Title
Reelin expression and secretion by olfactory ensheathing cells

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Publication Date
2015

Peer reviewed|Thesis/dissertation

UNIVERSITY OF CALIFORNIA

Los Angeles

Reelin expression and secretion by olfactory ensheathing cells

A dissertation submitted in partial satisfaction of the requirements for the degree Master of Science in Physiological Science

by

Rana R. Khankan

2015

ABSTRACT OF THE THESIS

Reelin expression and secretion by olfactory ensheathing cells

by

Rana R. Khankan

Master of Science in Physiological Science

Univeristy of California, Los Angeles, 2015

Professor Patricia Emory Phelps, Chair

Olfactory ensheathing cells (OECs) contribute to axon guidance and fascicle organization during the cyclical degeneration and regeneration in the olfactory system throughout life. OECs also support neurite outgrowth *in vitro* and axon regeneration following spinal cord injury. Reelin, a large extracellular matrix protein, is responsible for proper neuronal positioning of migrating neurons in the neocortex and spinal cord of the developing nervous system. Because OECs share phenotypic characteristics with Schwann cells and the absence of Reelin may impair peripheral nerve regeneration (Lorenzetto et al., 2008; Vincent et al., 2005), we asked if OECs express Reelin to mediate neural repair after injury. Here we show that olfactory bulb OECs produce and secrete Reelin. We also determined that Reelin secretion is a potential mechanism by which OECs can mediate process outgrowth. OEC-secreted Reelin did not enhance neurite outgrowth of postnatal cerebral cortical neurons, but did mediate enhanced outgrowth of DRG axons.

The thesis of Rana R. Khankan is approved.

Stephanie Ann White

Scott H. Chandler

Patricia Emory Phelps, Committee Chair

University of California, Los Angeles

2015

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ACKNOWLEDGEMENTS

This study acknowledges support from the National Science Foundation IOB-0924143 (PEP). We thank Dr. Stephanie White for providing western blotting reagents and equipment, Drs. Julie Miller and Christina Ghiani for assistance with western blotting protocols, Autumn Abadesco for Reelin immunohistochemistry, Alyssa Capili and Aly Mulji for tracing neurites, Katie Ingraham for assistance with DRG cultures, and Elizabeth Fraley, Elvira Khialeeva, Brian Mullen, and Dr. Ellen Carpenter for helpful discussions.

Introduction

The adult olfactory system is remarkably regenerative, as olfactory receptor neurons (ORNs) are continually generated and new synaptic connections are established throughout life. During normal turnover and following injury, newly generated ORNs project their axons from the olfactory epithelium through the cribriform plate to their glomerular targets in the olfactory bulb (Graziadei and Monti Graziadei, 1985). ORNs are regularly generated from a stem cell population within the olfactory epithelium and their growing axons are supported by olfactory ensheathing cells (OECs; Schwob, 1999; Doucette, 1990). Throughout the cycles of degeneration and regeneration in the olfactory system, OECs survive and maintain a conduit for the newly generated olfactory receptor axons to grow through as they project into the olfactory bulb (Williams et al., 2004; Li et al., 2005).

OECs are associated with both the peripheral and central components of the olfactory system. Peripherally, OECs are found in the lamina propria of the olfactory mucosa where they ensheath hundreds of axons (Li et al., 2005). The OECs located in the outer nerve layer of the main olfactory bulb likely contribute to axon guidance and fascicle organization during the axonal growth into the olfactory bulb (Crandall et al., 2000; Imai et al., 2009). OECs are distinctive glial cells that share phenotypic characteristics with both astrocytes and Schwann cells, yet more closely resemble Schwann cells (Doucette, 1991; Ramón-Cueto and Valverde, 1995; Vincent et al., 2005). For example, both OECs and Schwann cells originate from the neural crest, express similar immunological markers, and share transcriptional similarities (Barraud et al., 2010; Forni et al., 2011; Ramón-Cueto and Nieto-Samedro, 1992; Vincent et al., 2005). Following peripheral nerve injury, the loss of contact between axons and myelinating Schwann cells induces a nonmyelinating phenotypic change in Schwann cells (Fu and Gordon,

1997). These dedifferntiated nonmyelinating Schwann cells are proregenerative and upregulate the expression of glial fibrillary acidic protein (GFAP; Jessen et al., 1990), p75-nerve growth factor receptor (p75-NGFR; You et al., 1997), cell adhesion molecules (CAMs) such as L1 and N-CAM (Martini and Schachner, 1988), and neurotrophic factors, including brain derived neurotrophic factor (Meyer et al., 1992) and glial cell-line derived neurotrophic factor (Trupp et al., 1995). Another protein that is developmentally restricted to immature Schwann cells and whose expression is induced following sciatic nerve injury is Reelin (Panteri et al., 2006).

Reelin is a large extracellular matrix protein with multiple functions including proper neuronal positioning of migrating embryonic neurons in the neocortex and spinal cord. The Reelin-signaling pathway requires that secreted Reelin binds to the very-low-density lipoprotein receptor (Vldlr) and/or apolipoprotein E receptor 2 (Apoer2) to phosphorylate and activate the intracellular cytoplasmic adaptor protein Disabled-1 (Dab1; Rice et al., 1998; Trommsdorff et al., 1999). Phosphorylated Dab1 further activates additional kinases that are involved in multiple downstream cellular processes involving microtubule stabilization, synaptic plasticity, and neurite outgrowth (Herz and Chen, 2006). In embryonic wild-type mice, Schnaufer et al. (2009) reported that unidentified cells within the olfactory mesenchyme secrete Reelin, Vldlr mRNA is detected in the olfactory epithelium, and the Vldlr protein is found in the axon fascicles within the lamina propria and outer nerve layer of the olfactory bulb. Additionally, Reelin expression was induced in the olfactory bulb of adult mice following a chemical lesion of the olfactory epithelium (Okuyama-Yamamoto et al., 2005). Gene profiling studies of OECs have identified Reelin as a possible regulator of neurite outgrowth (Roet et al., 2011). Given that olfactory bulb-derived OECs express many of the same markers as nonmyelinating Schwann cells (Barnett et al., 1993; Ramón-Cueto and Nieto-Samedro, 1992) and promote the regeneration of injured

peripheral axons (Ramón-Cueto and Nieto-Samedro, 1994), we asked if OECs secrete Reelin and if Reelin secretion may be used by OECs to mediate neural repair after injury.

