climbing ability of transplanted spinal rats compared to media-treated controls (Kubasak et al., 2008; Ramón-Cueto et al., 2000; Takeoka et al., 2011; Ziegler et al., 2011). Despite these functional improvements. The lack of an OEC-specific marker has hampered the irrefutable identification of OEC transplants in spinal cord injury studies. Because studies were not able to identify OECs, there is very little currently known about the mechanisms by which they may facilitate axon regeneration and functional recovery.

To better understand how acutely transplanted OECs interact with the glial scar and surrounding inhibitory environment after a complete spinal cord transection, we used enhanced green fluorescent protein-labeled cells, control fibroblasts (eGFP-FBs) and OECs (eGFP-OECs). From eGFP transgenic rats (Perry et al., 1999). To assess the survival and engraftment, we determined the volume of GFP-positive cells in the lesion core between 1 and 8 weeks post-injury and found that that FBs did not survive as long as OECs. Next, we asked if the presence of OECs preserved neurons and provided a permissive environment for axons following injury. We found more neurons and axons in the lesion core and fewer inhibitory molecules in OEC-transplanted spinal cords. To determine if FBs and OECs differed in their modification of the lesion site, we examined astroglial scar formation and quantified the density of activated immune cells and their infiltration into the stumps. We administered the pharmacological immunosuppressant, cyclosporine-A (CSA), to enhance graft survival and determine whether or
not the beneficial effects of OECs were augmented. CSA treatment improved FB and OEC survival, which distinguished OECs as neuroprotective, growth permissive cells that can modulate the inhibitory environment and reduce secondary tissue damage following injury to promote axon regeneration.

Materials and Methods

Animals

All animal experimental procedures were approved by the Chancellor’s Animal Research Committee at UCLA and were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Animals were housed under standard conditions with ad libitum access to food and water. For primary FB and OEC cultures, we used eGFP rats generated by means of intracytoplasmic sperm injection of plasmid DNA that contains a ubiquitously expressed GFP reporter under the control of a synthetic CAG-promoter (Perry et al., 1999). Transgenic eGFP-expressing Sprague Dawley rats were bred to obtain heterozygote and homozygote litters that were confirmed with PCR (Perry et al., 1999). Homozygotic eGFP-expressing rats, 8-10 weeks old, were used to obtain FBs and OECs. Wildtype, postnatal day 8, rat pups were used in all cortical neurite outgrowth experiments. Rats were sacrificed with an overdose of ketamine-xylazine before the extraction of the olfactory bulbs, abdominal skin biopsies, or cerebral hemispheres. Female Sprague Dawley rats (Charles River Laboratories), 10-12 weeks of age, received cell transplants acutely following complete midthoracic spinal cord transection and were maintained 1, 2, 4, and 8 weeks post-injury.
Olfactory ensheathing cell cultures

Methods to prepare OEC primary and immunopurified cultures were similar to those of Ramón-Cueto et al. (2000) and identical to those recently reported (Khankan et al., 2015). OECs were dissected from the first two layers of the olfactory bulb and meninges and blood vessels were removed to reduce fibroblast contamination. Cells were dissociated in 0.1% trypsin (Gibco, Rockville, MD) and resuspended in a mixture of 1:1 Dulbecco’s Modified Eagle’s/Ham’s F12 medium (D/F medium, Gibco) supplemented with 10% Fetal Bovine Serum (FBS, Hyclone, Logan, UT) and 1% Penicillin Streptomycin (PS, Gibco; D/F-FBS). Dissociated OECs were plated into 25 ml culture flasks pre-coated with 0.05mg/ml Poly-L-Lysine (PLL; Sigma, St. Louis, MO). Primary OECs were maintained at 37°C with 5% CO₂ for 5-6 days and D/F-FBS medium was changed every 2 days.

Immunopurification was carried out using hydrophobic petri dishes coated overnight with Biotin-SP-conjugated AffiniPure goat anti-mouse IgG (1:1000; Jackson Immunoresearch Laboratories, West Grove, PA) in 50 mM Tris buffer at 4°C followed by another overnight incubation at 4°C with antibody against p75-nerve growth factor receptor (anti-p75-NGFR, 1:5; clone 192, Chandler et al., 1984) in 25mM PBS. Dishes were blocked with 0.5% Bovine Serum Albumin (Sigma) for 1 h. OEC primary cultures were dissociated with 0.25% trypsin-EDTA at 37°C for 3 min and D/F-FBS medium was added to inactivate trypsin. Following a medium rinse and centrifugation, resuspended cells were seeded onto pre-treated anti-p75-NGFR petri dishes and incubated at 37°C for 10 min. Unbound cells were removed with medium and a cell scraper was used to recover bound cells that were then subjected to a second round of immunopanning. Purified p75-NGFR-positive OECs were plated on PLL-coated culture flasks and incubated at 37°C with 5% CO₂ for 7 days with medium changed every 2 days. Purified OECs received D/F-
FBS medium supplemented with pituitary extract (20 μg/ml, Gibco) and forskolin (2 μM, Sigma). Mitogens were withdrawn 1-2 day prior to cell transplantation or use in neurite outgrowth experiments.

For in vitro neurite outgrowth experiments, wild-type or GFP-labeled OECs were seeded at a concentration of 32-35,000 cells onto 4-well glass slides pre-coated with PLL (0.05 mg/ml) 5 days following immunopurification. 48 hours later neurons were pipetted into the center of each well. Neurons were also cultured on control PLL + laminin (10 μg/ml; Invitrogen, Grand Island, NY) coated wells and all cultures were maintained for an additional 24 hours. Cultures were fixed with 4% paraformaldehyde in for 15 min, washed with buffer, and neurons were visualized with anti-β-3-tubulin (Covance, Berkeley, CA). Three independent culture experiments were conducted and 2 wells per experiment per variable were analyzed.

Fibroblast cell cultures

Skin biopsies from the abdominal wall were dissociated into fibroblast cultures as described by Takashima (1998). The dermis was separated from the epidermis and hypodermis mechanically. Dermal tissue was trypsinized (0.3%) for 10 min at 37°C, and rinsed with D/F-FBS medium. Cells were centrifuged at 365 g for 5 min before the pellet was resuspended and incubated in 12.5 mg/ml collagenase (Gibco) for 1 h at 37°C. The collagenase-cell mixture was filtered using a cell strainer, centrifuged, and then resuspended in D/F-FBS medium supplemented with Primocin (0.1 mg/ml, InvivoGen, San Diego, CA). Primary FBs were plated on PLL-coated culture flasks and maintained at 37°C with 5% CO2 and D/F-FBS medium changes every 2 days. FBs were passaged 1-2 times prior to cell transplantation (12-14 days in vitro).
Cortical neurite outgrowth assay

Cortical neurons were obtained from postnatal day 8 rat cerebral cortices. After removal of the leptomeninges, cerebral cortices were harvested in Hibernate-A medium (BrainBits, Inc., Springfield, IL) with P/S, L-glutamine (L-glu, Gibco), and B27 supplement (Gibco). Cortices were chopped and digested with warm papain (2 mg/ml, Worthington, Lakewood, NJ) in Hibernate-A medium with P/S and L-glu. Neurons were enriched with an OptiPrep step gradient (Axis-Shield, Norton, MA) and counted before they were added to the positive control, pre-coated laminin + PLL, the neutral control, PLL only, or either wildtype or transgenic OECs + PLL wells at a concentration of 100,000 cells per well.

Surgical procedures: spinal cord injury and cell transplantation

All surgeries were performed under aseptic conditions with the rats deeply anesthetized with isoflurane gas. 61 female Sprague Dawley rats, 10-12 weeks of age, received a complete spinal cord transection at level T8–T9 as described (Kubasak et al., 2008), and will be called spinal rats. An incision was made at levels T6 to L1, the paravertebral muscles were retracted, and a partial laminectomy at T8 and T9 was performed. The dorsal dura was exposed before the spinal cord was completely transected with micro-scissors. Two surgeons verified the transection was complete. 16 of the spinal rats were immunosuppressed with cyclosporine-A (CSA) 3 days before transection surgery and cell transplantation until the end of the study (Table 1).

Cells to be transplanted were harvested after a total of 12-14 days in vitro. Cells were rinsed with Hank’s Balanced Salt Solution without Ca\(^{2+}/Mg^{2+}\) and then dissociated with 0.25% trypsin-EDTA. Following a medium rinse and centrifugation, cells were resuspended at a concentration of 100,000 cells per μl in serum-free DMEM. FBs (29 rats) or OECs (32 rats) were
stereotactically injected into the spinal cord immediately after transection, both 1 mm rostral and caudal to the lesion site (Table 1). Approximately 50,000 cells were transplanted into 4 midline injection sites within each spinal cord stump for a total of 200,000 cells per stump. Rats were placed in an incubator until responsive and then housed individually. Spinal rats received manual bladder expression three times daily for two weeks and then twice daily thereafter at 12 h intervals. All animals were inspected for weight loss, dehydration, and urine was tested weekly using Multistix 10 SG reagent strips (Siemens, Malvern, PA).

Tissue preparation

Rats were anesthetized and intracardially perfused with 4% paraformaldehyde and post-fixed for 2 hrs at 4°C. Spinal cords were washed, dissected, cryoprotected with 30% sucrose, and embedded in OCT compound (Tissue-Tek, Torrance, CA). Injury sites were cryosectioned sagittally at a thickness of 25 µm, tissue sections were mounted in series onto 16 slides, and stored at 4°C in Millonig’s with azide buffer.

