BRAIN LIPID BINDING PROTEIN EXPRESSION IN LAMINA-PROPRIA OLFATORY ENSHEATHING CELLS IS REGULATED BY DELTA/NOTCH-LIKE EPIDERMAL GROWTH FACTOR-RELATED RECEPTOR

by

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The olfactory system exhibits remarkable regenerative ability in its neuronal population. The success of continuous neurogenesis is thought to be due, at least in part, to its unique glia – olfactory ensheathing cells (OECs). OECs bear characteristics of both peripheral and central glia, and serve to ensheath, guide and promote growth of olfactory receptor neurons (ORNs) throughout both development and adult life. Brain lipid binding protein (BLBP) is most highly expressed by radial glia during embryonic development. It is largely down-regulated in the adult CNS, but BLBP expression is retained in the adult by special subpopulations of glia, including OECs. BLBP expression is induced in radial glia via Notch signaling, but it is not known if these same mechanisms regulate BLBP expression in the adult CNS. Axonal-glial signaling is a dynamic process whereby closely apposed neuronal and glial cells regulate the growth, maintenance and plasticity of one another through direct cell-cell signaling. Delta/Notch-like EGF-related receptor (DNER) is a transmembrane protein expressed by Purkinje cells which has been implicated in the regulation of BLBP in Bergmann glia during cerebellum development through Notch1 deltex-dependent non-canonical signaling. We have found that DNER is expressed in more mature ORNs, and other exclusive subpopulations of cells within the CNS. OECs in close apposition with DNER-expressing ORNs in vivo appear to maintain the highest BLBP expression found in the nervous system through development and adulthood. Immunofluorescence shows that this close relationship between BLBP expressing cells and DNER expressing cells also
appears to be retained in specialized areas such as the hippocampus, retina and spinal cord, throughout mouse CNS development as well as in the mature system. Removing DNER or axonal input in vivo decreases the robustness of OEC BLBP expression, and the number of cells in OEC culture expressing BLBP decreases rapidly with time. OEC co-culture with a DNER expressing monolayer increases the number of OECs in vitro which express BLBP, providing evidence for the regulation of BLBP expression in OECs by DNER expression in apposing ORNs.
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<tbody>
<tr>
<td>A-type</td>
<td>Astrocyte-type</td>
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<tr>
<td>Ax</td>
<td>Axon bundle</td>
</tr>
<tr>
<td>B-DG</td>
<td>β-Dystroglycan</td>
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<tr>
<td>BET</td>
<td>Brain specific EGF-like transmembrane protein</td>
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<tr>
<td>bFABP</td>
<td>Brain fatty acid binding protein</td>
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<tr>
<td>BLBP</td>
<td>Brain lipid binding protein</td>
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<tr>
<td>BX</td>
<td>Bulbectomized</td>
</tr>
<tr>
<td>CA1</td>
<td>Cornu Ammonis 1</td>
</tr>
<tr>
<td>CA3</td>
<td>Cornu Ammonis 3</td>
</tr>
<tr>
<td>CBF-1</td>
<td>C-promotor binding factor-1</td>
</tr>
<tr>
<td>CMZ</td>
<td>Ciliary marginal zone</td>
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<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CSL</td>
<td>CBF1, Su(H) and Lag 1</td>
</tr>
<tr>
<td>D</td>
<td>Dorsal</td>
</tr>
<tr>
<td>DAPI</td>
<td>4'-6-Diamidine-2'-phenulindole dihyrochloride</td>
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<tr>
<td>DCX</td>
<td>Doublecortin</td>
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<tr>
<td>DG</td>
<td>Dentate Gyrus</td>
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<tr>
<td>DIV</td>