Materials and Methods

Animals and tissue preparation

All animal experimental procedures were approved by UCLA's Institutional Animal Care Use Committee and conformed to AVMA guidelines for research on vertebrate animals. Heterozygous mice were purchased from the Jackson Laboratory (B6C3Fe-*ala*-*Reln*^{rl}, Bar Harbor, ME), bred to generate wild-type (*Reln*$^{+/+}$) and Reelin-deficient mice (*Reln*$^{-/-}$), and PCR was used to identify genotypes as described (D'Arcangelo et al., 1996). We used enhanced green fluorescent protein (GFP)-expressing Sprague Dawley rats (Perry et al., 1999) to obtain purified olfactory bulb-derived OECs and isolate Reelin protein for western blotting.

Adult mice were transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (PB) at pH 7.4 and post-fixed overnight. Both olfactory bulbs were dissected, cryoprotected with 30% sucrose, and embedded in OCT compound (Tissue-Tek, Torrance, CA). Olfactory bulbs were cryosectioned sagittally at 40 µm thickness and stored free-floating in PB with 0.06% sodium azide at 4ºC.

Primary and purified OEC cultures

Olfactory bulbs were collected from 8-10 weeks old *Reln*$^{+/+}$ and *Reln*$^{-/-}$ mice or GFP-labeled rats and the leptomeninges were removed to reduce fibroblast contamination. Methods to prepare OEC primary cultures were adopted from Runyan and Phelps (2009) and Khankan et al. (2015). OECs were dissected from the first two layers of the olfactory bulb, olfactory nerve

layers were isolated, 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 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. A mixture of 1:1 DMEM and Ham's F12 (D/F medium, Gibco) supplemented with 10-15% Fetal Bovine Serum (FBS, Hyclone, Logan, UT) and 1% Penicillin/Streptomycin (P/S, Gibco; D/F-FBS-P/S medium) was used to inactivate trypsin prior to centrifugation. Dissociated cells were rinsed and centrifuged 3 times, and then plated into 12.5 cm^2 culture flasks pre-coated with 0.05 mg/ml poly-L-lysine (PLL, Sigma, St. Louis, MO). Cells were maintained at 37°C for 5-6 days and D/F-FBS-P/S medium was changed every 2 days.

Immunopurification was carried out using hydrophobic petri dishes coated overnight with Biotin-SP-conjugated AffiniPure goat anti-rabbit IgG (1:1000; Jackson ImmunoResearch, West Grove, PA) in 50 mM Tris buffer at 4°C followed by another overnight incubation at 4°C with rabbit anti-p75-nerve growth factor receptor (anti-p75-NGFR, 1:1500, Millipore, Temecula, CA) in 25mM PBS for mouse OECs. For rat OECs, dishes were coated overnight with Biotin-SP-conjugated AffiniPure goat anti-mouse IgG (1:1000; Jackson ImmunoResearch) in Tris buffer and then incubated overnight with a monoclonal antibody against 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. Prior to the addition of cells to be immunopanned, antibody-treated dishes were washed with PBS and DMEM.

OEC primary cultures were dissociated with 0.25% trypsin-EDTA at 37°C for 3 mins and D/F-FBS-P/S 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 mins. 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 either PLL-coated 4-chamber, polystrene-vessel culture slides (BD Falcon, San Jose, CA) and used as cellular substrates for the neurite outgrowth experiments or 12.5 cm^2 culture flasks and used for western blots. Purified OECs were incubated at 37°C with 5% CO_2 for 7 days and D/F-FBS-P/S medium supplemented with pituitary extract (20 μg/ml, Gibco) and forskolin (2 μM, Sigma) was changed every 2 days. Mitogens were withdrawn 2 days prior to use.

Brefeldin-A treatment

OECs that contained GFP (Perry et al., 1999) could be visualized directly under fluorescent illumination. Primary and purified GFP-labeled OEC cultures were rinsed 3 times with a mixture of 1:1 Dulbecco's Modified Eagle's/Ham's F12 medium (D/F medium, Gibco, Rockville, MD) prior to Brefeldin-A treatment. Brefeldin-A (5 μg/ml; Epicenter, Madison, WI; Kubasak et al., 2004) was mixed with D/F medium and added to OEC cultures for 2 hours at 37ºC while the control cultures received D/F medium only. Cultures were fixed with cold 4% paraformaldehyde in 0.1 M PB for 15 minutes at room temperature, rinsed 3 times with PB, and stored in PB with sodium azide at 4ºC until immunocytochemical staining.

Immunohistochemistry and immunocytochemistry

Olfactory bulb sections were labeled with mouse anti-Reelin (G10, Millipore). Free-floating sections were rinsed with PB buffer plus 0.9% NaCl (PBS) plus 0.5% Triton X-100 (PBST, Sigma), pre-treated with 3% H_2O_2 plus 10% methanol in PBS, and blocked with 10% normal donkey serum (NDS), 1% non-fat milk, 0.2% gelatin in PBST (Chin et al., 2007).

Sections were incubated overnight with mouse anti-G10 (1:1000), 3% NDS, and 0.2% gelatin in PBST. The following day tissue sections were rinsed with PBST and then incubated with donkey-SP-anti-mouse biotinylated secondary antibodies with 3% NDS plus 0.2% gelatin before amplification with Vectastain ABC standard kit (Vector laboratories, Burlingame, CA) and visualization with a 3, 3' diaminobenzidine (0.06%) reaction.

Cultured OECs treated with or without Brefeldin-A were labeled with mouse anti-G10 (1:1000) and rabbit anti-p75-NGFR (1:30K, Chemicon, Temecula, CA). Culture slides were rinsed with PBS, blocked with 5% normal goat serum for 1 h, and incubated with the appropriate primary antibodies overnight. The following day, slides were rinsed 3 times with PBS, incubated with species appropriate Alexa Fluor 594 (1:500) and/or 647 (1:100, Jackson ImmunoResearch) for 1 h at RT, and then cover slipped with Fluorogel (Electron Microscopy Sciences, Hatfield, PA).