Immunocytochemical procedures

One slide containing every 16th section was incubated with primary antibodies against Green Fluorescent Protein (GFP, 1:1000, Aves Labs, Inc., Tigard, OR), Glial Fibrillary Acidic Protein (GFAP, monoclonal 1:1000, BD Biosciences, San Jose, CA; polyclonal 1:10K, DakoCytomation, Glostrup, Denmark), and one additional marker to identify neurons (NeuN, 1:1000, Millipore, Billerica, MA), serotonergic axons (5-HT, 1:5000, ImmunoStar, Hudson, WI), immune cells (Iba-1, 1:5000, Wako Chemicals USA, Inc., Richmond, VA), native chondroitin sulfate proteoglycans (CS-56, 1:200, Sigma), water channel aquaporin 4 (AQP4, 1:1000, Sigma),
or fibronectin (FN, monoclonal 1:200, BD Biosciences; polyclonal 1:1000, Dakocytomation). Table 2 includes a full list of antibodies and concentrations used. Sections were permeabilized and placed in species appropriate serum as a presoak (donkey or goat serum; Sigma) before incubation with primary antibodies overnight. To visualize immunostaining, species appropriate Alexa Fluor 488, 555, 594, or 647 (1:100-500; Jackson ImmunoResearch, West Grove, PA) were used. Nuclei were stained with Hoechst dye (Sigma) for 5 min and sections were cover slipped with Fluorogel (Electron Microscopy Sciences, Hatfield, PA).

**Oil Red and GFAP Bright field Staining**

Sections were washed with ddH₂O for 5 min and subsequently rinsed with 70% ethanol for another 5 min. Sections were then incubated with Oil Red O stain (700mg of Oil Red powder dissolved in 100mL 70% ethanol, filtered twice, Sigma) for 30 min, and again washed with ethanol for 1 min and ddH₂O for 5 min. Sections were then rinsed with 0.1M Tris buffer containing 1.4% NaCl and 0.1% bovine serum albumin, followed by a 15 min in 0.1% Triton detergent buffer. Sections were then incubated in 5% donkey serum containing 0.1% Triton before incubation with primary antibody against GFAP overnight. The next day sections were incubated in Tris buffer with biotinylated rabbit secondary antibody, amplified by incubation with avidin-biotin complex, and visualized using diaminobenzidine (Dojindo laboratories, Rockville, MD).

**Lesion site analysis**

Photomicrographic montages of spinal cord sections were obtained using an Olympus AX70 microscope and Zen 2012 image capture software (Carl Zeiss) with the panorama module.
Confocal images were obtained with a Zeiss LSM 510 microscope and 25x oil objective. Analyses of the lesion volume, GFP area, neuronal proximity to the lesion site, and the length of 5-HT axons were conducted with the Neurolucida and Neurolucida Explorer software (version 10; MicroBrightField, Inc., Williston, VT).

The GFAP-negative area, GFP-positive area, and non-neuronal lengths were traced and quantified in every 8th section. Contours of the GFAP-negative area and the associated cavities were used to calculate lesion volume as in Kubasak et al. (2008). The total GFP area was calculated as the sum of the GFP-positive area in the GFAP-positive CNS stumps and the GFAP-negative lesion core. The percent of migration represents the ratio of the GFP-area in the lesion core over the total GFP-area measured. The length of the non-neuronal tissue was quantified as in Takeoka et al. (2011). For every 8th section, the distance between the neuron closest to the rostral and caudal GFAP-positive stumps was measured along with the total distance of the non-neuronal tissue and lesion core.

We analyzed serotonergic axons present in the rostral stump associated with the GFAP border of every 16th section were quantified. Axon sparing was measured as: 1) the total area of 5-HT-labeled axons within the GFAP-negative lesion core normalized to the lesion volume, 2) the number of 5-HT-positive axons and axon bundles that crossed the GFAP-positive border into the lesion core, and 3) the distance from the perpendicular rostral border to the furthest extending (or most caudally extending) 5-HT-labeled axon in the lesion core. Axon bundles were determined by their diameter and lack of punctate staining. Axons that did not extend beyond the GFAP-positive border (represented as zero) were measured and reported as negative lengths.

Micrographs were used to evaluate the mean pixel luminance from individual channels using the Zen 2012 annotations/measurements feature. For the Iba-1 analysis, five zones of each
spinal cord section were marked by rectangles (500,000 \( \mu m^2 \)) and labeled as: rostral stump, rostral border, lesion core, caudal border, or caudal stump. This analysis method was adapted from Wanner, et al. (2013) but the zone size and placements were modified. For the CS-56 analysis, eight zones of each spinal cord section were marked by squares (~6,500 \( \mu m^2 \)) and labeled as the GFAP-positive or GFAP-negative area within the rostral and caudal borders, and adjacent lesion core. Rectangles were aligned with the GFAP-positive border and cysts were avoided. Mean luminance measurements were made for Iba-1, CS-56, and GFAP immunoreactivity and normalized to background auto-fluorescence of each section. Results are presented as a fold-change from background and measurements from stump, border, and lesion core zones were averaged for each animal.

Neurite outgrowth imaging and analysis

Culture experiment images were obtained using a Zeiss LSM 510 confocal microscope. Approximately 10 randomly selected fields were acquired per well for neurite outgrowth analysis. Neuronal cell bodies and all neurites were traced using Neurolucida software (v. 10.31, MicroBrightField, Inc., Williston, VT) and then individual neurite lengths and cell body details were exported to Microsoft Excel (Redmond, WA) using Neurolucida Explorer 10.31.

Statistical analyses

Measurements from each section were averaged to obtain a mean per animal. The mean value per rat was then combined into a group mean for each time point and reported as mean ± SEM. All statistical comparisons were performed with JMP Software (version 10.0.0 for Microsoft Windows, SAS Institute Inc., Cary, NC). A two-way ANOVA was conducted to
compare the pooled effects of time and cell transplant groups. To compare group means across time points MANOVA followed by a two-way ANOVA was used. Where interactions were observed appropriate post-hoc tests (Student’s t-tests) were performed. Statistical significance was determined by $p < 0.05$.

**Results**

**Wildtype and GFP-OECs have similar growth promoting abilities**

OECs are unique glia of the olfactory system, but they express markers common to astrocytes, oligodendrocytes, and Schwann cells and consequently the lack of an OEC-specific marker has complicated the identification of OECs following spinal cord transplantation. For this study, we derived OECs from GFP-labeled rats generated by random transgene integration into the genome. To first assess if OECs cultured from GFP-rats differ from non-transgenic OECs, we examined their expression of typical OEC markers. Most GFP-positive OECs expressed established OEC markers (89% $p$-75NGFR$^+$; 96% S100$^+$; 100% Sox10$^+$). Then, we compared the growth promoting abilities of wild-type OECs with GFP-labeled OECs. We cultured postnatal cortical neurons on: 1) laminin (positive control), 2) PLL (neutral substrate), 3) PLL and OEC, and 4) PLL and GFP-OEC. Neurites grew long processes when cultured on laminin, but had limited outgrowth on PLL substrate alone (mean neurite length on laminin, 146±31 μm; PLL 54±9 μm; $p < 0.01$). The addition of OECs or GFP-OECs significantly enhanced neurite outgrowth compared to PLL alone (OEC 117±19 μm; GFP-OECs 118±29 μm; $p < 0.05$). Because neurite outgrowth did not differ between OECs and GFP-OECs ($p= 0.98$) the random insertion of the GFP gene does not appear to have altered the growth-promoting effects of OECs.
OECs survive longer than FBs following transplantation

Transplanted FBs and OECs both survived in both the lesion core and the GFAP-positive rostral and/or caudal stumps at 1 week post-injury (Table 1). Thus during week 1 both FBs and OECs migrated from the injection sites into the lesion core (Fig. 6A-B). Transplanted cells appeared healthy and maintained their characteristic morphology following transplantation (Fig. 6G-H). By 2 weeks post-injury, there was a clear loss of cells in the lesion core as FBs and OECs survived primarily within the spinal cord stumps (Fig. 6C-D). Additionally, GFP-positive debris was found in the lesion core and in the stumps at 2 and 4 weeks post-injury consistent with graft cell death. By 4 weeks post-injury, none of the rats with FBs had GFP-labeled cells, whereas OECs survived in 5 out of 8 transplanted rats (Fig. 6E-F). In 2 out of 5 transplanted rats, OECs were found both in the rostral or caudal stumps and failed to survive or migrate into the lesion core at 4 weeks post-injury, whereas in 3 out of 5 transplanted rats the majority of OECs were found in the lesion core (Fig. 6F). Additionally, more OECs survived in the lesion core at 4 than at 2 weeks (compare Fig. 6D and 1F). Finally, a few OECs were found within the stumps but none remained in the lesion core at 8 weeks post-injury (data not shown). Our data suggest that OECs survive longer than FBs following transplantation and that the OECs that survive in the stumps at 2 weeks migrate into the lesion core by 4 weeks post-injury.

Transplanted cells were injected 1 mm rostral and caudal from the transection site and migrated into the lesion core. To quantify FB and OEC survival and migration, the area of GFP-labeled cells was measured in the lesion core, and rostral and caudal stumps in every 8th spinal cord section. We report the percent of GFP-positive cells that survived following spinal cord transection and transplantation (Fig. 6J). At 1 week post-injury most FBs and OECs migrated into the lesion core (Fig.1J; FB 72 ± 5% vs OEC 79 ± 4%). Due to the decrease in survival of
transplanted cells at 2 weeks post-injury, we observed a similar reduction in the percent of GFP-labeled cells found in the lesion core of FB and OEC groups (Fig.1J; FB $3 \pm 3\%$ vs OEC $14 \pm 7\%$). By 4 weeks post-injury, the percent of GFP-labeled cells in the lesion core of OEC-treated rats was higher compared to FBs (Fig.1J; FB $0\%$ vs OEC $23 \pm 14\%; p < 0.05$), a finding that suggests OECs continue to migrate into the lesion core between 2 and 4 weeks.