<td>Day in vitro</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle medium</td>
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<tr>
<td>DNER, DNER-/-</td>
<td>Delta/Notch-like EGF-related receptor</td>
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<tr>
<td>E</td>
<td>Embryonic day</td>
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<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
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<tr>
<td>FABP</td>
<td>Fatty acid binding protein</td>
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<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<td>G</td>
<td>Glomerular layer</td>
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<td>GAP-43</td>
<td>Growth associated protein-43</td>
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<td>Glial fibrillary acidic protein</td>
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<td>Glutamate transporter protein-1</td>
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<td>GM</td>
<td>Grey matter</td>
</tr>
<tr>
<td>H</td>
<td>Hilus</td>
</tr>
<tr>
<td>INL</td>
<td>Inner nuclear layer</td>
</tr>
<tr>
<td>IPL</td>
<td>Inner plexiform layer</td>
</tr>
<tr>
<td>L</td>
<td>Lateral</td>
</tr>
<tr>
<td>LP</td>
<td>Lamina propria</td>
</tr>
<tr>
<td>LP-OEC</td>
<td>Lamina propria-olfactory ensheathing cell</td>
</tr>
<tr>
<td>LV</td>
<td>Lateral ventricle</td>
</tr>
<tr>
<td>M</td>
<td>Medial</td>
</tr>
<tr>
<td>MDV</td>
<td>Minimum essential medium eagle</td>
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<tr>
<td>ML</td>
<td>Mitral cell layer</td>
</tr>
<tr>
<td>ML</td>
<td>Molecular layer</td>
</tr>
<tr>
<td>MMP2</td>
<td>Matrix-metalloproteinase 2</td>
</tr>
<tr>
<td>NBX</td>
<td>Un-bulbectomized</td>
</tr>
<tr>
<td>NFL</td>
<td>Nerve fiber layer</td>
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<tr>
<td>NG2</td>
<td>Neuron glia antigen 2</td>
</tr>
<tr>
<td>NP</td>
<td>Nasal Pit</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>NST</td>
<td>Neuron specific tubulin/β-III tubulin/Tuj-1</td>
</tr>
<tr>
<td>O4</td>
<td>Oligodendrocyte marker 4</td>
</tr>
<tr>
<td>OB</td>
<td>Olfactory bulb</td>
</tr>
<tr>
<td>OB-OEC</td>
<td>Olfactory bulb-olfactory ensheathing cell</td>
</tr>
<tr>
<td>OE</td>
<td>Olfactory epithelium</td>
</tr>
<tr>
<td>OEC</td>
<td>Olfactory ensheathing cell</td>
</tr>
<tr>
<td>OMP</td>
<td>Olfactory marker protein</td>
</tr>
<tr>
<td>ON</td>
<td>Optic nerve</td>
</tr>
<tr>
<td>ONL</td>
<td>Olfactory nerve layer</td>
</tr>
<tr>
<td>ONL</td>
<td>Outer nuclear layer</td>
</tr>
<tr>
<td>ORN</td>
<td>Olfactory receptor neuron</td>
</tr>
<tr>
<td>OS</td>
<td>Optic stalk</td>
</tr>
<tr>
<td>P</td>
<td>Postnatal day</td>
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<tr>
<td>p75NTR</td>
<td>Low affinity nerve growth factor receptor</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PC</td>
<td>Purkinje cell layer</td>
</tr>
<tr>
<td>PenStrep</td>
<td>Penicillin-Streptomycin</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
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<tr>
<td>PNS</td>
<td>Peripheral nervous system</td>
</tr>
<tr>
<td>PSA-NCAM</td>
<td>Polysialic acid-Neural cell adhesion molecule</td>
</tr>
<tr>
<td>RMS</td>
<td>Rostral migratory stream</td>
</tr>
<tr>
<td>SCI</td>
<td>Spinal cord injury</td>
</tr>
<tr>
<td>S-type</td>
<td>Schwann cell-type</td>
</tr>
<tr>
<td>Su(H)</td>
<td>Suppressor of Hairless</td>
</tr>
<tr>
<td>SVZ</td>
<td>Subventricular zone</td>
</tr>
<tr>
<td>V</td>
<td>Ventricle</td>
</tr>
<tr>
<td>V</td>
<td>Ventral</td>
</tr>
<tr>
<td>VZ</td>
<td>Ventricular zone</td>
</tr>
<tr>
<td>WM</td>
<td>White matter</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type littermate control</td>
</tr>
<tr>
<td>β-DG</td>
<td>β-Dystroglycan</td>
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</table>
ACKNOWLEDGEMENTS