Tissue harvesting, protein isolation, and western blotting

Olfactory bulbs from adult *Reln*$^{+/+}$ and *Reln*$^{-/-}$ mice and olfactory nerve layers from adult Sprague Dawley rats were freshly dissected as described for primary OEC cultures. Cerebral cortices were harvested from adult *Reln*$^{+/+}$ and *Reln*$^{-/-}$ mice as described for primary neuron cultures. Olfactory and cortical tissue was collected, placed on ice, and homogenized in Ripa lysis buffer plus protease inhibitor cocktail (Sigma). Protein concentrations of brain and olfactory extracts were determined using the RC DC Protein Assay kit (Bio-Rad, Hercules, CA) as described (Miller et al., 2008). Whole cell lysates and conditioned medium (CM) were obtained from purified OEC cultures maintained in D/F medium. CM was collected from OEC

cultures before cells were incubated with Ripa lysis buffer and collected with a cell scraper. Protein samples were stored at -80ºC.

Protein samples (50 μg) were heated to 90–100°C for 3 min, resolved on 10% SDS-PAGE or 4–15% mini-protean TGX gels (Bio-Rad) run in Tris-Glycine-SDS (TGS) buffer (Bio-Rad) at 60V, and then transferred onto 0.22 μm PVDF/nitrocellulose membranes (Bio-Rad) in TGS with 20% methanol at 25V overnight at 4°C. Precision plus protein standards in dual color (Bio-Rad) were used to determine molecular weight. Membranes were cut, blocked with 5% non-fat dry milk (Bio-Rad) in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 1 h at room temperature on a shaker, and then incubated overnight at 4°C TBST plus 2.5% milk containing anti-Reelin (G-10, 1:750) or loading control mouse anti-GAPDH (1:100K, Millipore) antibodies. Following primary antibody incubation, blots were washed 3 times in TBST for 10 min while shaking and then probed with horseradish peroxidase-conjugated secondary antibodies anti-mouse IgG (1:2,000 for Reelin, 1:5,000 for GAPDH) in TBST. Immunoblots were developed using a chemiluminescence HRP detection kit (GE Healthcare, Piscataway, NJ,) and imaged with a Typhoon scanner (GE Healthcare).

Neuron cultures

Cortical neurons were obtained from postnatal day 8 *Reln*$^{+/+}$ and *Reln*$^{-/-}$ mice cerebral cortices with methods described in Khankan et al. (2015). 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 finely chopped and then digested with warm papain (2 mg/ml, Worthington, Lakewood, NJ) in Hibernate-A medium with P/S and L-glu. Cortical neurons were enriched with an OptiPrep step gradient (Axis-Shield,

Norton, MA) and counted before they were added at a concentration of 100,000 cells per well to each variable: pre-coated laminin + PLL (positive control), PLL only (neutral control), and either *Reln*$^{+/+}$ and *Reln*$^{-/-}$ OECs + PLL wells. Each experiment was conducted in triplicate.

Methods to prepare primary dorsal root ganglia (DRG) cultures were similar to those described by Runyan and Phelps (2009). P8 DRG from *Reln*$^{+/+}$ and *Reln*$^{-/-}$ mice were dissected in HBSS, then dissociated with 0.3% collagenase type I (Gibco) in HBSS without Ca^{2+}/Mg^{2+} for 30 min at 37°C followed by 0.1% trypsin for 10 min. DRG neurons were counted and added at a concentration of 100,000 cells per well to: 1) pre-coated laminin + PLL, 2) PLL only, or either 3) *Reln*$^{+/+}$ + PLL or 4) *Reln*$^{-/-}$ OECs + PLL wells. Each experiment was conducted in duplicate.

Imaging, outgrowth, and statistical analyses

Culture experiment images were obtained using an Olympus AX70 microscope and Zen 2012 image capture software (Carl Zeiss). 5-10 semi-randomly selected fields were acquired per well. Neuronal cell bodies and all neurites were traced using Neurolucida software (v. 10.31, MicroBrightField, Inc., Williston, VT) and individual neurite lengths were exported to Microsoft Excel (Redmond, WA) using Neurolucida Explorer 10.

Individual neurite lengths were summed to obtain the total tree length per neuron per image and then averaged and reported as the total neurite tree or axon length per well. The means per well were then combined into a group mean for each substrate 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). Two-way ANOVA were conducted to compare the effect of different substrates on neurite and axon outgrowth. Statistical significance was

determined by $p < 0.05$.

Results

OECs synthesize and secrete Reelin

In the main olfactory bulb of adult *Reln*$^{+/+}$ mice, Reelin-positive neurons are found in the mitral cell, external plexiform, and glomerular layers (Fig. 1A; Hack et al., 2002). The highly organized and laminated structure of the olfactory bulb suggests that these Reelin-expressing cells are mitral cells, periglomerular cells, and external tufted cells. The outer nerve layer, which consists mostly of olfactory receptor axons that are surrounded by OECs, was highly Reelin-immunoreactive (Fig. 1A). These data suggest that adult OECs within the ONL of the olfactory bulb may express Reelin.

Reelin is a large glycoprotein that is reportedly secreted by both neurons (D'Arcangelo et al., 1995; Kubasak et al., 2004) and glial cells (Schwann cells; Panteri et al., 2006). To determine if OECs synthesize and secrete Reelin, we treated primary and p75-NGFR purified OECs with or without Brefeldin-A, a fungal metabolite that specifically inhibits protein transport from the endoplasmic reticulum to the Golgi apparatus (Misumi et al., 1986). In Brefeldin-treated primary OEC cultures, spindle-shaped GFP-labeled OECs contain Reelin in their cytoplasm (Fig. 2A). Large contaminating cells present in primary cultures served as an intrinsic control as they are Reelin-immunonegative (Fig. 2A). Reelin-expression was also confirmed in Brefeldin-treated p75-NGFR purified OEC cultures (Fig. 2B-C). The majority of purified OECs colabeled with Reelin.