In the GFAP-positive stumps, FBs occupied restricted domains compared to OECs which intermingled with astrocytes at the astroglial scar-border (Fig. 6C-D). When in the lesion core, FBs formed a continuous layer of cells while OECs formed delicate trabecular-like networks (Fig. 6I; confocal image). To measure FB and OEC filling of the lesion core, the areas of GFP-labeled cells and fibronectin-positive extracellular matrix in the lesion core were measured in every $16^{th}$ section of the spinal cord to calculate graft and lesion volumes as reported (Kubasak et al., 2008). FBs filled approximately $83\pm17\%$ of the lesion volume by 1 week post-transplantation. At 2 weeks, however, few FBs remained within the lesion core ($3\pm3\%$ of the lesion volume). OECs filled $34\pm6\%$ of the lesion volume at 1 week and because most of the OECs remained within the rostral and caudal stumps at 2 weeks post-injury only $1\pm0.1\%$ of the lesion volume contained OECs. Interestingly, by 4 weeks post-transplant OECs appeared to migrate from the stumps into the lesion core and filled $8\pm5\%$ of the lesion volume. While these data suggest that FBs fill more of the lesion core than OECs at 1 week post-injury, there are considerable differences in size and cellular morphology between these two cell types. FBs are ovoid-shaped, form a contiguous carpet-like morphology, and occupy a larger area than OECs, which are spindle-shaped and align together into tubular networks (Fig. 6G-H). Because individual GFP-positive OECs could not be distinguished from each other, comparisons of cell
numbers were unreliable. These data suggest that FBs fill more of the lesion core than OECs, but this difference is dependent on the distinct morphology of the transplanted cells.

**OECs reduce neuronal degeneration following transection**

We next asked if the initial implantation of OECs had long lasting effects on tissue preservation and neuronal survival. To determine if OECs help preserve the neurons we measured the shortest distance between the GFAP-positive astroglial scar-border and NeuN-labeled neuronal cell bodies in the rostral and caudal stumps (Fig. 7A). The distance between the GFAP border and the nearest neuron did not differ between the rostral and caudal stumps and thus values from both stumps were combined to obtain the average neuron-free distance in the stumps. NeuN-labeled neuronal cell bodies were located closer to the astroglial scar-border in OEC than in FB-transplanted rats at 1 and 2 weeks post-injury (Table 3; Fig. 7C; 1 wk FB 1.2±0.1 mm vs OEC 0.5±0.1 mm \( p < 0.05 \); 2 wk FB 1.9±0.5 mm vs OEC 0.6±0.2 mm \( p < 0.01 \)). In addition, during the first 2 weeks post-injury the total distance between the closest rostral and caudal stump neurons was reduced in OEC compared to FB-treated spinal cords (Table 3; Fig. 7D; 1 wk FB 7.2±0.3 mm vs OEC 5.2±0.3 mm \( p < 0.05 \); 2 wk FB 7.4±1 mm vs OEC 4.1±0.6 mm \( p < 0.001 \)). While neuronal survival in the rostral and caudal stumps did not differ between transplant groups at 4 weeks post-injury, we observed spinal cord neurons ensheathed by GFP-positive OECs in the lesion core of OEC-treated rats (Fig. 7B). These results suggest that OEC transplantation promotes neuronal survival in the stumps and protects the surviving neurons within the lesion core.
OEC transplants reduce axonal dieback following transection

Immediately after a spinal cord injury, astrocytes become reactive and orient their processes to seal off the lesion, thereby blocking the “pathways” which could provide routes for regenerating axons (Li et al., 2005). To assess whether or not OEC transplantation could promote axon regeneration, we identified raphespinal tract axons in OEC and FB-treated spinal cords (Fig. 8A-D). This descending brainstem population of serotonergic (5-HT) axons is important for locomotion and previously we found that 5-HT axons that sprouted from the rostral stump into the lesion core following a complete transection are more likely to originate from raphespinal neurons in the brainstem rather than intraspinal 5-HT neurons (Takeoka et al., 2011). In OEC-treated spinal cords, we observed multiple 5-HT axons coalesce and associate with astrocytes at the GFAP-positive border (Fig. 8B, D). These 5-HT bundles aligned with astrocytes exhibiting rostro-caudally orientated processes that effectively provided “pathways” for severed axons to regenerate (Fig. 8B, D). Conversely in FB-treated spinal cords, astrocytic processes were oriented perpendicular to the rostro-caudal border and limited the extension of 5-HT axons into the lesion core (Fig. 8A, C). We quantified the average number of 5-HT bundles aligned with astrocytes at the scar-border and found more bundles in OEC compared to FB groups across all time points (axons per mm: FB 3±1 vs. 7±1, p < 0.01). At 2 weeks post-transection, the average number of bundles was four-fold greater in OEC than FB-treated rats (Table 3; Fig. 8F; axons per mm: FB 2±1 vs. OEC 8±1, p < 0.05). This suggests that OECs may modulate the configuration of reactive astrocytes adjacent to the lesion border and that rostro-caudally oriented astrocytes promote the extension of 5-HT axons beyond the scar-border.

Due to these changes in the astrocytic orientation, we also asked if more 5-HT axons were found in the lesion core of OEC than FB-treated rats. In OEC-treated spinal cords, we
found groups of 5-HT axons in the lesion core at 2 weeks post-injury (Fig. 8D). Because individual 5-HT axons could not be clearly distinguished from each other, we measured the area of the lesion core that contained 5-HT axons. Larger groups of 5-HT-positive axons were found in the lesion core of OEC than FB rats at 2 weeks post-injury (Fig. 8A-B; FB 3,054±1,750 µm² vs. OEC 25,775±9,869 µm², p < 0.05). When 5-HT axons extended beyond the scar-border, we measured the distance between the most caudal 5-HT axon and the rostral border. On average, axons projected further into the lesion core of OEC than FB-treated rats when all time points were combined (FB 21±51 µm vs. OEC 358±83 µm, p < 0.01). 5-HT axons were found twice as far in the lesion core of OEC compared to FB-treated cords at 1 week post-lesion (FB 263±147 µm vs. OEC 551±108 µm, p = 0.18). At the 2-week time point, most of the FB-treated rats exhibited axonal dieback into the rostral stump, however, 5-HT axons did extend into the lesion core but usually not more than 250µm (Table 3; Fig. 8F). Compared to FB-treated spinal cords, 5-HT axons extended further into the lesion core in OEC-treated rats at 2 weeks post-injury (Table 3; Fig. 8G; FB -116±108 µm vs. OEC 456±132 µm, p < 0.01). At 4 weeks post-injury, 5-HT axons persisted at the glial scar-border in FB-treated spinal cords whereas they were detected further from the astroglial scar-border in OEC-treated rats (Table 3; Fig. 8G; FB 5±66 µm vs. OEC 372±186 µm, p < 0.05). Moreover, the 5-HT axons that extended beyond the astroglial scar-border also aligned with OECs within the lesion core at 4 weeks post-transection (Fig. 8E). Our results suggest that the presence of OECs preserves 5-HT axons in the lesion core and reduces axonal dieback.
**OECs reduce inhibitory factors at the injury site**

Previous studies reported that after spinal cord injury OECs provide a permissive substrate and secrete trophic factors to stimulate axonal outgrowth. We asked if OECs also could reduce inhibitory factors present at the injury site, such as chondroitin sulfate proteoglycans (CSPGs) and myelin debris. To determine if OECs modify the inhibitory environment after SCI, we measured CS-56 immunofluorescence, which detects CSPGs, in the lesion core and adjacent astroglia scar-border in the rostral and caudal stumps of FB and OEC-treated spinal cords. The mean CS-56 luminance was greater in the lesion core (intensity mean value: FB 3.2±0.2 vs. OEC 1.6±0.1, p < 0.0001) and stumps (intensity mean value: FB 2.2±0.1 vs. OEC 1.2±0.1, p < 0.0001) of FB compared to OEC-treated rats when all time points were combined. At 2 weeks post-injury, CSPG immunoreactivity was higher in the lesion core and stumps of FB compared to OEC-treated spinal cords (Fig. 9A-B). The mean CS-56 luminance was greater in the lesion core of FB versus OEC-treated spinal cords at 2, 4, and 8 weeks post-injury (Table 4; Fig. 9C; intensity mean value: 2wk FB 3.2±0.1 vs. OEC 1.2±0.1, p < 0.0001; 4wk FB 2.7±0.3 vs. OEC 1.9±0.1, p < 0.01; 8wk FB 3.9±0.5 vs. OEC 1.4±0.2, p < 0.0001). The lesion core contained more CSPG immunoreactivity than the spinal cord stumps, and again CSPG levels were higher in the rostral and caudal stump borders of FB than OEC-treated spinal cords at 2 and 4, but not 8 weeks post-injury (Table 4; Fig. 9D; intensity mean value: 2wk FB 2.7±0.1 vs. OEC 1.2±0.1, p <0.0001; 4wk FB 2.1±0.3 vs. OEC 1.3±0.1, p <0.001; 8wk FB 1.6±0.2 vs. OEC 1.1±0.1, p =0.06). GFAP luminance measured from the same sections did not differ between FB and OEC groups (data not shown).

