Title
Expression of alpha 7 integrin mediates the effects of laminin on Olfactory Ensheathing Cells

Permalink
https://escholarship.org/uc/item/4pv7h1d9

Author
Ingram, Norianne Theresa

Publication Date
2013

Peer reviewed|Thesis/dissertation
Expression of alpha 7 integrin mediates the effects of laminin on Olfactory Ensheathing Cells

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

By

Norianne Theresa Ingram

2013
Abstract of the Thesis

Expression of alpha 7 integrin mediates the effects of laminin on Olfactory Ensheathing Cells

by

Norianne Theresa Ingram

Master of Science in Physiological Science
University of California, Los Angeles 2013
Professor Patricia Emory Phelps, Chair

Olfactory ensheathing cells (OECs) are unique glia found only in the olfactory system. OECs support neuronal turnover and axon outgrowth in adults and represent a promising cell-transplantation candidate for spinal cord injury repair. Current mechanistic hypotheses are based on the ability of OECs to stimulate neuronal outgrowth via both secreted and cell-contact mediated effects. Laminin is reported to stimulate the growth promoting abilities of OECs, and this study asks if OECs react to laminin using integrin receptors to enhance their function. We focus on the α7 integrin that is expressed by Schwann cells and implicated in peripheral nerve regeneration. Here we ask if adult OECs express α7 integrin and if the presence of α7 integrin mediates the OEC response on laminin by increasing their motility. We
found that α7 integrin colocalized with established OEC markers, SOX10, S100, and Aquaporin1. When adult OEC motility on laminin was tested, twice as many wildtype OECs migrated on laminin compared to α7 mutant OECs. These results show that α7 integrin mediates the OEC substrate preference for laminin, the first demonstration of a functional role for α7 integrin in OECs.
The thesis of Norianne Theresa Ingram is approved.

Rachelle Hope Watson

Scott H. Chandler

Patricia Emory Phelps, Committee Chair

University of California, Los Angeles

2013
# TABLE OF CONTENTS

| LIST OF FIGURES                                    | vi   |
| ACNOWLEDGMENTS                                   | viii |
| BODY OF TEXT                                     | 1    |
| TABLES AND FIGURES                               | 14   |
| REFERENCES                                       | 31   |
LIST OF FIGURES

Table 1 ........................................................................................................................................................................14
Primary Antisera Used

Figure 1........................................................................................................................................................................15
β-gal reaction product is found in OEC-rich regions of the olfactory system in α7lacZ/+/ and α7lacZ/lacZ mice.

Figure 2........................................................................................................................................................................17
In the olfactory bulb α7 integrin colocalizes with known OEC markers, SOX10, S100, and AQP1.

Figure 3........................................................................................................................................................................19
OECs in the olfactory nerve co-express α7/β-gal and SOX10.

Figure 4........................................................................................................................................................................21
α7/β-gal expression is distinct from, but closely associated with, the olfactory receptor neurons.

Figure 5........................................................................................................................................................................23
β-Dystroglycan expression in the primary olfactory system.
Immunopurification with p75-NGFR yields purified secondary OEC cultures.

OECs identified with p75-nerve growth factor (NGFR) after migrating through transwell inserts.

α7 integrin mediates OEC migration on a laminin substrate.
ACKNOWLEDGEMENTS

Foremost, I would like to thank Dr. Patty Phelps who is the most wonderful of mentors. As one of the hardest working people I know, she is truly an inspiration. Under her nurturing guidance I have been introduced to and taken my first steps into the world of science research. I could not have hoped for a better mentor to assist me with my master’s research project.

Next, I would like to thank Dr. Rachelle Crosbie-Watson and Dr. Scott Chandler for sitting on my committee.

Additional and special thanks to Dr. R. Crosbie-Watson and Dr. Jamie Marshall for helping with: mice, genotyping, protocols, antibodies, dystroglycan, ideas, and general questions related to alpha 7.

Thanks to the rest of Phelps’ Lab. In particular, thank you Rana for finding the alpha 7-null colony on campus, project/experiment ideas, and general mental health support. To Marianne, thank you for consulting on various protocol tweaks. Thank you to Khris, Hannah, Griselda, Katie, James, Danielle, Alyssa, and Andy. You guys kept a smile on my face even when things were difficult. A happy researcher makes happy data.

I’d like to thank the following non-persons: UCLA for giving me a place to call home, a road to knowledge, and the positive reinforcement to endure, God for creating all, including this wonderful magic we call Science, and rainbows for promising a colorful and bright tomorrow.
Finally, thank you to my parents and siblings. Thank you for keeping my life lively, for teaching me that the value of a smile is more than a dollar, and for supporting me no matter how crazy I get.
Introduction

Olfactory ensheathing cells (OECs) are unique glia that form a one-of-a-kind transition zone between the central and peripheral parts of the olfactory system. During embryonic development OECs migrate with the axons of peripherally born olfactory receptor neurons (ORNs), as these neurons make direct connections directly into the olfactory bulb (OB), a CNS structure. In addition to the initial synaptic connection into the OB, ORNs continue to be generated and project to the OB throughout life (Au et al., 2002). OECs play a key role in ORN turnover by assisting in the removal of degenerating neural remnants and guiding the replacement of new ORN axons (Su et al., 2013). These characteristics make OECs an attractive cell type for regenerative, cell-transplantation therapies.