To confirm that cultured OECs express and secrete Reelin, we performed western blotting. The G10 antibody recognizes full-length Reelin protein, which is approximately 400

kDa, and its two cleaved fragments at ~300 and ~180 kDa (de Bergeyck et al., 1998; Lambert et al., 1999; Lacor et al., 2000). The rat ONL and *Reln*$^{+/+}$ mouse olfactory bulbs showed the characteristic large molecular weight band at 400 kDa and another at 150 kDa (Fig. 3A, lanes 1-2). As expected, these bands were absent in mutant *Reln*$^{-/-}$ olfactory bulbs (Fig. 3A, lane 3). Additionally, Reelin-positive bands were present in blots of OEC whole cell lysates (WCL) and OEC CM (Fig. 3A, lanes 4-8). A 4-15% gradient gel allows for the visualization of all three Reelin isoforms (Fig. 3B). Brain extracts of *Reln*$^{+/+}$ (positive control) and *Reln*$^{-/-}$ (negative control) mice confirm the specificity of Reelin detection (Fig. 3B, lanes 1-2). All three Reelin isoforms at their corresponding molecular weights are present in rat ONL and OEC CM samples (Fig. 3B, lanes 3-6). These results indicate that cultured OECs synthesize and secrete Reelin.

Reelin and neurite outgrowth

In addition to its role in regulating neuronal positioning during embryonic development, the Reelin-signaling pathway promotes dendritogenesis of early postnatal hippocampal neurons (Niu et al., 2004; Matsuki et al., 2008). *Reln*$^{-/-}$ mice exhibit a reduction in the number and length of laminae II-III cerebral cortical neuron dendrites compared to those in *Reln*$^{+/+}$ mice (Hoe et al., 2009). Using a neuronal outgrowth assay, we asked whether or not the growth-promoting activities of OECs depend on the Reelin-signaling pathway. Because Gabaergic neurons in the cortex are Reelin-positive (Alcántara et al., 1998), we first assessed the length of neurite outgrowth of postnatal cortical neurons from *Reln*$^{+/+}$ and *Reln*$^{-/-}$ mice on a laminin (positive control) or PLL (neutral control) substrate. The average neurite tree length of neurites from *Reln*$^{+/+}$ and *Reln*$^{-/-}$ mouse cortices did not differ between the two substrates (Fig. 4A-B, E-F, I; Laminin: *Reln*$^{+/+}$ 81±7 μm, *Reln*$^{-/-}$ 58±13 μm; PLL: *Reln*$^{+/+}$ 91±31 μm, *Reln*$^{-/-}$ 99±27 μm). The

addition of Reelin-secreting OECs on a PLL substrate also did not increase the average total neurite length of *Reln*$^{+/+}$ or *Reln*$^{-/-}$ cortical neurons compared to the PLL controls (Fig. 4B-C, F-G, I; PLL + *Reln*$^{+/+}$ OECs: *Reln*$^{+/+}$ 68±17 µm, *Reln*$^{-/-}$ 64±14 µm). Similarly, cortical neurite length did not differ from control when *Reln*$^{+/+}$ or *Reln*$^{-/-}$ neurons were cultured with OECs from Reelin-deficient mice (Fig. 4B, D, F, H, I; PLL + *Reln*$^{-/-}$ OECs: *Reln*$^{+/+}$ 77±19 µm, *Reln*$^{-/-}$ 89±39 µm). The contact between cerebral cortical neurites and OECs did not differ between Reelin-expressing and deficient OECs (Fig. 4C-D, G-H). These data suggest that OEC-secreted Reelin does not alter neurite outgrowth of postnatal cortical neurons.

Because neuronal populations may respond differently to OECs, we also tested the effect of Reelin-secreting and Reelin-deficient OECs on axon outgrowth of postnatal DRG neurons. When cultured on laminin, the average total length of axons of *Reln*$^{+/+}$ DRG was significantly shorter than *Reln*$^{-/-}$ axons (Fig. 5A, E, I; *Reln*$^{+/+}$ 374±83 µm, *Reln*$^{-/-}$ 776±16 µm, $p < 0.05$). When on a neutral PLL substrate, the lengths of DRG axons did not differ (Fig. 5B, F, I: *Reln*$^{+/+}$ 105±18 µm, *Reln*$^{-/-}$ 124±44 µm). When Reelin-secreting OECs were added to a PLL substrate *Reln*$^{+/+}$ and *Reln*$^{-/-}$ DRG axon lengths were longer than the PLL controls (Fig. 5B-C, F-G, I; *Reln*$^{+/+}$ 597±50 µm, $p < 0.001$; *Reln*$^{-/-}$ 366±110 µm, $p < 0.05$). Additionally, when DRG axons were cultured with Reelin-deficient OECs *Reln*$^{+/+}$ and *Reln*$^{-/-}$ DRG axon outgrowth was greater than PLL controls (Fig. 5B, D, F, H, I; *Reln*$^{+/+}$ 343±47 µm, $p < 0.05$; *Reln*$^{-/-}$ 501±20 µm, $p < 0.05$). To determine if the presence of Reelin affects outgrowth of *Reln*$^{+/+}$ or *Reln*$^{-/-}$ DRG axons, we compared axon outgrowth with or without OEC-secreted Reelin. Axons of *Reln*$^{+/+}$ neurons were significantly longer when cultured with Reelin-secreting than Reelin-deficient OECs (Fig. 5C-D, I; *Reln*$^{+/+}$ OECs 597±50 µm vs *Reln*$^{-/-}$ OECs 343±47 µm, $p < 0.05$). Interestingly, axon outgrowth of *Reln*$^{-/-}$ DRG axons did not differ between OECs from *Reln*$^{+/+}$ or *Reln*$^{-/-}$ mice (Fig. 5G-H, I;

Reln$^{+/+}$ OECs 366±110 µm vs *Reln*$^{-/-}$ OECs 501±20 µm). These data suggest that OEC-secreted Reelin mediates DRG axon outgrowth.