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CHAPTER 1 : INTRODUCTION

1.1 The olfactory system

The primary olfactory neuraxis consists of both a peripheral and central nervous system component. The peripheral component is made up of the olfactory epithelium (OE) and the lamina propria (LP) which is separated from the central component, the olfactory bulb (OB), by the cribriform plate. The single neuronal subtype of the peripheral olfactory system, olfactory receptor neurons (ORNs) are bipolar neurons which span the primary neuraxis across the central-to-peripheral nervous system boundary. ORN cell bodies reside within the OE and their dendrites extend laterally into the nasal cavity; the axons of ORNs project through the lamina propria and across the cribriform plate where they synapse with their central nervous system target within the olfactory bulb (figure 1.1) (Farbman, 1992).

The pseudostratified columnar epithelium of the OE proper consists of relatively few cell types which support the developing, regenerating, and mature stages of the ORNs (Graziadei and Graziadei, 1979a, Graziadei and Graziadei, 1979b, Moulton, 1974). The mature OE can be roughly divided into three compartments corresponding to a hierarchy in ORN development; the apical, middle, and basal compartments (Calof et al., 1998). The cell bodies of the supporting sustentacular cells constitute the most apical layer; while the most primitive cells, the horizontal and basal putative stem cells, reside closest to the basal lamina; the cell bodies of the ORNs are situated in the middle compartment.
Figure 1.1 Basic diagram of the olfactory system
Horizontal and globose basal cells are the olfactory progenitors which sit close to the basal lamina of the olfactory epithelium in the peripheral olfactory system. These give rise to the regenerating cell types in the olfactory system. Olfactory receptor neurons have their cell bodies within the olfactory epithelium, supported by sustentacular cells. Olfactory ensheathing cells (OECs) begin ensheathing ORNs as they exit the basal lamina (LP-OECs) until they reach the nerve fiber layer of the olfactory bulb in the CNS (OB-OECs). Here the ORNs defasciculate and contact their appropriate targets. Figure adapted from (Barnett and Chang, 2004).
Each of these cell types are easily defined by position, morphology and antigenic profile (Schwob, 2002).

Olfactory receptor neurons develop from horizontal and globose basal cells, which are in direct contact with the basal lamina of the epithelium; these cells also serve as bipotential progenitors in the adult system (Graziadei and Graziadei, 1979a, Graziadei and Graziadei, 1979b, Carter, MacDonald and Roskams, 2004, Murdoch and Roskams, 2007). Immature ORN cell bodies lie closest to the basal lamina, with the more mature ORN cell bodies situated more apically. Nascent ORNs extend their dendrites apically through the supporting sustentacular cell body layer into the nasal cavity where ORN dendrites can interact with odorant molecules. The unmyelinated axons of ORNs fasciculate into discrete axon bundles in the lamina propria, and then project through the cribriform plate where ORN axons synapse with their target mitral/tufted cells in the glomeruli of the olfactory bulb (Farbman, 1992). Immature ORNs are commonly marked by immunohistochemistry by GAP43 expression, a membrane bound protein (Spencer et al., 1992); while mature ORNs are marked by OMP, olfactory marker protein, a cytoplasmic protein which is expressed only in ORNs and not progenitors, around the time that the ORNs make contact with their synapses (Margolis et al., 1991).

The lamina propria (LP) just medial to the epithelium contains several cell types surrounding ORN axon bundles. A variety of connective tissue cell types such as macrophages and leukocytes as well as fibroblasts reside within the LP and provide structural and immune support to the overlying OE. The LP also
supports its neighboring OE by housing mucous secreting Bowman’s glands, and a
large network of blood vessels both of which are necessary for ongoing support as
well as quick response to environmental insult (Farbman, 1992). Olfactory
ensheathing cells (OECs) are responsible for secretion of extracellular matrix and
the basal lamina of the LP (Gong and Shipley, 1996). The designation ‘OE’ often
refers collectively to the entire peripheral component of the olfactory system: the
olfactory epithelium proper, as well as the lamina propria.

OECs are a dynamic glial subtype which reside in the lamina propria of the
peripheral nervous system as well as the lateral boundary of the olfactory bulb in
the CNS where they form part of the glial limitans (Doucette, 1984, Doucette,
1993). From the point at which the ORN axons enter the basal lamina, until they
defasciculate from the inner nerve layer of the olfactory bulb just central to the
cribiform plate, OECs maintain constant direct physical contact with ORN axons.
Each OEC ensheathes 50-100 unmyelinated axons (Field, Li and Raisman, 2003).
OECs support ORN axonal growth and regeneration by continuously providing
target guidance, physical and trophic support, as well as secretion of growth-
permissive extracellular matrix (Ramon-Cueto and Avila, 1998). The specific role
of OECs varies with anatomical location as they support ORN axons through three
distinct morphological domains of the olfactory neuraxis. The first domain begins at
the interfacing of the OE and LP where OECs support ORN neurite outgrowth into
the LP. OECs and ORNs then continue through the connective tissue of the
extracranial and then intracranial regions where OECs support ORN axon growth
and fasciculation into bundles. OECs and ORNs continue into the CNS up to the
nerve fiber layer which is the area between the cribriform plate and the olfactory bulb where OECs support ORN axon defasciculation and targeting onto mitral/tufted cells within the olfactory bulb (Whitesides and LaMantia, 1996).

The peripheral olfactory system develops from a placode on the outside of the body which invaginates to form a pit during embryonic day 9 of development. Nascent ORNs, ensheathed by OECs, migrate through the mesenchyme towards the developing olfactory bulb (Baker and Bronner-Fraser, 2001). ORN regeneration begins in the periphery of the olfactory system and is continuous in adulthood.