Next we asked if the areas occupied by FBs or OECs caused a reduction in CSPGs in the stumps and lesion core. At 2 weeks post-injury, we found a 50% reduction in CSPG luminance
in areas directly occupied by OECs compared to FBs (Fig. 9A-B, GFP vs CS-56 insets). Interestingly, disrupted astrocytic border formation was evident as more CSPG-labeling overlapped with FB territories but not OEC areas (Fig. 9A-B). In contrast, astrocyte processes encircled and sequestered CSPG-labeled cells within cavities in the stumps of OEC-treated spinal cords (Fig. 9B). These data show that OECs reduce the presence of inhibitory CSPGs at the injury site, but do not alter astrocyte border formation as seen with FBs.

Next we asked if OECs could alter the presence of myelin debris, another inhibitory factor found in and around the injury site. Using Oil Red O, a histological stain that identifies neutral lipids including myelin debris, we qualitatively observed an increase in oil red staining in FB compared to OEC groups. Myelin debris extended further into the rostral and caudal stumps of FB versus OEC-treated spinal cords at 2 weeks post-injury (Fig. 10A-B). The presence of myelin debris in the stumps may result from increased demyelination and resultant oligodendrocyte loss in FB but not OEC-treated spinal cords. These data suggest that OEC transplantation can modulate two different components of the inhibitory environment at the lesion site, CSPG and myelin debris, both of which contribute to stunted axon regrowth following spinal cord injury.

**OECs reduce immune cell activation and infiltration following complete transection**

Our results suggest that the presence of OECs mediate the survival of neurons and limit dieback of 5-HT axons, yet very few OECs survive at 8 weeks post-injury in this transplant model. Cell transplantation therapies are adversely affected by the immune response which has 2 separate peaks at 2 and 8 weeks following spinal cord injury (Beck et al., 2010). To evaluate the interactions between transplant survival and activated microglial/macrophages and astrocytes,
antibodies against ionized calcium-binding adaptor molecule (Iba-1) and GFAP were used. We measured the mean Iba-1 and GFAP luminance in 5 zones: the rostral and caudal scar-borders and stumps, and the lesion core (Fig. 11A). Iba-1 levels in the lesion core did not differ between FB or OEC-transplanted rats at 1, 2, 4, and 8 weeks. Although the lesion core did not vary between transplant groups, a sharp increase in Iba-1 luminance was observed in the rostral border and stump of FB compared to OEC-treated rats at 2 weeks post-injury (Fig. 11B, D; intensity mean value: 3.1±0.6 vs. 1.3±0.3, border p < 0.01, stumps p < 0.05). Iba-1 levels also were greater in the caudal stump of FB compared to OEC-treated rats at 2 weeks post-injury (Fig. 11C, E; intensity mean value: FB 3.0±0.4 vs. OEC 1.8±0.3, p < 0.05). Interestingly, the OEC-transplanted group appeared to delay the increase in Iba-1 levels between 1 and 2 weeks post-injury in the rostral but not the caudal border and stump (compare slope in Fig. 11B, D to C, E). At 4 and 8 weeks the Iba-1 levels in the borders and stumps of OEC-treated rats increased to levels similar to the FB group (Fig. 11B-E). These trends suggest that OECs delay and reduce the initial activation of Iba-1-positive immune cells compared to FBs.

Following spinal cord injury, reactive astrogliosis limits immune cell infiltration and reduces secondary tissue damage (Faulkner et al., 2004; Wanner et al., 2013). To evaluate if the astrocytic response was altered in FB and OEC-transplanted rats post-injury, we measured GFAP luminance within the 5 zones delineated in Fig. 11A. At all 4 time points and in both treatment groups, we observed a distinct GFAP-negative zone surrounded by GFAP-positive scar borders. GFAP luminance levels did not differ between FB and OEC groups at any time point (data not shown) and therefore neither FB nor OEC-transplantation altered the levels of GFAP expression.

Although GFAP luminance measurements were not altered following FB or OEC transplantation, a closer examination of the scar-borders revealed differences. The astroglial scar
border was well-defined by 2 weeks post-injury in all transplanted rats, but the orientation and arrangement of astrocytic processes varied by transplant group and time post-injury (Fig. 12A-D). In FB-treated rats, astrocytic processes appeared disorganized and resulted in disrupted border formation as evident by more diffuse globoid Iba-1-positive cells within the GFAP-positive areas of the scar-border (Fig. 12A, C). In contrast, astrocyte processes are organized in parallel and then form a distinctive barrier that encircles and sequesters Iba-1-positive immune cells in OEC-treated spinal cords (Fig. 12B, D). The distribution of Hoechst nuclear staining also demonstrates that the astrocytic border formation was altered in FB compared to OEC-treated rats (Fig. 12A-D, insets). To quantitatively assess if immune cell infiltration was limited as a result of border formation following cell transplantation, we analyzed the differences in mean Iba-1 and GFAP luminance in the stumps relative to the lesion core. A greater difference was found in mean Iba-1 luminance between FB and OEC-treated spinal cords at 2 weeks post-injury suggests higher levels of immune cell infiltration into the stumps (Fig. 12E; intensity mean value: 1.9±0.5 vs. 0.5±0.3, p < 0.01). Most GFAP is found in the stumps with little in the lesion core and therefore a large difference in GFAP luminance was expected between the stumps and lesion core. FB and OEC-treated rats did not differ in those measurements (Fig. 12F). The data highlight the relationship between scar-border formation and immune cell infiltration and suggests that OECs may reduce immune cell activation and infiltration.

**Immunosuppression using cyclosporine-A enhances graft cell survival**

We show that OECs can modulate the host immune response at 2 but not at 8 weeks post-injury and suspect that this loss is due to poor transplant survival due to immune-mediated rejection. To determine if the survival of transplanted cells could be enhanced by
immunosuppression, cyclosporine-A (CSA) was administered prior to and up to their termination by perfusion. CSA administration enhanced the survival of both transplanted cell types at 2 and 8 weeks post-injury (Table 1). Most transplanted cells in CSA-treated rats at 2 weeks post-injury were found in the lesion core, whereas few cells survived in the lesion core of non-immunosuppressed rats (Compare Fig. 13A-B to 1C-D). At 2 weeks-post injury, FBs and OECs in CSA-treated (FB + CSA; OEC + CSA) rats both migrated into the lesion core to similar levels (Fig. 13E; FB 84±10 %, OEC 75±4 %). Transplants in CSA-treated rats also were found in the lesion core at 8 weeks post-injury, but fewer rats had surviving cells (Table 1; Fig. 13C-D, E; FB 48±27 %, OEC 14±8 %). Of interest, transplanted cells formed a bridge between the rostral and caudal stumps as a result of their prolonged survival due to CSA-treatment (Fig. 13A-D). The CSA treatment significantly increased the percent of the lesion volume filled by FBs 29±12 % and OECs by 41±8 % compared to non-CSA treated rats at 2 weeks post-injury (FB 3±3 %, p < 0.01; OECs 1±0.1 %, p < 0.0001). Compared to the non-CSA treated rats at 8 weeks post-injury in which no FBs or OECs survived, transplanted cells occupied a modest percent of the lesion (FB 8±9 %; OEC 4±3 %).

To determine if enhanced cell survival was due to suppression of the innate immune response following CSA administration, we measured Iba-1 luminance levels as in Fig. 11A. CSA reduced Iba-1 levels in both transplant groups at 2 and 8 weeks post-injury with a greater reduction in FB- than OEC-treated rats. Additionally, we assessed immune cell infiltration by reporting the difference in mean Iba-1 luminance in the stumps and lesion core of transplanted rats at 2 and 8 weeks post-injury. At both time points, immune cell infiltration was markedly reduced in FB and OEC-transplanted spinal cords after CSA treatment at 2 weeks post-injury (Fig. 13F; intensity mean value: 2wks FB + CSA 0.6±0.1 vs. OEC + CSA -0.2±0.1; 8wks FB +
CSA 0.6±0.4 vs. OEC + CSA 0.2±0.1). Interestingly, CSA-treatment significantly reduced Iba-1 luminance levels of FB but not OEC-treated rats compared to their CSA untreated controls (Fig. 13F; intensity mean value: FB 1.9±0.5 vs. FB + CSA 0.6±0.1, p < 0.05). These data suggest that CSA administration reduced the innate immune response and prolonged the survival of both transplanted FBs and OECs at the 2-week time point.

Enhanced-OEC survival promotes survival of neurons and axon regeneration

To determine if CSA-enhanced OEC survival can further promote a beneficial tissue healing response, we assessed neuronal survival and 5-HT axon regeneration at 2 and 8 weeks post-injury. Neurons were found close to the astroglial scar-border following CSA treatment (Fig. 14A), as shown in non-CSA-treated rats (Fig. 7A). The neuron-free distance and the total distance between rostral and caudal stump neurons were comparable in FB and OEC groups with or without CSA administration both at 2 and 8 weeks post-injury (Table 5). While the extent of tissue preservation in the stumps did not differ following CSA-enhanced OEC survival, neurons survived in the GFAP-negative lesion core zones of OEC-transplanted CSA-treated rats at 2 weeks post-injury (Fig. 14B). Collectively, our data show that the survival of OECs in the lesion core can directly preserve neurons.