Discussion

This study shows that Reelin is expressed in the olfactory nerve layer of the adult mouse olfactory bulb, an area that contains a large number of OECs. Next we generated biochemical and anatomical evidence that OECs synthesize and secrete Reelin. When we examined the effects of OEC-Reelin secretion on process outgrowth, our data demonstrated that OEC-secreted Reelin mediates enhanced outgrowth of DRG axons, but not the neurites of cortical neurons.

Neurite and axonal outgrowth

Although we detected a robust effect of Reelin expression on DRG axonal outgrowth, we failed to observe a similar response in cerebral cortical cultures. This was surprising because some cortical neurons express the downstream components of the Reelin-signaling pathway, i.e., Vldlr and Apoer2 receptors and intracellular adaptor signaling molecule Dab1 (D'Arcangelo et al., 1999; Trommsdorff et al., 1999; Jossin and Goffinet, 2007; Abadesco et al., 2014), and therefore would likely respond to Reelin-secreted by OECs. Reelin activates Dab1 and downstream PI3K and Akt pathways that mediate dendritic growth of embryonic hippocampal and cortical neurons (Jossin and Goffinet, 2007; Niu et al., 2004). Our results are consistent, however, with a previous report that OEC CM does not increase neurite elongation of postnatal corticospinal tract neurons (Witheford et al., 2013). One possible explanation may be that embryonic and postnatal neurons differ in their response to Reelin or that Reelin-enhanced neurite outgrowth is age-dependent.

Rat OECs enhance neurite outgrowth of postnatal cortical neurons in a contact-dependent manner (Khankan et al., 2015). Yet despite the direct contact between co-cultured OECs and mouse cerebral cortical neurons, neurite outgrowth was not enhanced in the current study. DRG axon outgrowth was enhanced on OECs when compared to PLL substrate alone, yet outgrowth did not depend on alignment of DRG axons with OECs. This implies that different neuron populations may vary in their response to OECs. Interestingly, we observed similar levels of outgrowth when cerebral cortical or DRG neurons were cultured with recombinant Reelin or Reelin-secreting OECs (data not shown). This suggests that glial-derived Reelin and recombinant Reelin may have similar effects and that process outgrowth may not depend on the relationship between the Reelin secreting cells and neurons.

Alternative functions of Reelin

Reelin-induced phosphorylation of Dab1 activates multiple kinases involved in various cellular processes, such as the migration of neuronal precursors and neurogenesis (Hack et al., 2002; Won et al., 2006). OEC-secreted Reelin may have different functions as well depending on the cellular population involved. The localization of Reelin within OECs and the presence of the OECs next to a highly proliferative stem cell population in the olfactory epithelium suggests that alternative functions of OEC-secreted Reelin may exist. In fact, Reelin may regulate pathfinding of Vldlr-expressing embryonic (Schnaufer et al., 2009) and mature ORNs (Nickell et al., 2012) as Reelin expression is increased following olfactory bulb lesion (Okuyama-Yamamoto et al., 2005). Yet the function of Reelin/Vldlr-mediated signaling within immature and mature ORNs remains unclear.

Other cell membrane components involved in cell adhesion, cell migration, and neurite outgrowth such as integrins (Andressen et al., 1998; DeFreitas et al., 1995; Dulabon et al., 2000), are known to interact with Reelin. Because integrins are established laminin receptors, a Reelin interaction with integrins is suggested by our findings that *Reln*$^{-/-}$ DRG axons were significantly longer than *Reln*$^{+/+}$ axons when cultured on laminin. Lorenzetto et al. (2008) proposed that Reelin may interact with Beta-1 integrin present on the surface of Schwann cells (Fernandez-Valle et al., 1994) and on axons (Tomaselli et al., 1993) to provide a signal that stimulates axonal outgrowth following peripheral nerve injury. Interestingly, in our neuron-OEC co-cultures DRG axons did not interact with OECs as frequently as cerebral cortical neurons. Thus, axon-substrate or axon-glia interactions may be regulated by Reelin/integrin signaling.

Our interest in the function of OEC-secreted Reelin stems from previous studies using OEC transplants as a therapy following spinal cord injury (Kubasak et al., 2008; Takeoka et al., 2011; Ziegler et al., 2011). OEC transplantation into the spinal cord may provide an exogenous source of Reelin that can phosphorylate Dab1-containing spinal cord neurons. Corticospinal tract axons and many cells in the spinal cord express Dab1 including sympathetic and parasympathetic preganglionic neurons, somatic motor neurons, a number of dorsal horn neurons, and oligodendrocytes (Phelps et al., 2002; Yip et al., 2004; Villeda et al., 2006; Siebert and Osterhout, 2011; Abadesco et al., 2014). Further investigation of the role of OEC-secreted Reelin on various neuronal and glial populations will serve to increase our understanding of the mechanisms by which OECs mediate repair following spinal cord injury.

Figure legends:

Figure 1. Reelin expression in the adult olfactory bulb.

A: In a sagittal section, Reelin-labeled cells in the laminated adult mouse olfactory bulb include mitral cells, periglomerular cells, and external tufted cells. The ONL contains olfactory ensheathing cells that are Reelin-expressing. ONL: outer nerve layer, GL: glomerular layer, EPL: external plexiform layer, MCL: mitral cell layer, IPL: internal plexiform layer, GCL: granule cell layer. Scale bars A-C = 100 μm.

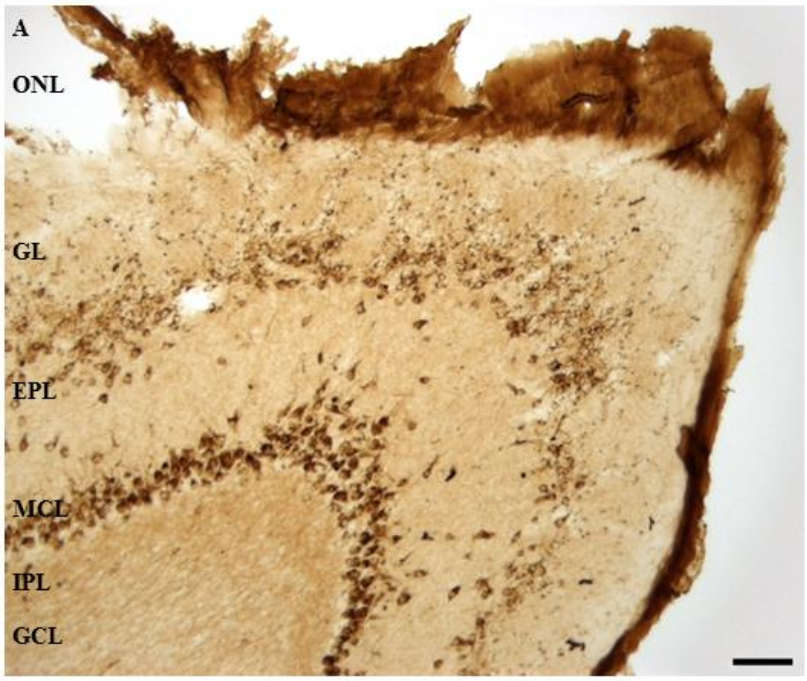

Figure 1

Figure 2. Reelin expression in cultured OECs.