OECs have been credited with the ongoing plasticity and regeneration that is rare in the overall mature CNS, but robust and ongoing in the mature olfactory system. Constant regeneration is a requirement for the olfactory system, as it is subject to the damage and stress of direct physical contact with the external environment within the nasal cavity. The typical ORN lifespan in the adult rat was once thought to range from 4-6 weeks but this is directly modulated by the ‘dirtiness’ of the olfactory environment. Higher ORN turnover is associated with ‘dirty’ or odorant-rich environments (Farbman, 1992, Murray and Calof, 1999, Hinds, Hinds and McNelly, 1984). However, ORN lifespan is more variable than once thought, and ORNs can also display significant longevity sometimes persisting for up to one year (Hinds, Hinds and McNelly, 1984). This regulated regeneration provides potential insight into adult neural regeneration which is a process lacking in most other regions of the CNS, particularly after injury or degeneration due to disease.
The olfactory bulb is an area which, like its peripheral companion, is continually replenished with new neurons. The olfactory bulb develops from the embryonic telencephalon, and is populated in a secondary wave of developmental neurogenesis which slows into a continuous rate of regeneration of granular and periglomerular interneurons in the adult. Neurogenesis initiates in the subventricular zone along the side of the lateral ventricle where progenitors reside. The resident neural stem cells, named B-cells, show astrocyte like characteristics and surround nascent neuroblasts while maintaining close contact to the ependymal cells at the edge of the ventricle (Seri et al., 2006). Neuroblasts then migrate out of the SVZ and into the rostral migratory stream (RMS); neuroblasts continue to migrate along one another in a process called ‘chain migration’ in a large stream of cells clearly visible during postnatal development; at this stage the RMS is bounded by astrocytes on either side of thoroughfare. In the adult, this stream of cells narrows considerably and becomes more intimately ensheathed by astrocytes (Seri et al., 2006, Ming and Song, 2005). Once they reach the olfactory bulb, the neuroblasts migrate tangentially into the bulb and differentiate into periglomerular or granule neurons (Ming and Song, 2005).

1.2 Olfactory ensheathing cells: Identity, heterogeneity and phenotype shifts

As described above, OECs play a very important role within both the developing and the mature olfactory system. They provide a glial environment which allows the stable support of mature functional ORNs while simultaneously dynamically assisting ORN regeneration, targeting and controlled death. OECs are
also the only glial cell type known to cross the PNS:CNS boundary and are therefore considered to be a very plastic glial subtype.

Because they span both the PNS and CNS, OECs resemble glial types from both systems, particularly Schwann cells and astrocytes. They were originally named olfactory nerve Schwann cells due to their morphology in vivo (Gasser, 1956, De Lorenzo, 1957). However all three cell types come from developmentally exclusive origins: OECs arise from the olfactory placode (Chuah and Au, 1991) with Schwann cells arising from the neural crest (Farbman and Squinto, 1985) and astrocytes arising from radial glia of neuroepithelial origin (Kriegstein and Gotz, 2003). Though closely related to both cell types, OECs exhibit a morphology, behavior, and transcriptional profile most closely related to Schwann cells (Wewetzer et al., 2002, Vincent et al., 2005) (figure 1.2).

Several lines of evidence clearly set OECs apart from Schwann cells as a distinct glial cell type. First, Schwann cells and OECs associate with different numbers of axons; unmyelinating Schwann cells associate with 1-10 axons, whereas OECs associate with 50-100 unmyelinated axons respectively (Doucette, 1984, Jessen and Mirsky, 2005). OECs also have distinctly different behaviors in vitro, and in vivo after transplantation with regards to their cell-cell interactions. Most notably OECs freely interact with astrocytes and do not cause astrocytic hypertrophy as Schwann cells do (Barnett and Riddell, 2004, Lakatos, Franklin and Barnett, 2000) allowing for better OEC integration and remodeling of the injury site that is crucial for repair after injury. Dye loading shows that Schwann cells are
Figure 1.2 OECs resemble both astrocytes and Schwann cells *in vitro*, but transcriptional profiling reveals OECs are a distinct cell population

(A-C) OEC morphology as probed with commonly used OEC markers (A) p75NTR, (B) S100β, and (C) GFAP. Within the same culture OECs show a heterogeneity in morphology: the arrow points to an S-type, or Schwann cell like OEC. The arrowhead points to an A-type, or astrocyte-like OEC. Scale bar is 50 microns in each picture. (Au and Roskams, 2003). (D-E) Venn diagrams from transcriptional profiling of OECs, astrocytes, and Schwann cells respectively. (D) shows transcripts which are enriched by >1.5 fold whereas (B) indicates transcripts which are depleted >1.5 relative to a reference RNA pool of fibroblast and neonatal rat brain RNA. These diagrams indicate that OECs and Schwann cells are more closely related to each other than astrocytes, but are still distinguishable from both cell types by a modest number of unique transcripts. (Richter and Roskams, 2008).
extensively coupled through gap junctions; OECs rarely couple with more than one other cell (Barnett and Riddell, 2004). Furthermore, an OEC can be specifically defined by EM as a large cell with electron dense cytoplasm, an irregular nucleus and scattered intermediate filaments (Field, Li and Raisman, 2003).