Due to the unique characteristics of OECs, they were among the early cell transplantation therapies tested in complete spinal cord transection models. Enhanced functional recovery is reported in both rat and canine transplantation models for spinal cord injury (Ramón-Cueto et al., 2000, Takeoka et al., 2011, Ziegler et al., 2011, Granger et al., 2012). To date the mechanisms that stimulate OEC-induced regeneration are not well understood, but tissue culture experiments suggest parallel mechanisms. Previous data show enhanced axonal outgrowth due to OEC secretion of brain-derived neurotrophic factor (BDNF) in vitro (Runyan and Phelps, 2009). In addition to BDNF, OECs secrete a variety of other neurotrophins including nerve growth factor, glial derived neurotrophic factor, and ciliary neurotrophic factor (Lipson et al., 2003). Besides secreted factors, neurite outgrowth and survival is increased if OECs directly make contact with neurons in culture (Khankan et al., 2010, Liu et al., 2010). To understand how OEC-neurite contacts might stimulate outgrowth, we examined cell adhesion molecules that are expressed by OECs.

Cell adhesion molecules dictate the associations between different cell types and also interactions between cells and the local extracellular matrix (ECM) components.
Microarray data indicate that several cell adhesion molecules are candidates for mediating OEC-contact interactions, such as α7 integrin, cadherin 4, and neural cell adhesion molecule 2 (Roet et al., 2011). Velling et al. (1996) reported the presence of α7 integrin protein in the olfactory nerve layer of the OB, but did not identify the cell types. Due to the concentration of OECs in the olfactory nerve layer, we suspect that α7 integrin may be associated with this unique glia.

Integrins are a subfamily of cell adhesion molecules involved in bidirectional signaling between a cell and its environment. Integrins are composed of two transmembrane subunits: an α subunit and a β subunit. While the β isoforms are often ubiquitous in specific tissues, the α subunit usually mediates the substrate interaction of the integrin complex and drives specific intracellular effects. In particular, α7 integrin combines with the most common β isoform, β1, to form a functional receptor for laminin (Hynes et al., 1992). The α7β1 integrin is best known as a contributor to congenital types of muscular dystrophy. In the musculoskeletal system, α7β1 integrin acts as a co-receptor with the dystroglycan (DG) complex for laminin. Deficiencies in α7 integrin, DG, and/or laminin can all lead to muscular dystrophy (Jimenez-Mallebrera et al., 2005).

DG is a calcium-dependent, adhesion complex known to stabilize muscle cells to the surrounding ECM components, most commonly laminin. DG is composed of one α and one β subunit, but is not an integrin. The binding of the DG complex to ECM molecules is mediated by α-DG, whereas β-DG forms the transmembrane domain of the DG complex. Dystrophin binds β-DG on its cytoplasmic side where a tyrosine phosphorylation site determines its level of affinity to dystrophin. The extracellular domain of β-DG associates with α-DG (Sgambato and Brancaccio, 2005). The DG complex and α7 integrin are reported to have varying levels of colocalization in muscle and nervous system (Chernousov et al., 2007) cell types and must be carefully evaluated.

Both α7 integrin and DG are co-receptors for laminin, one of the most abundant components of the ECM. Laminin is composed of three distinct chains: α, β, and γ, and the
chains self-assemble to help form and stabilize the ECM in the presence of divalent cations. Laminins also provide environmental cues to cells that express many different receptors. Laminin triggers various cellular functions such as adhesion, migration, differentiation, proliferation, apoptosis, and gene expression (Aumailley and Smyth, 1998). OECs express the mRNA of several laminin isoforms including: laminin γ1, laminin α5, and laminin B3 (Roet et al., 2010). Additionally, laminin α1 is expressed in both the accessory OB and olfactory nerve layer (ONL, Velling et al., 1996). These findings suggest that the OECs both secrete laminin and bear its receptors. Whether or not olfactory receptor neurons express laminin is unknown. Several studies localize laminin in the olfactory nerve layer and glomerular layer of the olfactory bulb, but do not identify the cells that secrete it (Liesi, 1985, Kafitz and Greer, 1997). These studies also suggest that the up-regulation of laminin after injury contributes to the regenerative potential of the adult nervous system. Although it is not known if ORNs secrete laminin, elaborate gene trap experiments demonstrated that many CNS-derived neurons do make and potentially secrete laminin (Yin et al., 2003).