A-C: Rat OEC cultures treated with Brefeldin-A to inhibit protein transport. A: Reelin immunoreactivity was detected in GFP-labeled OECs (arrows), but not in other olfactory bulb cell types (arrowhead) found in primary cultures. B-C: GFP-labeled OECs immunopurified with anti-p75-NGF receptor express Reelin. Scale bars = 40 μm.

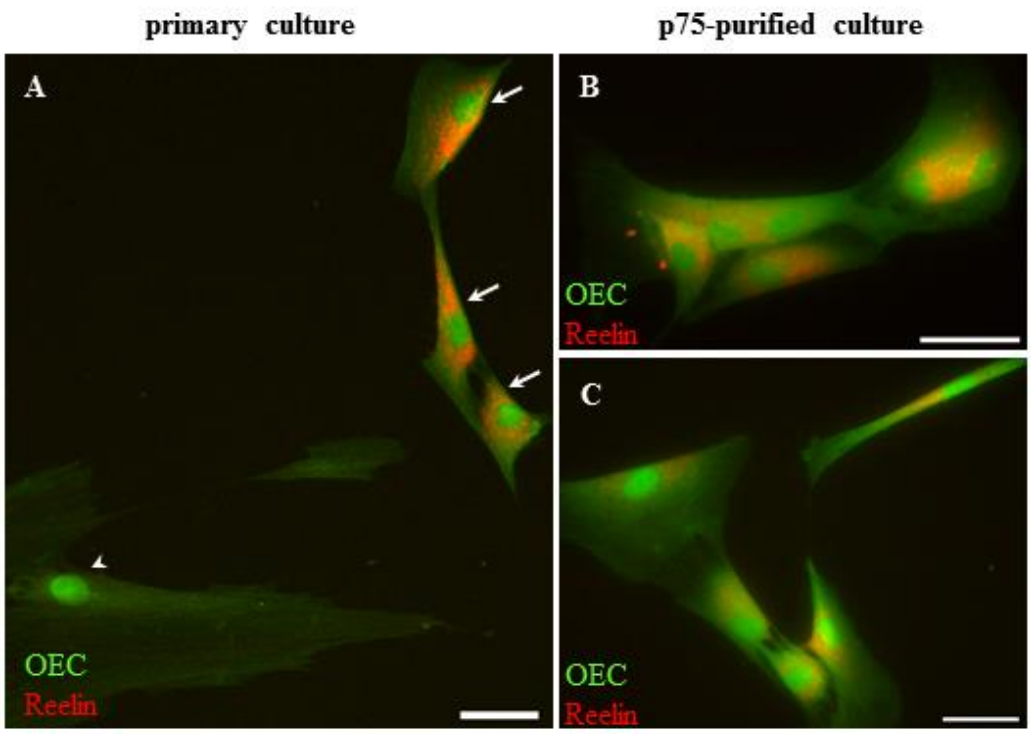
primary culture
p75-purified culture
A
B
C
OEC
Reelin
OEC
Reelin
OEC
Reelin

Figure 2

Figure 3. Reelin is expressed and secreted by olfactory ensheathing cells.

A: Western blot confirms the expression of Reelin in rat olfactory nerve layer (ONL; lane 1), and *Reln*$^{+/+}$ and *Reln*$^{-/-}$ olfactory bulbs used as positive and negative controls, respectively (lanes: 2 and 3). OEC whole cell lysates (WCL; lanes: 4, 6, and 8), and OEC conditioned medium (CM; lanes: 5 and 7). B: All three Reelin isoforms (400, 300, and 150 kDa) were visualized using a 4-15% gradient gel. *Reln*$^{+/+}$ and *Reln*$^{-/-}$ mouse cortices were used as controls (lanes: 1 and 2). Reelin was detected in the rat ONL (lane 3) and different OEC-CM samples (lanes: 4, 5, and 6). GAPDH was used as a loading control for tissue homogenates (A, lanes 1-4, 6, 8; B, lanes: 1-3).

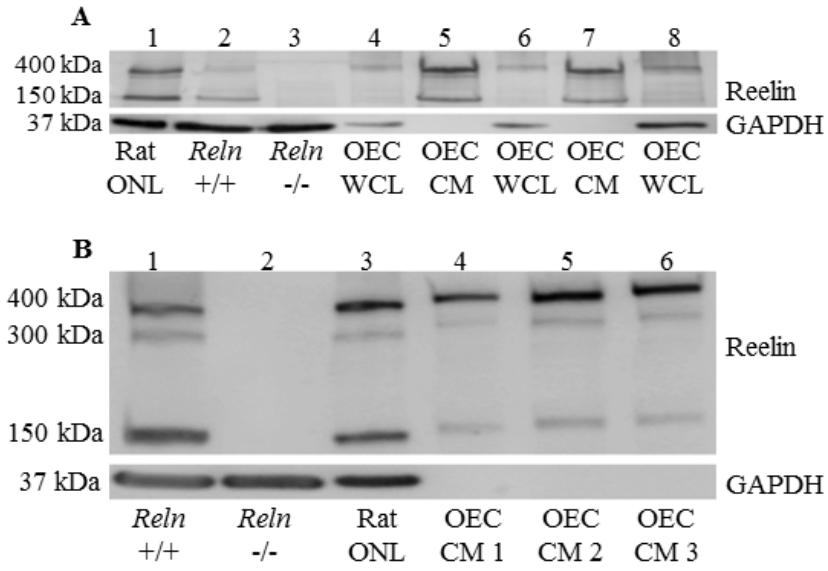
A
1 2 3 4 5 6 7 8
400 kDa
150 kDa
Reelin
37 kDa
GAPDH
Rat ONL
Reln +/+
Reln -/-
OEC WCL
OEC CM
OEC WCL
OEC CM
OEC WCL
B
1 2 3 4 5 6
400 kDa
300 kDa
Reelin
150 kDa
37 kDa
GAPDH
Reln +/+
Reln -/-
Rat ONL
OEC CM 1
OEC CM 2
OEC CM 3

Figure 3

Figure 4. Cortical neurite outgrowth is independent of OEC-secreted Reelin.