In the adult system, observations of OEC cell morphology in vivo lead to the first indications as to their role in maintenance and regeneration. In the LP, where OECs are in continuous contact with the basal lamina, they are large cells with spindly processes enveloping ORN axons. When OECs reach the olfactory nerve layer (ONL) of the olfactory bulb, they divide the first cranial nerve into many small branches as the ORN axons defasciculate and contact their target cells in the olfactory bulb (De Lorenzo, 1957, Barber and Lindsay, 1982). In vitro, OECs were first broadly described as dichotomous ‘spindly’ versus ‘flat’ cells (Barber and Lindsay, 1982); this description was later refined to ‘S-type’, referring to a spindly Schwann-cell like morphology; and ‘A-type’ referring to a flattened stellate morphology similar to astrocytes (Barnett, Hutchins and Noble, 1993) (figure 1.2 A-C).

This S versus A-type description also corresponds with a differential antigenic profile both in vitro and in vivo based on anatomical location or specific cell context. While there has been some discussion as to whether or not these heterogeneous cells were two distinct cell types, it is now generally accepted that OECs represent multiple cell populations from the same lineage (Franceschini and Barnett, 1996). This point was reinforced by evidence that OEC heterogeneity appears to be rapidly and reversibly changeable when in culture. Factors such as
laminin, cyclic AMP, endothelin, neuregulin gene products or glial growth factor 2
added or removed from the culture conditions can cause rapid change in OEC cell
morphology, expression and secretion (Pixley, 1992, Chuah and Teague, 1999,
Vincent, West and Chuah, 2003, Pollock et al., 1999, Chuah et al., 2000). This is
furthermore supported by analysis of gene-ontology in cultured versus freshly
dissociated OECs which show a significant difference in the gene expression profile
between these two groups (Franssen et al., 2008). These points of evidence
solidify that OEC identity may be variable, but only within a defined range of
parameters which can be experimentally manipulated.

Despite the work towards their characterization, an OEC specific marker has
yet to be identified and a combination of antigenic markers is typically used to
define OECs. Transcriptional profiling shows that OECs are related to both
astrocytes and Schwann cells, but are also unique, and supports previous work
which suggests that OECs most resemble embryonic pre-myelinating Schwann
cells (Wewetzer et al., 2002, Vincent et al., 2005, Franssen et al., 2008) (figure 1.2
D-E). The most established OEC markers are the low affinity nerve growth factor
receptor (p75NTR), the calcium binding cytoskeleton associated protein S100 or its
subunit S100β, and the intermediate filament glial fibrillic acid protein (GFAP)
(Barber and Lindsay, 1982, Barnett, Hutchins and Noble, 1993, Ramon-Cueto,
Perez and Nieto-Sampedro, 1993). Other commonly used markers include but are
not limited to vimentin, nestin, neural cell adhesion molecules (PSA-NCAM),
fibronectin and oligodendrocyte marker O4 (Ramon-Cueto and Avila, 1998,
Franceschini and Barnett, 1996, Pixley, 1996). A new marker for OECs has been
recently published; β-Dystroglycan is a membrane associated protein which marks
the point at which OECs interact with the basal lamina (Takatoh et al., 2008).

Being that OECs are a morphologically heterogeneous population, it is not
surprising to find that protein expression levels vary as well. In general, different
morphologies in vitro are associated with differential expression of the established
OEC markers. Flattened A-type cells often express high levels of fibrous GFAP as
well as PSA-NCAM; whereas diffuse GFAP and higher p75NTR expression are
associated with the spindly S-type OECs in vitro (Sonigra et al., 1999). Expression
of some proteins, such as p75NTR, can increase with time in culture when OECs
lose contact with ORN axons; others drop over time in culture such as the
production of matrix-metalloproteinase 2 (MMP2) (Franceschini and Barnett, 1996,
Au and Roskams, , Pastrana et al., 2006). In vivo, OEC expression patterns of
proteins such as GFAP, S100, and p75NTR show both spatiotemporal gradients
and variability from cell to cell depending on the immediate cell context (Astic,
Pellier-Monnin and Godinot, 1998). All taken, OEC protein expression is incredibly
plastic and context dependent, making these cells difficult to define.