OEC + CSA treatment enhances 5-HT axon regeneration

To assess if enhanced FB or OEC survival due to CSA treatment could preserve axons and promote axon regeneration, we identified raphespinal tract axons and 1) counted the number of axons that crossed the border, 2) quantified the 5-HT area in the lesion core, and 3) measured the distance between the border and the furthest extending 5-HT axon in OEC and FB-treated
spinal cords with and without CSA (Fig. 14C-D). CSA-treatment did not enhance the number of 5-HT axons that could cross the border in either FB or OEC-treated rats at 2 or 8 weeks post-injury (Table 5). In fact, OEC-transplantation alone maintained a 2-fold greater number of 5-HT axons that cross the border than all treatment groups (Table 3, 5; axons per mm: 2wk 8±1, p < 0.05; 8wk 8±1, p < 0.01). Larger 5-HT-positive areas were found in the lesion core of CSA than non-CSA-treated rats, however, only those that received FBs showed a significant increase after CSA administration at 2 weeks post-injury (2wk FB 3,054±1,750 µm² vs. FB + CSA 19,719±5,707 µm², p < 0.05). Surprisingly, CSA administration did not increase the distance of the furthest extending 5-HT axon compared to non-CSA-treatment in either FB or OEC-transplant groups at 2 weeks post-injury (Table 5; Fig. 14E) While at 8 weeks post-injury, 5-HT-positive axons were found further in the lesion core of OEC + CSA-treated rats than in any other treatment group (Table 5; Fig. 14E; FB + CSA -117±166 µm; OEC + CSA 362±137 µm; p < 0.05). These data suggest that OEC transplantation promotes the extension of 5-HT axons beyond the scar-border and that CSA-enhanced survival of OECs preserves these axons in the lesion core.

Enhanced-OEC survival reduces inhibitory CSPGs at the lesion site

To determine if CSA-enhanced OEC survival can reduce inhibitory factors in the lesion site beyond that observed with non-CSA-treated rats, we assessed CSPG content using CS-56 luminance levels in the lesion core, and rostral and caudal stumps. We observed a time-dependent CSA treatment effect on the levels of CSPG in the lesion core (intensity mean value: 2wks CSA 2.7±0.3 vs. none 2.3±0.2, p < 0.05; 8wks CSA 1.9±0.2 vs. none 2.7±0.5, p < 0.05), but not the stumps. At 2 weeks post-injury, OEC + CSA treatment increased CSPG levels in the
lesion core compared to OEC treatment alone (Fig. 14F; intensity mean values: OEC 1.2±0.1; OEC + CSA 2.1±0.3, \( p < 0.05 \)). However, CSPG levels significantly decreased in FB + CSA compared to FB-treated rats at 8 weeks post-injury (Fig. 14F; intensity mean values: FB 3.9±0.5; FB + CSA 2.4±0.1, \( p < 0.001 \)). Most noticeably, CSPG levels remained lower in OEC + CSA compared to FB + CSA groups at 2 and 8 weeks post-injury (Table 4; Fig. 14F; 2wks \( p < 0.01 \); 8wks \( p = 0.05 \)). In addition, CSPG levels were also reduced in OEC + CSA compared to FB + OEC groups in the stump borders at both time points (Table 4; Fig. 14G; 2wks \( p < 0.05 \); 8wks \( p < 0.05 \)). These data suggest an OEC-specific reduction of CSPG levels at the lesion site irrespective of CSA treatment.

**Discussion**

Spinal cord injury produces a temporally regulated, pathological cascade of cellular changes that involve both central and peripheral cells that form a layered scar and create an inhibitory environment at the lesion site (Burda and Sofroniew, 2014; Cregg et al., 2014). Here we found that FBs and OECs differ in their modification of the lesion site after complete spinal cord transection. OECs directly preserve neurons and 5-HT axons in the lesion core, reduce inhibitory CSPGs and myelin debris, and limit immune cell infiltration to reduce secondary tissue damage. When CSA was used to enhance OEC survival the innate immune response was reduced together with secondary tissue damage. The prolonged presence of OECs protected surviving neurons, decreased inhibitory CSPGs, and facilitated the regeneration of 5-HT axons at the lesion site. When combined, our results provide evidence that OECs are neuroprotective and mediate axon regeneration by modifying the inhospitable environment that forms after SCI.
OEC do not alter the formation of the scar-border

After injury, astrocytes respond by forming a protective barrier around the damaged CNS to limit further cell loss, restrict the spread of inflammation, and promote blood-brain barrier repair (Bush et al., 1999; Faulkner et al., 2004). Insufficient border formation following injury resulted in increased spread of immune cells and neuronal loss (Wanner et al., 2013). We show that FBs, but not OECs, alter astrocyte barrier formation and subsequently FB-transplanted rats have more immune infiltration and tissue damage than rats transplanted with OECs. Indeed, immune cell infiltration peaked at 2 weeks in FB-treated rats, but not until 4 weeks in OEC-treated rats. Additionally, reactive astrocyte processes in FB-treated rats were disorganized and did not sequester immune cells and invading fibroblasts within the GFAP-positive spinal cord stump. Bundesen et al. (2003) reported that segregation and reformation of the glial limitans after injury may depend on cell contact-mediated repulsion between ephrin-B2-expressing astrocytes and EphB2-expressing meningeal FBs at the lesion interface. Thus the transplantation of skin FBs into the injured spinal cord, may have interfered with ephrinB/EphB signaling, delayed astrocytic boundary formation, and prolonged the period of cellular invasion. In contrast to FBs, OECs express ephrin-B2 during development and may participate in olfactory receptor neuron axon sorting to their appropriate glomeruli (St. John and Key, 2001). Additionally, when OECs were added to an astrocyte-meningeal fibroblast scar-like culture model they interact with both cell types and did not alter astrocyte reactivity. GFAP-negative fibroblast zones are encircled by reactive astrocytic processes in scar-like cultures and these zones are reminiscent of those that occur following SCI (Khankan et al., 2015). These findings demonstrate that OECs, compared to FBs, do not impede astrocyte border formation and may actually contribute to the glial limitans.
Reactive astrocytes at the scar-border of FB-treated spinal cord stumps were unable to organize their processes to surround invading Iba-1-positive inflammatory cells and CSPG-positive meningeal fibroblasts in a manner reminiscent of STAT3-deficient mice (Wanner et al., 2013). STAT3-dependent mechanisms are mediated by heterodimerization of a tripartite receptor complex composed of ciliary neurotrophic factor (CNTF) receptor-alpha, leukemia inhibitory factor (LIF) receptor-beta, and glycoprotein130 (gp130), an interleukin-6 (IL-6) signal transducer (Hibi et al., 1990; Stahl and Yancopoulos, 1994). The cytokines that participate in the activation of this receptor complex by binding to their respective receptor components are CNTF, LIF, and IL-6 (Stahl and Yancopoulos, 1994). Interestingly, OECs express CNTF *in vivo* and *in vitro* (Asan et al., 2003; Wewetzer et al., 2001), upregulate the expression of LIF and/or IL-6 after olfactory bulbectomy (Nan et al., 2001) and may regulate cytokine signaling in response to bacterial infection (Herbert et al., 2012). Additionally, CNTF, LIF, and IL-6 receptors are expressed by OECs and may be involved in an OEC-mediated regulatory role of cytokine secretion (Wewetzer et al., 2001; Nan et al., 2001). Thus several studies support the idea that OECs may collaborate with astrocytes to limit immune cell infiltration and secondary tissue damage via activation of the gp130/STAT3 pathway.

**OECs are immunomodulatory and neuroprotective**

Beck et al. (2010) quantitatively show that there is a time-dependent multiphasic inflammatory response in which macrophages first peak at 7 and then again at 60 days following a spinal cord contusion injury. In FB and OEC-treated rats, we observed a similar time-dependent increase in Iba-1 luminance levels that peaked at 4 weeks post-injury. Interestingly, the activation of immune cells was delayed in OEC-treated rats as Iba-1 levels did not begin to
rise until 2 weeks and was more noticeable in the rostral compared to the caudal stump. Eventually, however both FB and OEC-treated rats reached the same levels by 4 weeks post-injury. This delay in immune activation may have contributed to the prolonged survival of OECs compared to FBs and suggests an intrinsic immune modulatory function of OECs. Harris et al. (2009) found that bacterial infection causes OECs to rapidly increase the expression of iNOS and production of nitric oxide (NO). Additionally, the expression of iNOS requires activation of the transcriptional factor nuclear factor kappa-B (NF-κB) which controls cytokine and chemokine expression and mediates apoptosis (Li and Verma, 2002). Therefore, transplanted OECs may produce NO and modulate cytokine production and inflammation in the lesion site following SCI (López-Vales et al., 2004).

The OEC-mediated immune modulation appears to reduce secondary tissue damage due to macrophage infiltration, limit myelin debris due to oligodendrocyte loss, and preserve neurons close to the scar-border. Because the effects of reduced immune cell infiltration in OEC-treated rats cannot be separated from the reduction in secondary tissue damage that results in neuronal and oligodendroglial cell loss, it is difficult to determine if OECs directly support neurons and reduce oligodendrocyte loss. However, we do provide evidence that the presence of OECs in the lesion core protects and ensheaths neurons at 4 weeks post-injury. Furthermore, we show no differences in the ability of OECs to preserve neurons close to the scar-border with or without CSA treatment. This suggests that OECs have a direct neuroprotective effect on neurons independent of the immune response.