Neurons from postnatal day 8 *Reln*$^{+/+}$ (A-D) and *Reln*$^{-/-}$ (E-H) cerebral cortices (CTX) grown on laminin (A, E), PLL (B, F), PLL + *Reln*$^{+/+}$ OECs (C, G), or PLL + *Reln*$^{-/-}$ OECs (D, H). A-D: CTX neurons (white) from *Reln*$^{+/+}$ mice extend neurites of comparable length on laminin (A), PLL (B), PLL + *Reln*$^{+/+}$ OECs (C, green), or PLL + *Reln*$^{-/-}$ OECs (D, green). E-H: *Reln*$^{-/-}$ neurites (white) do not differ in total tree length when grown on laminin (E), PLL (F), PLL + *Reln*$^{+/+}$ OECs (G, green), or PLL + *Reln*$^{-/-}$ OECs (H, green). I: The average total neurite tree length of *Reln*$^{+/+}$ (blue) or *Reln*$^{-/-}$ (red) cortical neurons. A total of 850 cortical neurons were traced. Each experiment (n = 3 or 4) is represented by a blue (*Reln*$^{+/+}$) or red (*Reln*$^{-/-}$) dot and mean ± SEM values are reported. β3tub, β3-tubulin; OEC, olfactory ensheathing cell. Scale A–H = 25 μm.

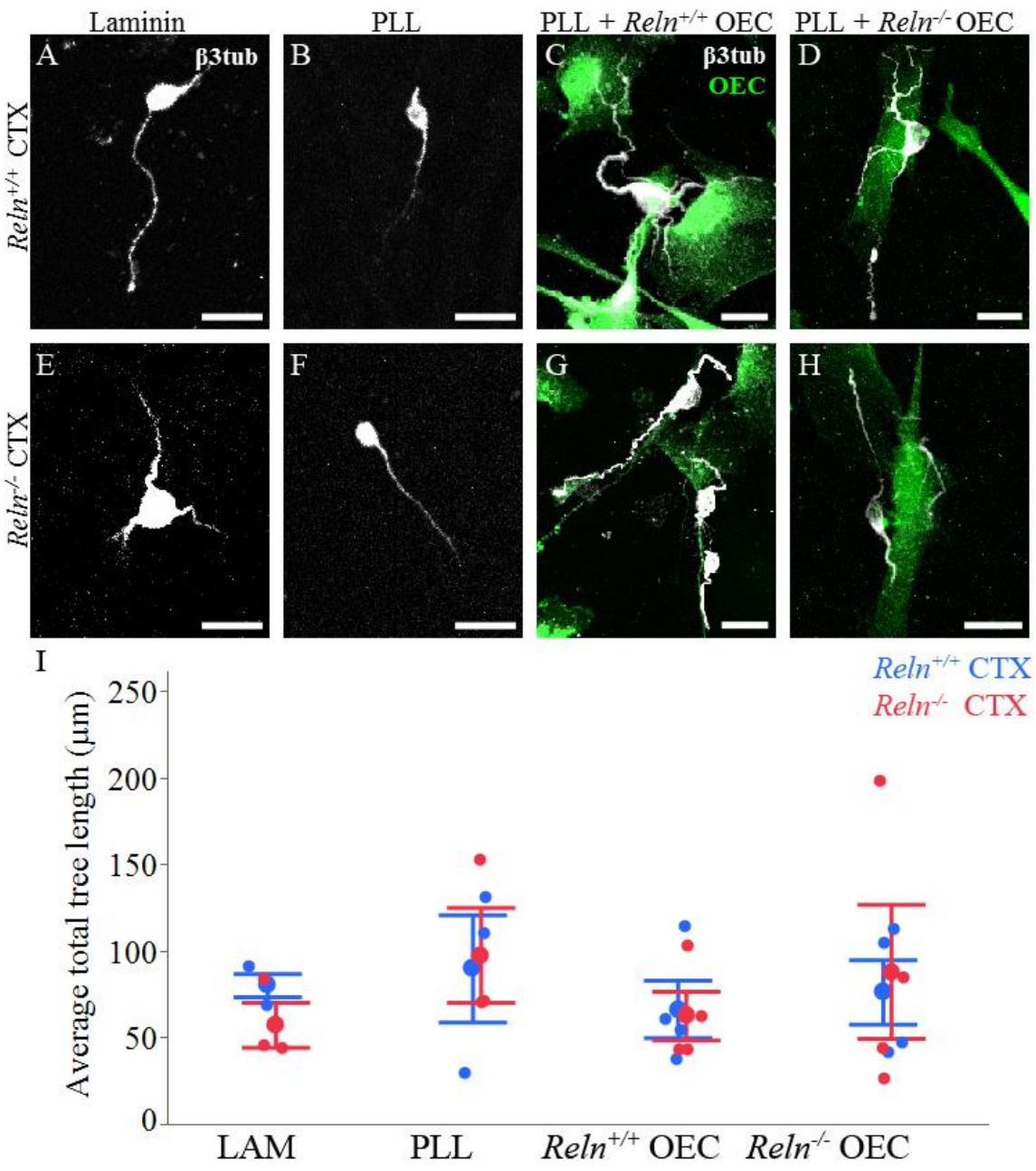
Laminin
PLL
PLL + Reln+/+ OEC
PLL + Reln-/- OEC
A
β3tub
B
C
β3tub
OEC
D
Reln+/+ CTX
E
F
G
H
Reln-/- CTX
I
Reln+/+ CTX
Reln-/- CTX
Average total tree length (µm)
250
200
150
100
50
0
LAM
PLL
Reln+/+ OEC
Reln-/- OEC

Figure 4

Figure 5. OEC-secreted Reelin enhances dorsal root ganglia axonal outgrowth.