The α7 integrin is also found in the nervous system, where it is involved in regulating neurite outgrowth and regeneration (Gardiner et al., 2004; Mercado et al., 2004; Werner et al., 2000). Of interest, α7 integrin expression is seen in Schwann cells (Previtali et al., 2003a), which are derived from the neural crest. Neural crest cells are highly migratory and respond to guidance and differentiation cues along their pathway to reach their targets. Kil and Bronner-Fraser (1995) reported that α7 integrin is expressed in several populations of migrating neural crest including the dorsal root ganglia and cranial neural crest. Such findings suggest that other cranial neural crest-derived cells such as OECs may also express α7 (Barraud et al., 2010, Armati and Mathey, 2013).

Although Schwann cells co-express both α7 integrin and DG, these laminin receptors may play distinct roles during Schwann cell differentiation (Chernousov et al., 2007). Reportedly, the B1-type integrin receptors mediate the effects of laminin during
axon sorting, whereas the DG complex participates during myelination (Previtali et al., 2003b). The loss of α7 integrin function in Schwann cells also causes deficits in peripheral nerve regeneration after injury, but not in the initial peripheral nerve formation or myelination (Previtali et al., 2003a). If OECs do express α7 integrin and DG, they may also have different functions when exposed to laminin.

In this study we first ask if α7 integrin co-localizes with established OEC markers. For these experiments we use mice with a lacZ reporter inserted into exon 1 of α7 integrin (Flintoff-Dye et al., 2005). The coexpression of DG is also examined. Our second goal is to test if adult OECs in a transwell assay use α7 to mediate their migration on laminin. Taken together, these experiments examine if the α7 integrin is a key signaling molecule involved in the function of OECs.

**Materials and Methods**

**Animals and tissue preparation**

The α7 integrin mouse line was created with the insertion of the lacZ reporter into the first exon in the α7 integrin gene locus (Flintoff-Dye et al., 2005). Mutant mice (α7lacZ/lacZ) express only β-galactosidase (β-gal), rather than one (α7lacZ/+ or two (α7+/+) functional α7 integrin subunits. Pairs of α7lacZ/+ mice were bred to yield all three genotypes. Genotypes were confirmed by PCR as described in Flintoff-Dye et al. (2005).

Adult mice underwent transcardial perfusion with 4% paraformaldehyde followed by a 4 h postfix. The olfactory bulbs and nasal epithelium were dissected, cryoprotected, and embedded into OCT (Sakura Finetek, Torrance, CA). Olfactory bulbs and the attached epithelium were sectioned sagittally, 15-20 μm thick, and slide mounted. Horizontal sections of nasal epithelium were used to examine cross sections of the olfactory nerve fascicles.
**X-gal histochemistry**

Fixed sections from all 3 genotypes were washed in 0.12M Millonig's buffer 3 times for 5 min and then incubated for 45 min in 50% X-gal mixer/ 50% Millonig's buffer. X-gal stain (Khialeeva et al., 2011) was added to the sections and incubated for 4-5 h at 37°C. After 4-5 h, strong blue staining was observed in both α7^{lacZ/+} and α7^{lacZ/lacZ} sections.

**Immunohistochemistry (IHC)**

To localize α7 integrin in OECs, we carried out double-labelling experiments on adult olfactory bulbs and epithelium with anti-β-gal, which marks α7-expressing cells, and established OEC markers: SOX10, a neural crest nuclei marker (Barraud et al., 2010); S100, a glial marker (Au et al., 2002); and Aquaporin1, a water channel (Shields et al., 2010).

Rabbit anti-β-gal was used to identify α7 expression in α7^{lacZ/lacZ} mice. β-gal IHC was conducted first with either standard immunofluorescence or if both primary antibodies were raised in rabbit, with tyramide signal amplification (TSA; Perkin-Elmer, Waltham, MA). Standard fluorescent β-gal IHC was carried out with 0.1M TRIS + 1.4% NaCl + 0.1% BSA buffer (TBS). Sections were incubated for 1 h with 5% normal donkey serum (NDS) and left overnight in rabbit anti-β-gal (Table 1). The following day, sections were washed in TBS, and incubated in donkey anti-rabbit conjugated with Alexa 488 (1:200-250; Invitrogen, Grand Island, NY) for 1 h. Sections were washed with TBS, and followed by the second IHC protocol.

Experiments using antibodies in different species did not require special IHC considerations. These included the following antibodies: goat anti-SOX10, goat anti-OMP, a marker for mature olfactory receptor neurons, and/or mouse anti-β-DG, which recognizes the β subunit of the dystroglycan complex (Table 1). 0.1M Phosphate buffered saline (PBS) was used for buffer rinses. After blocking with 5% NDS, primary antibodies were incubated overnight at 4°C. After buffer rinses, sections were incubated 1 h in
donkey anti-goat secondary conjugated with Alexa Fluora 594 (1:800; Invitrogen). Sections were washed in buffer and then coverslipped with Fluorogel (EMS, Hatfield, PA).