CSA, a potent immunosuppressant that blocks T-cell activation and subsequently regulates other immune cells (Ho et al., 1996), reduced Iba-1 infiltration in FB and OEC-treated rats to the same levels. CSA treatment reportedly is neuroprotective following spinal cord
compression injury (Ibarra et al., 2003) and thus our findings that CSA reduced the neuron-free distance in FB-treated rats to the same levels as OEC treatment alone was not surprising. Yet, neurons were not found in the lesion core of FB + CSA-treated rats suggesting that the neuroprotective mechanism by which OECs preserve neurons differs from that of CSA. Therefore, the addition of CSA to OEC-treated rats exerted an additive effect by enhancing the survival of OECs in the lesion core where they protected and ensheathed neurons at 2 weeks post-injury. Thus, OEC-mediated immune modulation reduces secondary tissue damage and limits oligodendrocyte loss, and when combined with the direct presence of OECs protects neurons in the lesion core.

**OEC-axon interactions facilitate axon regeneration**

Infiltrating blood-derived macrophages are responsible for axonal dieback in a dorsal column crush model of spinal cord injury (Evans et al., 2014). Our data support OEC-mediated immunomodulation and direct OEC-axon alignment as possible mechanisms that maintained 5-HT axons in the lesion. First, 5-HT axonal dieback was observed only in FB-treated rats. Second, at the 4-week peak of immune infiltration in OEC-treated rats 5-HT axons were found further in the lesion core than in FB-treated rats and these axons directly aligned with OECs. Third, the preservation of 5-HT axons was lost by 8 weeks post-injury when OECs were no longer present in the lesion core. Lastly, CSA treatment enhanced the survival of OECs and maintained 5-HT axons further in the lesion core than OEC-treatment alone. Collectively, these results suggest that the immunomodulatory role of OECs depends on their presence and that OECs facilitate 5-HT axon regeneration via contact-mediated mechanisms.
Previously we found that OEC-neurite alignment independently enhanced neurite outgrowth in a scar-like model of spinal cord injury (Khankan et al., 2015). OECs express cell adhesion molecules (CAMs) that improve neurite and axonal outgrowth such as NCAM and L1-CAM (Roet et al., 2013; Witheford et al., 2013). OECs also express Neuropilin-1, a Semaphorin 3A receptor, that is important for neurite growth support and when associated with L1-NCAM, changes semaphorin 3A-induced chemorepulsion to chemoattraction (Castellani et al., 2000; Roet et al., 2013). In the current study, we observed direct alignment between OECs and 5-HT axons in the lesion core. Because OECs express multiple axon guidance and adhesion molecules, it is likely that these OEC-axonal interactions may both orient axons and enhance their regeneration following spinal cord injury.

**OECs reduce inhibitory CSPGs following SCI**

A well-known feature of the inhibitory glial scar is the up-regulation and secretion of CSPGs by astrocytes and fibroblasts following SCI (Fitch and Silver, 2008; McKeon et al., 1995; Morgenstern et al., 2002). Inflammatory cell infiltration also is associated with increased CSPGs after injury (Fitch and Silver, 1999). Several studies report that OECs reduce CSPG immunoreactivity in the injured spinal cord (Lakatos et al., 2003; Barbour et al., 2013). With our 8 week data we also show a correlation between the loss of OECs and the increase in CSPG density. In fact, the OEC + CSA treatment maintained axons further in the lesion core than any other treatment combinations. This suggests that while a reduction in CSPGs and immune infiltration is effective at preserving 5-HT axons, the presence of OECs in the lesion core augments this effect. Collectively, these results provide strong evidence that the OEC-mediated reduction of inhibitory molecules and inflammatory cells at the lesion site preserves 5-HT axons.
Astrocytes and microglia/macrophages respond to a traumatic injury by activating complex molecular signaling cascades, and the response of multiple neural and peripheral cells generates both a chemical and mechanical barrier to axon regeneration. OECs can modify the astrocyte response by aligning astrocytic processes in the rostrocaudal orientation to provide pathways that would enhance 5-HT axon growth (Li et al., 2005), reduce the presence of inhibitory molecules at the lesion site, and maintain a barrier that limits secondary immune damage and protects neurons. Despite the reduction in surviving OECs at 4 weeks post-injury, the number of 5-HT axons crossing the border still increases and these axons were found further in the lesion core. This finding suggests that engrafted OECs influenced the lesion microenvironment at an early point yet have long lasting effects on regeneration. Collectively, these results suggest that OEC transplantation following SCI mediates neuroprotective and immunomodulatory mechanisms and creates an environment that supports axonal regeneration.
Table 1. The number of transplanted rats in which GFP-positive cells were identified in the lesion core and/or spinal cord stumps post-injury. OECs survive longer than FBs following transplantation. CSA administration enhanced the survival of transplanted GFP-positive cells.

<table>
<thead>
<tr>
<th>Weeks post-injury</th>
<th>Treatment</th>
<th>n=</th>
<th>Rats with engrafted cells</th>
<th>Proportion of rats with engrafted cells and their location</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 week</td>
<td>FB OEC</td>
<td>4</td>
<td>4 of 4</td>
<td>Stumps and lesion core</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>5 of 5</td>
<td>Stumps and lesion core</td>
</tr>
<tr>
<td>2 weeks</td>
<td>FB OEC</td>
<td>6</td>
<td>2 of 6</td>
<td>Stumps: 1 of 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>3 of 5</td>
<td>Lesion Core: 1 of 2</td>
</tr>
<tr>
<td></td>
<td>FB + CSA OEC</td>
<td>4</td>
<td>4 of 4</td>
<td>Stumps and lesion core</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>4 of 4</td>
<td>Lesion Core: 1 of 3</td>
</tr>
<tr>
<td>4 weeks</td>
<td>FB OEC</td>
<td>5</td>
<td>0 of 5</td>
<td>None present</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8</td>
<td>5 of 8</td>
<td>Stumps: 2 of 5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Lesion Core: 3 of 5</td>
</tr>
<tr>
<td>8 weeks</td>
<td>FB OEC</td>
<td>6</td>
<td>0 of 6</td>
<td>None present</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6</td>
<td>0 of 6</td>
<td>None present</td>
</tr>
<tr>
<td></td>
<td>FB + CSA OEC</td>
<td>3</td>
<td>2 of 3</td>
<td>Stumps and lesion core</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>3 of 5</td>
<td>Lesion Core: 2 of 3</td>
</tr>
</tbody>
</table>
Table 2. Markers and primary antibody concentrations used to identify various neural, non-neural, and peripheral cells.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Antibody</th>
<th>Concentration</th>
<th>Vendor</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFP-OECs</td>
<td>Green fluorescent protein (GFP)</td>
<td>Chicken 1:1000, Mouse 1:200</td>
<td>Aves labs, Inc. BD Biosciences</td>
</tr>
<tr>
<td></td>
<td>P75-NGFR</td>
<td>Mouse 1:50</td>
<td>Hybridoma-192</td>
</tr>
<tr>
<td></td>
<td>S100</td>
<td>Rabbit 1:1000</td>
<td>DakoCytomation</td>
</tr>
<tr>
<td></td>
<td>Sox-10</td>
<td>Goat 1:20</td>
<td>R&amp;D Systems</td>
</tr>
<tr>
<td>Astrocytes</td>
<td>Glial fibrillary acidic protein (GFAP)</td>
<td>Rabbit 1:10,000, Mouse 1:1000</td>
<td>DakoCytomation BD Biosciences</td>
</tr>
<tr>
<td></td>
<td>Water channel aquaporin 4 (AQP4)</td>
<td>Rabbit 1:1000</td>
<td>Sigma</td>
</tr>
<tr>
<td>Macrophages/microglia</td>
<td>Ionized calcium binding adaptor molecule-1 (Iba-1)</td>
<td>Rabbit 1:5000</td>
<td>Wako Chemicals USA, Inc.</td>
</tr>
<tr>
<td>Neurons</td>
<td>Neuron-specific nuclear protein (NeuN)</td>
<td>Mouse 1:1000</td>
<td>Millipore</td>
</tr>
<tr>
<td>Serotonergic axons</td>
<td>Serotonin (5-HT)</td>
<td>Goat 1:5000</td>
<td>ImmunoStar</td>
</tr>
<tr>
<td>GFP-FBs</td>
<td>GFP</td>
<td>Chicken 1:1000, Mouse 1:200</td>
<td>Aves labs, Inc. BD Biosciences</td>
</tr>
<tr>
<td>Meningeal fibroblasts</td>
<td>Fibronectin (FN)</td>
<td>Rabbit 1:1000, Mouse 1:200</td>
<td>DakoCytomation BD Biosciences</td>
</tr>
<tr>
<td>Extracellular matrix</td>
<td>Native chondroitin sulfate proteoglycan (CS-56)</td>
<td>Mouse 1:200</td>
<td>Sigma</td>
</tr>
</tbody>
</table>
Table 3. Quantitative analyses of neuronal and serotonergic axon preservation after FB or OEC transplantation following spinal cord injury. Neurons are preserved closer to the scar-border in OEC compared to FB-transplanted rats. OECs preserve 5-HT axons in the lesion core and reduce axonal dieback.