Lamina Propria OECs (LP-OECs) are the OECs within the peripheral part of
the olfactory system. LP-OECs are of special interest because they are much more
easily accessible than Bulb derived-OECs (OB-OECs) located in the CNS; LP-OEC
cell dynamics and relative proximity to the progenitors in the olfactory system
suggests that LP-OECs may be a more primitive population of OECs more suited to
responding to injury or disturbance (Murdoch and Roskams, 2007). Though they
largely resemble their centrally derived counterparts, LP-OECs in vitro show faster
proliferation, expansion, and migration than OB-OECs and can be cultured to a later passage before showing signs of senescence; (Murdoch and Roskams, 2007, Au and Roskams, 2003, Richter and Roskams, 2008, Richter et al., 2005). In addition to these kinetics, LP-OECs tend toward higher expression of more developmentally related proteins such as vimentin, CREB-binding protein, PACAP, Notch 3 and CD44 (Au and Roskams, 2003).

1.3 OECs and central nervous system injury

Because of the role they play in situ supporting axonal regeneration, OECs have been targeted as a potential transplant therapeutic for central nervous system injury such as stroke and spinal cord injury (SCI). After CNS injury, there is not only the scar and cavity of the injury itself, but the adult environment naturally inhibits axon growth, and adult neurons are often limited in their ability to initiate growth programs (Kwon and Tetzlaff, 2001). SCI occurs in two distinct phases. Primary injury is the physical rupture and hemorrhaging which immediately severs axons; this is followed by Wallerian degeneration of damaged fibers and the considerable immune response that creates a large cystic cavity lined by a glial scar littered with growth-inhibitory myelin debris (Barnett and Riddell, 2007). It has been speculated that the biggest issue after injury is the astrocytic scar lining the cavity. Astrocytes naturally play a dual role of providing a permissive substrate for nerve fibers, but also establishing a tight basal lamina at their end-feet essentially sealing off nervous system tissue from outside insult; the latter response is crucial to maintaining nervous system integrity after injury but simultaneously produces a
barrier to regeneration (Faulkner et al., 2004). The Pathway hypothesis of repair suggests that transplanted glial cells may be able to breech this protective boundary by integrating with the existing glial scar and establishing permissive glial tracts for fiber regeneration (Raisman and Li, 2007).

OECs have been extensively investigated as a potential transplant therapeutic for this above purpose. OECs are a relatively easily accessible cell type for autotransplant, particularly LP-OECs, from which attempts to collect and culture LP-OECs from the human nasal cavity have been made (Bunge, 2002, Bianco et al., 2004). OECs have been shown to express and secrete a variety of growth factors, ECM components and signaling proteins suitable to support of axonal outgrowth (Wewetzer et al., 2002). OECs support axon outgrowth in vitro from a variety of neuronal subtypes from different areas of the CNS including the cerebellum, cortex and retina (Sonigra et al., 1999, van den Pol and Santarelli, 2003, Kafitz and Greer, 1999). Though OECs do not myelinate the small diameter axons of ORNs in situ, OEC have been shown to myelinate with peripheral myelin once transplanted into regions of focal demyelination (Franklin, 2003, Franklin and Gilson, 1996).

Despite the lack of specific information regarding OEC-axon interactions in the spinal cord and other injuries, excitement over the possibility of OECs as a therapeutic has prompted several transplantation efforts in human SCI (Feron et al., 2005, Ibrahim et al., 2006). However, at this time, well controlled clinical studies can tell us with certainty that OEC transplant into human chronic SCI injury are safe; the preliminary data as yet does not tell us much about the potential for axonal
regeneration supported by OECs in this paradigm (Mackay-Sim et al., 2008). Initial transplant experiments in rats were promising and showed that there was regeneration across the dorsal root entry zone where normally peripheral ingrowth is inhibited after injury; this was followed by evidence of functional recovery and long range fiber regeneration following OEC transplant (Ramon-Cueto and Nieto-Sampedro, 1994, Ramon-Cueto et al., 1998, Ramon-Cueto et al., 2000, Li et al., 2004). However, similar experiments from other groups have had different results; some dispute the above results, attributing them to spared fibers and not actual regeneration (Gomez et al., 2003, Ramer et al., 2004a, Riddell et al., 2004). What is known is that OEC transplantation into the spinal cord does have several positive impacts, including secretion of growth factors, enhancement of sprouting from spared fibers, sparing from secondary damage and local effects on angiogenesis (Richter et al., 2005, Williams, Franklin and Barnett, 2004, Chuah et al., 2004).