Dorsal root ganglia (DRG) neurons (white) from postnatal day 8 *Reln*$^{+/+}$ (A-D) and *Reln*$^{-/-}$ (E-H) mice grown on laminin (A, E), PLL (B, F), PLL + *Reln*$^{+/+}$ OECs (C, G), or PLL + *Reln*$^{-/-}$ OECs (D, H). A-D: *Reln*$^{+/+}$ DRG axonal outgrowth on laminin (A), PLL (B), PLL + *Reln*$^{+/+}$ OECs (C, green), or PLL + *Reln*$^{-/-}$ OECs (D, green). E-H: *Reln*$^{-/-}$ DRG extend axons on laminin (E), PLL (F), PLL + *Reln*$^{+/+}$ OECs (G, green), or PLL + *Reln*$^{-/-}$ OECs (H, green). I: The average axon length of *Reln*$^{+/+}$ (blue) or *Reln*$^{-/-}$ (red) DRG neurons is compared on 4 substrates. Approximately 318 neurons were traced and analyzed. Each experiment (n = 2) is represented by a blue (*Reln*$^{+/+}$) or red (*Reln*$^{-/-}$) dot and means ± SEM values are reported. ¥, §, and * $p < 0.05$, *** $p < 0.001$. β3tub, β3-tubulin; OEC, olfactory ensheathing cell. Scale A–H = 25 μm.

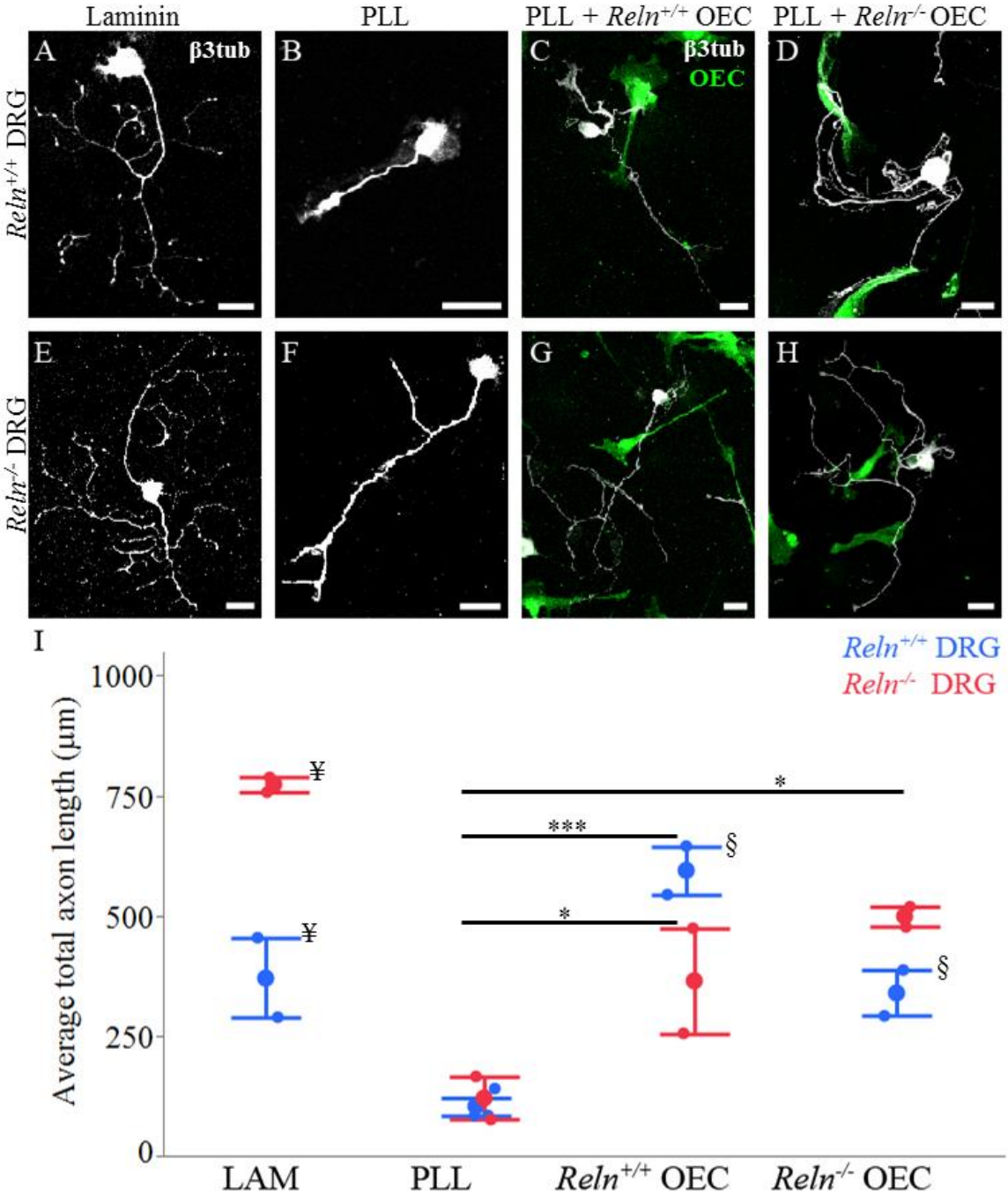
Laminin
PLL
PLL + Reln+/+ OEC
PLL + Reln-/- OEC
A
β3tub
B
C
β3tub
OEC
D
Reln+/+ DRG
E
F
G
H
Reln-/- DRG
I
Reln+/+ DRG
Reln-/- DRG
Average total axon length (µm)
1000
750
500
250
0
¥
¥
*
***
*
§
§
LAM
PLL
Reln+/+ OEC
Reln-/- OEC

Figure 5

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