β-gal was also localized with the TSA fluorescein kit using TNT buffer (0.1M TRIS-HCl + 0.15M NaCl + 0.05% Tween) when experiments required two primary antibodies generated in the same species. All antibody and blocking incubation steps used TNB (0.1M TRIS-HCl + 0.15M NaCl + blocking reagent from TSA kit) buffer. After washes, 20% avidin (avidin-biotin blocking kit, Vector, Burlingame, CA) in TNB was added for 1 h. Rabbit anti-β-gal in 20% biotin (avidin-biotin blocking kit, Vector) was added to TNB for overnight incubation. After a buffer wash in TNT, sections were incubated in donkey anti-rabbit IgG (1:500; Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 h, rinsed, Streptavidin-HRP was added for 1 h (SA-HRP; 1:250; TSA kit), and reacted with fluorescein for 4 min.

To keep primary antibodies derived from the same species from cross-reacting, the first TSA experiment was followed by a citric acid treatment (Tóth and Mezey, 2007, and adapted by Shields et al., 2010). First sections were fixed for 15 min with 4% paraformaldehyde. After rinsing in buffer, sections were placed in 10mM citric acid, pH 6.0 and heated in a microwave at 100% power for 2.5 min and 50% power for an additional 2 min and cooled. Rabbit anti-S100 and rabbit anti-AQP1 both required additional signal amplification (Table 1). These experiments followed the TSA protocol above except that TBS buffer was used instead of TNT or TNB. After primary incubation, donkey anti-rabbit IgG (1:500) was incubated for 1 h, followed by Strepavidin conjugated to Alexa 594 (1:1200, Invitrogen).

**Dissociated OEC cultures**

Primary OEC cultures were generated from olfactory bulbs dissected from 2 mice per genotype using methods adapted from Ramón-Cueto et al. (2000) and Runyan and Phelps et al. (2009). D/F media with 15% FBS was changed daily. After 5-6 days in
vitro, primary OEC cultures were immunopurified using rabbit anti-p75-NGFR (1:1500-2000, Table 1). After purification OECs were seeded in 4-chamber, polystrene-vessel culture slides (BD Falcon, San Jose, CA) coated with poly-L-lysine (PLL) to characterize the cell types in the cultures, and on transwell inserts (see below) to test migratory ability.

**Migration assay and analysis**

To test the effects of laminin and α7 integrin on OEC migration, transwell inserts (0.8 μm pore, 24-well; BD Falcon) were coated with either PLL (Sigma, St. Louis, MO) or PLL coated with laminin (10 μg/ml, Invitrogen). Experimental groups (n=4 culture dates) were: 1) α7+/+ OECs grown on laminin or PLL, 2) α7lacZ/+ OECs with laminin or PLL, and 3) α7lacZ/lacZ OECs with laminin or PLL. Inserts were placed in 24-well plates with 400 μl of D/F media plus 15% FBS beneath the insert to stimulate migration (Kostidou et al., 2007). Purified OECs were added to the top of the insert in serum-free D/F media (20-25,000 cells/300 μl) and placed in a 37° C incubator with 5% CO2. After 24 h 200 μl of fluid was removed from the bottom of each well, and 400 μl of fresh D/F media with 15% FBS was added to stimulate further migration. After 48 h, the top of each insert was scraped to remove non-motile cells, and fixed with 4% paraformaldehyde for 10 min. OECs on the inserts were identified with rabbit anti-p75 NGFR (Table 1), and the Hoechst nuclei marker (1:300, Sigma). Inserts were photographed at 4x, and all nuclei were quantified (800-9000 cells/insert) using Neuralucidia computer software (MBF Bioscience, Williston, Vermont) to mark and count nuclei.

After counting all nuclei present, the number of nuclei on laminin was normalized by generating a ratio of the number of nuclei present on laminin, divided by the number of nuclei from the same genotype on PLL. We called this the “migratory potential ratio.” A migratory potential ratio is 1 when the migration on each substrate is equivalent. Computations were carried out in JMP 9.0 (SAS Institute, Cary, NC) using an analysis of
variance (ANOVA) model to determine differences between the migratory potential ratio of each genotype.

Photography

Fluorescent and bright field images were taken on an Olympus microscope or with a Zeiss LSM 510 confocal microscope. Confocal images were taken with 25x and 63x oil immersion objectives and collected in Z-stacks taken at 1-3 µm thick. Images were assembled with Adobe Photoshop and modified only to match exposure levels for comparisons.