Perhaps the most important OEC affect on SCI is on the glial scar. OECs are known to associate freely with astrocytes, and it has been suggested that transplantation causes a rearrangement of astrocytes at the lesion border, making the scar boundary more permissive to regeneration and even creating channels for extension of growing axons (Lakatos, Franklin and Barnett, 2000, Richter and Roskams, 2008, Li et al., 2004, Ramer et al., 2004a, Ramer et al., 2004b).

Why is there so much variation amongst similar OEC transplantations into SCI? Three main points have been cited as the likely source of this variation (Richter and Roskams, 2008). First, the various studies have used OECs cultured from different anatomical or developmental sources; namely LP versus OB-OECs.
and embryonic versus post-natal or adult OECs; cell kinetics and plasticity can vary widely in this spatiotemporal plane. LP-OECs in particular have only recently been investigated after transplant into spinal cord lesion (Ramer et al., 2004a, Ramer et al., 2004b, Lu et al., 2001). Similarly, culturing paradigms and in vivo investigations discussed earlier have pointed to the heterogeneity of these cells and their sensitivity to small changes in the immediate environment. Therefore, even slight variations in culturing and transplantation paradigms can potentially shift the OECs to phenotypes relatively more or less capable of supporting axonal growth and regeneration. Another general issue with the use of OECs as a transplant therapeutic is that the differences between their native environment in the olfactory system and the spinal cord are considerable, most notably in scale. The distances traveled during embryonic development or adult regeneration in the olfactory system is nowhere near the distances required for regeneration after injury in a structure such as the spinal cord. Nor does regeneration in the spinal cord occur in pre-formed glial tracts as it does in the established olfactory system (Richter and Roskams, 2008). Furthermore, OECs have been shown to have varying effects on different neuronal subtypes, and we have not yet fully understood the mechanism by which OECs selectively promote the growth of certain fibers and not others (Richter et al., 2005). An effort to pinpoint specific mechanisms and signaling events which determine the relative plasticity of these cells, both in culture and after transplantation, may help us in understanding the disparity of results and refine OECs as an effective transplant therapeutic.
1.4 Development, repair and regeneration in plastic areas of the adult CNS

Radial glia are the cells which provide the scaffolding for the inside-out generation of the brain that occurs via neuronal migration over considerable biological distances (Rakic, 1971b). The vast majority, 80-90% of embryonic neuronal precursors, migrate along radial glial processes to establish the brain (Hatten, 1999). Radial glia are derived from neuroepithelium and have a characteristic radial-process bearing morphology which consists of one endfoot on the ventricular surface of the neural tube, and a process which extends to the pia. Radial glia persist during neuronal precursor migration until they transform into astrocytes (Kriegstein and Gotz, 2003). BLBP induction in radial glia is coincident with their neurogenic phase, with induction of BLBP expression occurring in a spatiotemporal pattern that overlaps with the primary wave of neurogenesis in the developing CNS (Anthony et al., 2004).

After embryonic development is complete, there is a secondary phase of neurogenesis and migration in the postnatal brain, in areas such as the cerebellum and retina, and specialized glia that resemble their radial glial predecessors persist in these areas (Hatten, 1999). In the established adult system, we now know that new neurons are continuously produced and integrated into specific areas of the adult vertebrate brain (Altman, 1969). Regeneration is not limited to the olfactory system; in broader terms, the plastic areas of the adult CNS can be divided into two categories; those that are continuously regenerating and those that are subject to enhanced repair after damage. Specialized glia that resemble OECs in antigenicity, morphology and function reside in these areas, among them are astroglia of the
hippocampus, Müller glia of the retina, and Bergmann glia of the cerebellum. These in turn, bear a strong resemblance to their developmental forebear, the radial glia. Some of these cells, along with OECs, have been suggested as a population of glia termed ‘Aldynoglia’ which are developmentally delayed on the spectrum from radial glia to astrocyte, and are therefore specialized to their resident system where they provide support for growth or plasticity (Gudino-Cabrera and Nieto-Sampedro, 1999).