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Group</th>
<th>1 Week</th>
<th>2 Weeks</th>
<th>4 Weeks</th>
<th>8 Weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neuron-free distance in rostral and caudal stumps (mm)</td>
<td>FB</td>
<td>1.23 ± 0.08</td>
<td>1.90 ± 0.52</td>
<td>0.68 ± 0.09</td>
<td>1.23 ± 0.15</td>
</tr>
<tr>
<td></td>
<td>OEC</td>
<td>0.51 ± 0.10</td>
<td>0.65 ± 0.18</td>
<td>0.55 ± 0.06</td>
<td>0.70 ± 0.12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p = 0.05</td>
<td>p = 0.0004</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total distance between rostral and caudal stump neurons (mm)</td>
<td>FB</td>
<td>7.24 ± 0.25</td>
<td>7.40 ± 1.0</td>
<td>4.35 ± 0.50</td>
<td>5.80 ± 0.70</td>
</tr>
<tr>
<td></td>
<td>OEC</td>
<td>5.18 ± 0.34</td>
<td>4.11 ± 0.63</td>
<td>4.54 ± 0.45</td>
<td>4.85 ± 0.48</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p = 0.04</td>
<td>p = 0.0007</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-HT axons at the scar-border per mm</td>
<td>FB</td>
<td>6 ± 2</td>
<td>2 ± 1</td>
<td>3 ± 2</td>
<td>3 ± 1</td>
</tr>
<tr>
<td></td>
<td>OEC</td>
<td>9 ± 1</td>
<td>8 ± 1</td>
<td>5 ± 2</td>
<td>8 ± 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p = 0.0114</td>
<td>p = 0.0578</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Average 5-HT area in the lesion core (µm²)</td>
<td>FB</td>
<td>10,655 ± 4,389</td>
<td>3,054 ± 1,750</td>
<td>1,829 ± 950</td>
<td>2,691 ± 1,759</td>
</tr>
<tr>
<td></td>
<td>OEC</td>
<td>81,921 ± 43,404</td>
<td>25,775 ± 9,869</td>
<td>7,853 ± 3,603</td>
<td>5,956 ± 2,807</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p = 0.034</td>
<td>p = 0.034</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Average distance between the furthest 5-HT axon and the rostral scar-border (µm)</td>
<td>FB</td>
<td>263 ± 147</td>
<td>-116 ± 108</td>
<td>5 ± 66</td>
<td>11 ± 40</td>
</tr>
<tr>
<td></td>
<td>OEC</td>
<td>551 ± 108</td>
<td>456 ± 132</td>
<td>372 ± 186</td>
<td>43 ± 109</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p = 0.0045</td>
<td>p = 0.046</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 4. Quantitative analyses of chondroitin sulfate proteoglycan (CSPG) immunoreactivity measured as mean CS-56 luminance. OECs reduce the presence of inhibitory CSPGs at the injury site.

<table>
<thead>
<tr>
<th>Weeks post-injury</th>
<th>Location</th>
<th>Treatment group</th>
<th>Intensity mean value (CS-56)</th>
<th>Treatment group</th>
<th>Intensity mean value (CS-56)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>Lesion core</td>
<td>FB OEC</td>
<td>3.2 ± 0.1 1.2 ± 0.1</td>
<td>FB + CSA OEC + CSA</td>
<td>3.4 ± 0.4 2.1 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>Stumps</td>
<td>FB OEC</td>
<td>2.7 ± 0.1 1.2 ± 0.1</td>
<td>FB + CSA OEC + CSA</td>
<td>2.4 ± 0.3 1.6 ± 0.4</td>
</tr>
<tr>
<td>4</td>
<td>Lesion core</td>
<td>FB OEC</td>
<td>2.7 ± 0.3 2.1 ± 0.1</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td></td>
<td>Stumps</td>
<td>FB OEC</td>
<td>1.9 ± 0.1 1.3 ± 0.1</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>8</td>
<td>Lesion core</td>
<td>FB OEC</td>
<td>3.9 ± 0.5 1.4 ± 0.2</td>
<td>FB + CSA OEC + CSA</td>
<td>2.4 ± 0.1 1.6 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>Stumps</td>
<td>FB OEC</td>
<td>1.6 ± 0.2 1.1 ± 0.1</td>
<td>FB + CSA OEC + CSA</td>
<td>1.8 ± 0.3 1.2 ± 0.2</td>
</tr>
</tbody>
</table>
Table 5. Quantitative analyses of neuronal and serotonergic axon preservation after CSA administration and FB or OEC transplantation following spinal cord injury. Neurons are preserved close to the scar-border in FB and OEC-treated rats. CSA treatment preserves 5-HT axons in the lesion core and reduces axonal dieback. 5-HT-positive axons were found further in the lesion core of OEC + CSA-treated rats than in any other treatment group.

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Treatment group</th>
<th>2 weeks post-injury</th>
<th>8 weeks post-injury</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neuron-free distance in rostral and caudal stumps (mm)</td>
<td>FB + CSA</td>
<td>1.3 ± 0.3</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>OEC + CSA</td>
<td>0.7 ± 0.1</td>
<td>0.8 ± 0.2</td>
</tr>
<tr>
<td>Total distance between rostral and caudal stump neurons (mm)</td>
<td>FB + CSA</td>
<td>6.0 ± 1.1</td>
<td>5.1 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>OEC + CSA</td>
<td>3.6 ± 0.5</td>
<td>4.1 ± 1.0</td>
</tr>
<tr>
<td>5-HT axons at the scar-border per mm</td>
<td>FB + CSA</td>
<td>2 ± 1</td>
<td>1 ± 0</td>
</tr>
<tr>
<td></td>
<td>OEC + CSA</td>
<td>4 ± 0</td>
<td>3 ± 1</td>
</tr>
<tr>
<td>Average 5-HT area in the lesion core (µm²)</td>
<td>FB + CSA</td>
<td>19,719 ± 5,707</td>
<td>6,097 ± 2,989</td>
</tr>
<tr>
<td></td>
<td>OEC + CSA</td>
<td>33,013 ± 8,295</td>
<td>9,142 ± 3,100</td>
</tr>
<tr>
<td>Average distance between the furthest 5-HT axon and the rostral scar-border (µm)</td>
<td>FB + CSA</td>
<td>145 ± 84</td>
<td>-117 ± 166</td>
</tr>
<tr>
<td></td>
<td>OEC + CSA</td>
<td>201 ± 5</td>
<td>362 ± 137</td>
</tr>
</tbody>
</table>
**Figure 6. Survival and migration of transplanted cells.** A-F: After acute transplantation, GFP-labeled FBs (A, C, E) or OECs (B, D, F) were present within the lesion core (asterisks) and/or GFAP-positive (cyan) rostral and caudal stumps between 1 and 4 weeks after injury. Anti-GFP immunolabeling identified large numbers of engrafted FBs and OECs in the lesion core (white arrowheads) and stumps (white arrows) at 1 week post-transplant (A-B). At 2 weeks, neither FBs nor OECs survived in the lesion core, but GFP-positive cells are present in the stumps (white arrows, C-D). No FBs survived at 4 weeks (E), whereas OECs are present in the lesion core (white arrowhead) and stumps (white arrow) at 4 (F) but not 8 weeks after transplant. G-H: Confocal images reveal engrafted FBs (G) as dense sheets of cells (yellow arrowheads), whereas OECs (H) formed thin tubular tracks of cells (yellow arrows). I: Confocal image shows intermingled spindle-shaped OECs (inset) at 4 weeks post-injury within a trabecular network of fibronectin-positive (red) extracellular matrix within the lesion core. J: OECs migrate and occupy more of the lesion core relative to the total GFP volume than FBs at week 4 (* = p < 0.05), but do not differ at other time points. Each animal is represented by a circle and group means ± SEM values are represented in black for FBs and green for OECs. Spinal cords are oriented with rostral to the left and dorsal to the top and the lesion cavity is marked with an asterisk in this and subsequent figures. Scale bars A-F = 1 mm, G-H = 50 μm, I = 200 μm. GFAP, glial fibrillary acidic protein; GFP, green fluorescent protein.
Fig. 6
Figure 7. OECs reduce neuronal degeneration post-transplantation. A: An OEC-transplanted spinal cord 2 weeks post-injury has the astroglial scar-border identified with GFAP (cyan) and neurons (yellow arrows) marked with NeuN (red). No OECs were present in this spinal cord section. The distance between neurons (yellow arrows) and the nearest astrocyte scar-border (c) and the total distance between rostral and caudal neurons (d) were measured. B: Confocal image near the rostral scar-border contains neurons (white arrows) ensheathed by OECs within the GFAP-negative lesion core at 4 weeks post-transection. C-D: The neuron-free regions in the rostral and caudal stumps (labeled ‘c’ in A) and the total distance between the rostral and caudal stump neurons (labeled ‘d’ in A) were greater in FB than OEC-treated rats. Each animal is represented by a circle and group means ± SEM values shown in black (FBs) and green (OECs). *p < 0.05; **p < 0.01. Scale bars A = 500 μm and B = 50 μm.
Fig. 7

A. 2 Weeks

B. 4 Weeks - OEC

C. Response time distance between the rostral section and distal section (weeks post-injury)

D. Total distance between the rostral and distal section (weeks post-injury)
**Figure 8. OECs reduce serotonergic axonal dieback.** A-D: Representative images (A-B) and enlargements (C-D) show serotonergic axons (5-HT, red) that extend across (yellow arrowheads) the GFAP-positive rostral border and into the lesion core (*, yellow arrows) in FB- (A, C) and OEC-transplanted (B, D) spinal cords. E: Confocal image shows that 5-HT axons bundle together, cross the astroglial-scar border (white arrowheads), align with OECs (white arrows), and extend into the GFAP-negative lesion core (inset). F-G: On average, more 5-HT-labeled axon bundles cross the rostral border in OEC-treated rats (F). The distance between the scar border and the most caudally extending 5-HT axon was greater in OEC- than FB-treated spinal cords (G). Each animal is represented by a circle. Group means ± SEM values are shown in black (FBs) and green (OECs). *p < 0.05; **p < 0.01. Scale bars A-B = 200 μm; C-E = 50 μm; inset = 10 μm.
**Figure 9. OECs reduce inhibitory chondroitin sulfate proteoglycans (CSPGs) in the lesion core.** A-B: Spinal cord injury sites labeled for astrocytes (GFAP, cyan), CSPGs (CS-56, red), and GFP-labeled FBs (green, A) or OECs (green, B) at 2 weeks post-injury. GFP-labeled FBs (white arrows) overlap with CSPG-labeling (yellow) but not GFAP-immunolabeling (cyan). Astrocyte processes (white arrowheads) fail to encircle CSPG-positive cells in FB-treated rats (A). GFP-labeled OEC (yellow arrows) do not overlap with CSPG (red) immunostaining and astrocyte processes surround CSPG-positive cells into distinct domains (yellow arrowheads). Individual channels of the entire image are displayed on the right to show antibody distribution. 