Results

α7 integrin localization in the primary olfactory system

The olfactory bulbs appear the same size and shape in all three genotypes, and therefore, the deletion of α7 integrin does not cause major anatomical defects. To determine the overall distribution of α7 integrin expression in the olfactory system, we examined the β-gal distribution in sections of olfactory bulbs and nasal mucosa. No β-gal reaction product was detected in the wildtype (α7+/+) sections (Fig. 1A). Both α7$^{lacZ/+}$ (Fig. 1B) and α7$^{lacZ/lacZ}$ (Fig. 1C) sections contained intense blue staining in the olfactory nerve layer (ONL) and nasal lamina propria (LP), areas known to contain OECs. The olfactory nerve expresses α7 integrin (Fig. 1D-E, cranial nerve I, arrows) as it courses from the lamina propria through the cribiform plate of the ethmoid bone to join the ONL of the olfactory bulb. In the glomerular layer OECs are restricted to areas around the glomeruli, as only ORN axons enter the glomerular structures (Au et al., 2002). β-gal precipitate also associated with blood vessels as α7 integrin is detected on vascular smooth muscle cells (Yao et al., 1997). In short, blue reaction product is mainly confined to the anatomical areas of the olfactory system that are reported to contain OECs (Au et al., 2002, Doucette, 1990).
**α7/β-gal expression is found in OECs**

Next we used double immunofluorescence to determine if the α7/β-gal product colocalized with different OEC markers. Wildtype mice only had scattered β-gal immunofluorescence in the glomerular layer that did not colocalize with the OEC nuclear marker, SOX10 (Fig. 2A-C). In contrast, α7lacZ/lacZ olfactory bulbs showed overlapping expression of β-gal and SOX10 immunoreactivity in the ONL (Fig. 2D-F). Two additional OEC markers, S100 (Fig. 2G-I) and Aquaporin 1 (AQP1; Fig. 2J-L; Shields et al., 2010), also colocalized with α7 integrin in the olfactory nerve layer. Figs. 2H and 2K both show that OEC processes cover the olfactory nerve and enter the glomerular layer (arrowheads), but do not enter the glomeruli, and thus exhibit the typical pattern of OECs.

We also examined the cellular expression pattern of α7/β-gal within the olfactory nerve (Fig. 3). SOX10 identifies the OEC nuclei present in the α7lacZ/lacZ mice (Fig. 3B, C), and β-gal reactivity outlines the cell bodies of OECs (Fig. 3A, 3B). The thin OEC processes cover the olfactory nerve are lightly β-gal immunoreactive (Fig. 3A).

**Mature olfactory receptor neurons do not express α7 integrin**

To exclude the possibility the α7 integrin is expressed on the axons of the ORNs, we asked if β-gal would colocalize with olfactory marker protein (OMP). OMP is highly expressed by mature ORNs in the olfactory epithelium, their axons in the ONL, and in the glomeruli where they synapse. In α7lacZ/lacZ olfactory bulbs there was a close association between β-gal and OMP-labelled processes but the patterns were distinctly different. Both β-gal and OMP fluorescence are found in the ONL, but only OMP enters the glomeruli (Fig. 4A-C). Cross sections of the olfactory epithelium show OMP expression in cell bodies of ORNs (Fig. 4E, 4F), and in the lamina propria. Fascicles are surrounded by α7/β-gal expression and contain OMP-positive nerve bundles (Fig. 4D-F, arrows; Choi et al., 2008).
These results demonstrate that α7 integrin is restricted to the glial compartment, and is not expressed by the axons of mature ORNs.

**β-dystroglycan is found in the primary olfactory system**

In both muscle tissue and peripheral glia, the α7 integrin and the dystroglycan (DG) complex are co-receptors that bind to the laminin in basement membranes (Hultgårdh-Nilsson and Durbeej, 2007, Rooney et al., 2006, Previtali et al., 2003a). In their microarray study Roet et al. (2010) reported that OECs express the mRNA for dystroglycan. We therefore asked if α7/β-gal and β-dystroglycan (β-DG) colocalized in the olfactory bulb. The ONL contained large circular structures that expressed high levels of β-DG compared with the more extensive α7 integrin expression throughout the ONL (Fig. 5A-C). The β-DG appeared limited to the outer layer of the olfactory nerve fascicles as they enter the ONL. Surprisingly, some OEC cell bodies were directly adjacent to the β-DG expression (Fig. 5A-C), but were not clearly colocalized.

In contrast to the olfactory bulb, the nasal mucosa contained more extensive β-DG expression including light reactivity in the olfactory epithelium and strong labelling of the axon bundles in the lamina propria (Fig. 5D-F). Vascular smooth muscle cells are reported to express both α7 integrin and DG (Hultgårdh-Nilsson and Durbeej, 2007) and are present on blood vessels (insets, Fig. 5D-F). Mice with the α7lacZ reporter show DG spanning the outer membrane, whereas the α7/β-gal appears cytosolic. Fasciculated axon bundles are strongly for β-DG-positive along the outer borders, which are composed of OECs (Fig. 5G-I, arrowheads).

**α7 integrin mediates OEC migration on laminin substrate**

To determine if α7 integrin functions in OEC motility, we cultured OECs from the olfactory bulbs derived from all three genotypes (Fig. 6A-C). After immunopurification the secondary cultures contained approximately 86 ± 9% p75-labeled OECs (Fig. 6D-F), and
then were plated on transwell inserts to evaluate any differences in migration. Once the inserts were stained, we observed many OECs with extended membrane protrusions, in which individual cells seemed to contact other OECs (Fig. 7, arrowheads). The OECs observed on PLL inserts had similar morphologies as those seen in Fig. 7.

To quantify the stimulatory effects of laminin on OECs we first counted all the nuclei present on inserts coated with PLL or PLL plus laminin. Because OECs do migrate on the PLL substrate, we normalized the results of laminin stimulation by the baseline migration quantified on PLL. Data are expressed as a “migratory potential ratio”: within each genotype, the number of nuclei present on laminin inserts is divided by the number on PLL-only inserts. A migratory potential ratio of 1 shows that OECs have no preference for the laminin substrate versus PLL, while numbers larger than 1 indicate a substrate preference and increased migration. On average twice as many wildtype OECs migrated on laminin than on the PLL substrate (2.14 ± 0.76), α7lacZ/+ OECs showed some preference for laminin (1.45 ± 0.23), while α7lacZ/lacZ OECs had no preference (0.92 ± 0.19). Significantly more wildtype OECs migrated on laminin than α7lacZ/lacZ OECs (p<0.01, Fig. 8). There were no differences between wildtype and α7lacZ/+ OECs or α7lacZ/+ OECs and α7lacZ/lacZ OECs. From this data we suggest that laminin is a migratory stimulant for OECs and that α7 integrin is, in part, responsible for mediating this effect.

Discussion

Using mice that express β-gal in lieu of α7, we showed that α7/β-gal colocalized with established OEC markers (SOX10, S100, and AQP1), but not with OMP, a marker for mature olfactory receptor neurons. These results demonstrate that OECs express the α7 integrin, a laminin receptor. Additionally, we tested if α7 integrin affects OEC motility on laminin. Interestingly, the migratory potential of wildtype OECs was twice that of α7lacZ/lacZ OECs. From these findings we conclude that α7 integrin mediates laminin-induced migration of adult OECs. Because there is a baseline level of migration for all genotypes
tested on PLL alone, there must be additional, parallel migratory mechanisms that do not involve laminin. This is consistent with the literature, in which several neurotrophic factors are reported to stimulate migration in OECs and related cell types (Cao et al., 2007, Windus et al., 2007).

OECs are closely related to Schwann cells. Both types of glial are derived from neural crest populations (Barraud et al., 2010, Armati and Mathey, 2013), share numerous surface and intracellular markers, and are implicated in nerve regeneration (Werner et al., 2000). The presence of α7 integrin on Schwann cells is well established (Previtali et al., 2003a, Chernousov et al., 2007), but the present study is the first to confirm α7 integrin expression on OECs. In addition to α7 integrin expression on Schwann cells and OECs, laminin, is reported to stimulate migration in both cell types in vitro (Tisay and Key, 1999, Yamauchi et al., 2004). The motility of OECs during migration is characterized by “lamellipodial waves” (Windus et al., 2007). These lamellipodial waves are protrusions of the plasma membrane of OEC processes that seem to direct cell-to-cell interactions and migration. Windus et al. (2007) reported that glial cell line-derived neurotrophic factor (GDNF) greatly enhances the activity of the OEC waves, while selective inhibitors JNK and SRC kinases decrease the formation of lamellipodial waves. SRC is a downstream target of integrin cascades that involve the phosphorylation of the focal adhesion kinase (FAK, Das et al., 2013). Immunoprecipitation experiments with α7 integrin detected FAK and the integrin adaptor molecule talin in Schwann cells (Chernousov et al., 2007). Thus, α7 integrin activation by laminin could presumably activate the FAK-SRC pathway to alter lamellipodia development and migration in OECs. The FAK-SRC pathway is implicated in both the early formation of adhesion as well as the destabilization of focal adhesions for continued migration (Das et al., 2013).

Recently, Windus et al. (2010) reported functionally heterogeneous subtypes of OECs, and their differences appeared to be regulated by their lamellipodial waves and subsequent cell-cell interactions. Specifically, the peripheral OECs from the olfactory...
mucosa used their lamellipodia to adhere to and form cell-cell contacts, while central OECs did not adhere until they matured and never to the extent seen with peripheral OECs. Differential expression of DG in the olfactory system, with higher concentrations on peripheral OECs and lower levels on central OECs, may reflect different OEC functions as is seen with Schwann cell subtypes (Chernousov et al., 2007).