C-D: The mean luminance of CSPGs was greater in the lesion core (C) and the rostral and caudal stumps (D) of FB (black) than of OEC (green) transplanted spinal cords at 2, 4, and 8 weeks post-injury. The mean of each animal is plotted as a circle and group means ± SEM values are represented as black (FBs) or green (OECs) lines. Scale bars A-B = 200 μm. *p < 0.05, **p < 0.01, ***p < 0.001.
Fig. 9
**Figure 10. OECs reduce myelin debris at 2 weeks post-injury.** More Oil Red O-labeled myelin debris (red) is present in the lesion core (*) and stumps (brown) of FB (A) than OEC-treated spinal cords (B). Myelin debris (black arrows) extended beyond the lesion border and into white matter axonal tracts in FB-treated (A) rats whereas less was found in OEC-transplanted (B) rats. The astroglial scar-border is marked with GFAP (brown) immunostaining. Scale bar A-B = 1 mm.
Fig. 10
Figure 11. OECs reduce immune cell activation during the first 2 weeks post-injury. A: Lesion site of an OEC-transplanted spinal cord 2 weeks post-injury was labeled for microglia/macrophages (Iba-1, red) and astrocytes (GFAP, cyan). OECs were not present in this section. White boxes represent the zones in which Iba-1 and GFAP immunofluorescence intensities were measured. B-E: Graphical representation of the mean Iba-1 luminance in the rostral border (B), caudal border (C), rostral stump (D), and caudal stump (E). At 2 weeks Iba-1 intensities were greater in FB than OEC-treated spinal cords (Mean ± SEM; *p < 0.05, **p < 0.01). Each animal is represented by a circle and group means ± SEM values are represented in black (FBs) and green (OECs). Scale bar = 500μm.
Fig. 11
**Figure 12. OECs reduce immune cell infiltration into the spinal cord stumps.** A-D: Within the rostral (A, B) and caudal border (C, D) zones, astrocytes (GFAP, cyan) and activated macrophages/microglia (Iba-1, red) are immunolabeled and nuclei are stained (Hoechst, yellow) from representative FB- (A, C) and OEC-treated (B, D) spinal cords at 2 (A, B) and 4 weeks (C, D) post-injury. The GFAP scar-border (cyan, insets) is clearly defined at 2 (A, B) and 4 weeks (C, D) post-injury. The distribution of Iba-1-positive immune cells (red, insets) between the GFAP-positive scar-border compared to the lesion core (*) differs between FB (A) and OEC (B) sections. Spherical Iba-1 immune cells are clustered and contained into distinct zones (yellow arrows) by astrocyte processes in rats that received OECs-transplants (B-D), but these are rarely seen in FB-treated rats. In FB-treated rats, astrocytes processes cluster together and are oriented parallel (white arrowsheads) to the lesion border whereas they are organized perpendicularly (yellow arrowheads) in OEC-treated rats. Hoechst (yellow, insets) nuclear staining shows the distribution of invading cells within the stump in FB (inset A, C) compared to OEC (inset B, D) treated rats. E-F: The difference in mean Iba-1 (E) and GFAP (F) luminance in the rostral and caudal stumps (stump and border zones in Fig. 11A) relative to the lesion core. At 2 weeks, more Iba-1-positive cells infiltrated the stumps of FB than OEC-transplanted spinal cords (E; **p < 0.01). GFAP luminance served as a control and displayed similar levels of astrocyte reactivity between FB and OEC-treated spinal cords (F). Each animal is represented by a circle and group means ± SEM values are shown in black (FBs) and green (OECs) Scale bars A, B = 200 μm and C, D = 100 μm.
Fig. 12
Figure 13. Cyclosporine-A (CSA) administration improved transplanted cell survival at 2 and 8 weeks post-transplantation. A-D: With CSA immunosuppression both FB (A, C) and OEC (B, D) transplants survived and migrated into the lesion core (*) at 2 (A, B) and were maintained 8 weeks (C, D) post-injury. GFP-labeled cells were found in the stumps (white arrows) and lesion core (white arrowheads) in both FB and OEC-treated rats. E: Comparison of the percent of GFP in the lesion core of FB (left) and OEC (right) transplanted rats without (FB, black; OEC, green) or with CSA (FB, orange; OEC, purple). The volume of GFP-labeled FBs and OECs in the lesion core was larger with than without CSA administration. F: The difference in Iba-1 luminance relative to the lesion core demonstrates that immune cell infiltration in CSA-(FB, orange; OEC, purple) compared to non-CSA-treated (FB, black; OEC, green) spinal cords of both FB (left) and OEC (right) transplanted rats. Group means ± SEM values are shown in black (FBs), orange (FB + CSA), green (OECs), and purple (OEC + CSA). *p < 0.05, **p < 0.01, and ***p < 0.001. Scale bars A-D = 1 mm.
Fig. 13

A. FB + CSA

B. OEC + CSA

2 Weeks

GFAP  GFP

8 Weeks

E. Percent of GFP in the lesion core

F. Difference in mean Bi-luminance

Weeks post-injury

Weeks post-injury
Figure 14. OEC survival in CSA-treated rats reduced neuronal degeneration and enhanced serotonergic axonal regeneration. A-B: With combined CSA and OEC treatments, neurons (red, white arrows) are detected close to the astrocyte scar-border (cyan) at 2 weeks post-injury (A). Section A contains numerous GFP-labeled OECs (green) shown in B. Neurons (red) marked with white arrowheads (A, B) survived in the lesion core (*) in OEC + CSA rats at 2 weeks post-injury. The enlargement in B shows that OEC (green) surround these neurons (red, white arrowheads) that survive in the GFAP-negative lesion core. C-D: At 8 weeks post-injury, 5-HT axons (red, yellow arrowheads) were found in the GFAP-positive (cyan) rostral stump after FB + CSA treatment (C), whereas axons (were found beyond the scar-border (dashed line) in OEC + CSA (D) treated rats. E: The average distance between the furthest extending 5-HT axon and the scar border was greater in 8 week CSA-treated OEC-transplanted rats (purple) than any other treatment group (FB, black; FB + CSA, orange; OEC, green). F-G: The mean luminance of CSPGs was greater in the lesion core (F) and stumps (G) of FB + CSA (orange) than in OEC + CSA (purple) treated spinal cords at 2 and 8 weeks post-injury. Within the lesion core (F), FB-transplanted rats have reduced levels of CSPGs with (orange) than without CSA (black) at 8 weeks post-injury whereas OECs (green) have lower levels of CSPGs than OEC + CSA (purple) at 2 weeks post-injury. Each animal is represented by a circle and group means ± SEM values are shown in black (FBs), orange (FB + CSA), green (OECs), and purple (OEC + CSA). *p < 0.05, **p < 0.01, and ***p < 0.001. Scale bars A-B = 500 μm; C-D = 200 μm.
References


Chapter 4. Conclusions

Our aim is not to determine if OECs can promote regeneration, but rather it is to determine how they can promote axon regeneration. First using an in vitro model, we investigated the contact-mediated mechanisms by which OECs mediate neurite regeneration. We used an astrocyte and meningeal fibroblast scar-like culture model of spinal cord injury to test the effect of OEC treatment on postnatal cortical neurite outgrowth. OECs enhanced neurite outgrowth in an inhibitory scar-like environment by their unique ability to enter astrocyte and fibroblast zones and promote neurite alignment along their surfaces. We also provided evidence that OEC-neurite alignment augments neurite outgrowth beyond that which growth-permissive astrocytes are able to provide. Together, OECs provided a spatially restricted and contact-dependent growth-promoting effect on neurites that is independent of the scar-like environment.

In our short-term in vivo study implanting control FBs and OECs after a complete spinal cord transection, we found that FB and OEC transplantations modify a number of features in the lesion site. OECs directly preserve neurons and 5-HT-labeled axons in the lesion core, reduce inhibitory CSPGs and myelin debris, and limit the immune cell infiltration that causes secondary tissue injury. We also showed that the survival of FBs and OECs can be enhanced with CSA administration. Spinal cords treated with CSA have a reduced overall innate immune response concomitant with the reduction of further secondary tissue damage. The enhanced survival of OECs protects neurons in the inhibitory lesion core at 2 weeks post-injury, maintained a reduction in inhibitory factors, and provided pathways for regenerating 5-HT axons at the lesion site at 8 weeks post-injury. Our data provide evidence that OECs are neuroprotective, immunomodulatory, and mediate axon regeneration by modifying the inhospitable environment that forms after SCI.