Mammalian CNS neurons are known to have a low intrinsic ability to undergo regeneration following injury while neurons in the PNS are able to regenerate to a greater degree. Differences in the regenerative capacity of peripheral and central neurons likely reside in the properties of the glia that surround the injured neurons. In the PNS, Schwann cells express α7 integrin (Previtali et al., 2003a, Chernousov et al., 2007), and after trauma, both α7 integrin and laminin expression increase in the injury site. In contrast, CNS injuries do not induce increases in α7 integrin expression or its ligand, and regeneration is limited here (Werner et al., 2000, Liesi, 1985). One promising CNS therapy for spinal cord injury involves the transplantation of OECs, as they improve functional recovery in various injury and animal models (Ramón-Cueto et al., 2000, Takeoka et al., 2011, Ziegler et al., 2011, Granger et al., 2012). How OECs induce axon regeneration is not yet understood, but secreted neurotrophins and cell adhesion molecules are both currently implicated. Based on these findings that OECs express α7 integrin, future experiments will determine if α7 integrin is a mediator of the cellular processes controlling OEC-based neurite regeneration.
### Table 1: Primary Antisera Used

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Immunogen</th>
<th>Source; Catalogue #</th>
<th>Species</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>B-galactosidase (B-gal)</td>
<td>B-gal from E. coli.</td>
<td>MP Biomedicals (Solon, OH); 55976</td>
<td>Rabbit</td>
<td>1:10,000</td>
</tr>
<tr>
<td>SOX10</td>
<td>E. coli-derived recombinant human SOX10</td>
<td>R&amp;D Systems (Minneapolis, MN); AF2864</td>
<td>Goat</td>
<td>1:75</td>
</tr>
<tr>
<td>S100</td>
<td>S100 from cow brain</td>
<td>Dako A/S (Glostrup, Denmark); Z0311</td>
<td>Rabbit</td>
<td>1:30,000</td>
</tr>
<tr>
<td>Aquaporin 1 (AQP1)</td>
<td>19aa synthetic peptide (aa 251-269) from C-terminus</td>
<td>Chemicon (Temecula, CA); AB3065</td>
<td>Rabbit</td>
<td>1:5,000</td>
</tr>
<tr>
<td>Olfactory marker protein (OMP)</td>
<td>Rodent OMP</td>
<td>Wako (Richmond, VA); 544-10001</td>
<td>Goat</td>
<td>1:5,000</td>
</tr>
<tr>
<td>Platelet endothelial cell adhesion molecule (PECAM-1)</td>
<td>Rat PECAM</td>
<td>BD Pharmingen (San Jose, CA); 550300</td>
<td>Mouse IgG</td>
<td>1:75</td>
</tr>
<tr>
<td>B-dystroglycan (B-DG)</td>
<td>15aa synthetic peptide from C-terminus of human B-DG</td>
<td>Developmental Studies Hybridoma Bank; MANDAG2 clone 7D11</td>
<td>Mouse IgG</td>
<td>1:750</td>
</tr>
<tr>
<td>p75 nerve growth factor receptor (p75)</td>
<td>Extracellular fragment from third exon in mouse p75 (aa 43-161)</td>
<td>Chemicon (Millipore, Billerica, MA); AB1554</td>
<td>Rabbit</td>
<td>Immunopurification: 1:1,500-2,000 IHC: 1:5,000</td>
</tr>
</tbody>
</table>
Figure 1: β-gal reaction product is found in OEC-rich regions of the olfactory system in α7lacZ/+ and α7lacZ/lacZ mice.

Areas with high α7 integrin expression contain β-gal reaction product after the X-gal histochemical experiments. A: Wildtype mice have no blue reaction product. B, C: Mice with one (B) or two copies (C) of α7lacZ have intense blue reaction product that is localized to the olfactory nerve layer (ONL) in the olfactory bulb and the lamina propria (LP) in the nasal mucosa, areas populated with OECs. Blue precipitate is also detected in blood vessels. D, E: Black arrows in the enlargements point to the olfactory nerve (cranial nerve I) as it courses through the cribriform plate (c) into the olfactory bulb. F: Horizontal section of α7lacZ/lacZ olfactory mucosa illustrates that reaction product is intense in the LP (arrows) compared to the olfactory epithelium (OE). Scale A-C: 200 μm; D-E: 50 μm; F: 100 μm
**Figure 2:** In the olfactory bulb α7 integrin colocalizes with known OEC markers, SOX10, S100, and AQP1.

A-C: No specific labeling for α7/β-gal (green) is detected in the olfactory nerve layer (ONL) of wildtype olfactory bulbs. SOX10 (red) identifies OEC nuclei concentrated in the ONL. D-F: Immunofluorescence depicts strong overlap of α7/β-gal (green) with SOX10 (red nuclei) in α7lacZ/lacZ olfactory bulbs. Arrows indicate cells that express both α7/β-gal and SOX10. G-I: Both the glial marker S100 (red) and α7/β-gal (green) appear colocalized in α7lacZ/lacZ ONL. OEC processes extend into the glomerular layer (arrowheads), but do not enter the glomeruli (GL). J-L: The water channel AQP1 (red) is highly expressed in the ONL. AQP1 and α7/β-gal fluorescence (green, arrowheads) are expressed in the GL, but not within the glomeruli. Scale A-L: 50 µm.
Figure 3: OECs in the olfactory nerve co-express α7/β-gal and SOX10.

A-C: A confocal image (4.0 µm-thick Z-stack) reveals individual cells within the olfactory nerve. α7/β-gal immunofluorescence (green) is seen at high levels within the OEC cell bodies (arrows) and at low levels in their thin processes. SOX10 (red) marks OEC nuclei. All SOX10-positive cells co-localize with β-gal. Scale A-C: 20 µm.
Figure 4: α7/β-gal expression is distinct from, but closely associated with, the olfactory receptor neurons.

A-C: An image of an α7lacZ/lacZ olfactory bulb shows that α7/β-gal (green) and olfactory marker protein (OMP, red) are expressed in different patterns within the ONL. OMP immunoreactivity fills the interior of the glomeruli (GL), while β-gal expression is excluded. D-F: Fascicles of the olfactory nerve (arrows) within the lamina propria (LP) contain OMP-positive (red) axon bundles extending from ORN cell bodies in the olfactory epithelium (OE). β-gal fluorescence (green) wraps around the OMP-positive (red) axon bundles, typical interaction between OECs and ORN axons. Scale A-C: 50 µm; D-F: 20 µm
Figure 5: β-Dystroglycan expression in the primary olfactory system.

A-C: Immunofluorescence of β-Dystroglycan (β-DG, green) in the olfactory bulb is concentrated on the perimeters of large fascicles in the ONL, whereas α7/β-gal (red) is distributed broadly in the ONL. α7/β-gal clearly labels OEC cell bodies (arrows) that appear distinct from the β-DG immunoreactivity. D-F: β-DG reactivity is extensive within the nasal mucosa. Light β-DG is seen in the α7lacZ/lacZ olfactory epithelium (OE) and strong labeling in the LP surrounding the olfactory nerve fascicles. The α7/β-gal signal (red, arrowheads) is associated with the fascicles in the lamina propria (LP). Insert: Cross section of a blood vessel surrounded by vascular smooth muscle cells that express β-DG (green) and β-gal. Colocalization is limited due to the restriction of the immunogens to different cellular compartments. G-I: Enlargements of olfactory nerve fascicles in D-F show that the outer perimeter of the fascicles are double-labelled with β-DG and α7/β-gal (yellow, arrowheads). Fascicle cross sections illustrates typical OEC pattern of wrapping around bundles of unlabelled axons. Scale A-F: 20 µm; D-F inset: 20 µm; G-I: 10 µm.
Figure 6: Immunopurification with p75-NGFR yields purified secondary OEC cultures. A-C: Primary dissociated cultures from wildtype (A), α7lacZ/+ (B), and α7lacZ/lacZ olfactory bulbs (C) show mixed cell types including p75 (green) and SOX10- (red) labeled OECs and other unidentified cells (blue nuclei, arrows). D-F: After immunopurification secondary cultures primarily contain p75 and SOX10 labeled OECs. Scale A-F: 50 µm.
Figure 7: OECs identified with p75-nerve growth factor (NGFR) after migrating through transwell inserts.

A-C: OECs purified with rabbit anti-p75-NGFR from all three genotypes migrate on transwell inserts coated with laminin. D-F: Enlargements of OECs in A-C illustrate OECs with numerous membrane protrusions (arrowheads) present as they come into close proximity to other OECs. Scale A-C: 50 µm; D-F: 50 µm.
Figure 8: $\alpha_7$ integrin mediates OEC migration on a laminin substrate. Cells were fixed 48 hours after plating on transwell inserts. Migration was assessed in each genotype by counting the number of cells that migrated through the insert on laminin and dividing by the number of cells that migrated on PLL-coated inserts (termed migratory potential ratio). Wildtype OECs ($\alpha_7^{+/+}$) migrated with a ratio of $2.14 \pm 0.76$, the ratio of OECs from $\alpha_7^{lacZ/+}$ mice was $1.45 \pm 0.23$, and $\alpha_7^{lacZ/lacZ}$ OECs did not prefer to migrate on laminin compared to PLL ($0.92 \pm 0.19$). The migratory potential ratio of $\alpha_7^{+/+}$ OECs was significantly greater than that of $\alpha_7^{lacZ/lacZ}$ OECs (**$p<0.01$). OECs from $\alpha_7^{lacZ/+}$ mice did not differ from either $\alpha_7^{+/+}$ or $\alpha_7^{lacZ/lacZ}$ OECs.
References


Doucette, R (1990) Glial influences on axonal growth in the primary olfactory system. Glia
3: 433-49.