Two areas of the vertebrate nervous system undergo continuous and robust neural regeneration throughout adulthood, these are the olfactory system, and the dentate gyrus of the hippocampus (Farbman, 1992, van Praag et al., 2002). Neurogenesis in the dentate gyrus of the hippocampus occurs in vertebrates including humans (Eriksson et al., 1998). In the rodent, regeneration is robust, with six percent of the neuronal population being regenerated monthly (Cameron and McKay, 2001). These new granule neurons are born in the dentate gyrus where the hilus meets the granule cell layer of the hippocampus, from cells which bear astrocytic characteristics. These cells are commonly referred to as radial astrocytes, type I progenitors, or B cells; they divide and interact closely with their progeny which are the immediate neuronal precursors to the resident granule cells also named D cells or type II progenitors (Seri et al., 2004, Steiner et al., 2006). Both under normal conditions or after insult, the astrocytic progenitors proliferate to maintain or repopulate precursor and neuronal populations (Seri et al., 2001). Like radial glia B-cells maintain their glial morphology and characteristics while dividing to produce neuronal precursors (Ihrie and Alvarez-Buylia, 2008). B-cell radial
processes which penetrate the granule layer are closely apposed to their progeny and are postulated to ensheath these nascent precursors, providing support for neuronal maturation and short migration into the granule cell layer where the neurons extend a process to contact pyramidal cells in the CA3 (*Cornu ammonis*) and hilar regions (Ming and Song, 2005, Seri et al., 2004).

Two regions within the higher vertebrate which show no active regeneration but do show potential for enhanced repair due to their resident glial population are the cerebellum and the retina, which both contain specialized glia: the Bergmann glia and Müller glia respectively. Both cell types retain a radial glial-like morphology with processes interacting closely with neuronal counterparts. Both retain radial glial like behaviors also shared with OECs with regards to regulating neurogenesis in their respective systems (Bellamy, 2006, Metea and Newman, 2006, Hartl et al., 2007). Both Bergmann glia and Müller glia share markers of radial glia and neural stem cells and show functional evidence of being the resident progenitors or stem cells of their respective areas (Alcock, Scotting and Sottile, 2007, Moshiri, Close and Reh, 2004).

In the cerebellum, Bergmann glia postnatally guide the migration of granule neurons; this occurs long after the migration of Purkinje cells along radial glia to their final location in the purkinje cell layer, immediately next to Bergmann glial cell bodies (Rakic, 1971a, Misson et al., 1988). Once the system is established, Bergmann glia stretch their processes across the molecular layer of the cerebellum as well as radially towards the pial surface and assist in the migration of granular neurons (Bellamy, 2006). However, if Purkinje cells are grafted into an adult host
which does not produce any Purkinje cells, then the grafted cells migrate in a backwards pattern from that seen in development; they migrate from the cerebellar surface to the Purkinje cell layer along host Bergmann glia (Sotelo and Alvarado-Mallart, 1987). When in direct contact with migrating Purkinje cells, Bergmann glia antigenic expression transiently changes, with the cells upregulating Nestin, a marker of immature or progenitor cells. Notably, Bergmann glia show the same antigenic shift after experimentally induced damage (Sotelo et al., 1994). This evidence leads to the suggestion that though the cerebellum is not undergoing continuous regeneration in the adult CNS, Bergmann glia, like OECs maintain the ability to assist in migration and system regeneration, by undergoing a phenotypic shift to allow these changes to occur.

The retina of lower vertebrates has a ciliary marginal zone (CMZ) which undergoes continuous regeneration throughout postnatal life, and is capable of regeneration after injury. However by postnatal development, histogenesis in higher vertebrates is largely complete with no postnatal contribution from the CMZ (Moshiri, Close and Reh, 2004). Retinal development proceeds in a central to peripheral wave, with the late stage progenitors in the last wave of development developing into either rods or Müller glia, in a Notch-signaling dependent manner (Turner and Cepko, 1987, Hojo et al., 2000). Mature Müller glia span the retina from the vitreal surface to the retinal pigmented epithelium and are quiescent. However, after retinal damage in the fish, there is widespread Müller glial proliferation; these cells then acquire a progenitor-like gene profile which suggests de-differentiation before they give rise to both Müller glia and other retinal cell types.
including ganglion cells, which are normally produced before Müller glia in development (Fischer and Reh, 2002, Fischer and Reh, 2001). In the mammalian retina, very small numbers of cells reenter the cell cycle after retinal damage, but these cells can be maintained in culture (Dyer and Cepko, 2000, Lewis et al., 1992). The evidence suggests that Müller glia are also able to respond to changes in microenvironment and assist with regeneration, even regenerating those cells which appear before Müller glia themselves normally develop.

1.5 BLBP is a marker for glial plasticity and neuronal migration

Brain lipid binding protein (BLBP), also called Fatty acid binding protein 7 (FABP7) or Brain-Fatty acid binding protein (bFABP) is part of a family of intracellular fatty acid binding proteins which bind long chain hydrophobic ligands, including eicosanoids and retinoids. This family is divided into four major subfamilies: liver, heart, intestinal and cellular retinoid binding proteins, all which maintain the same tertiary structure (Hanhoff, Lucke and Spener, 2002). A brain specific fatty-acid binding-protein was searched out when it was found that fatty acid binding proteins from the heart, to which BLBP is most closely related, promoted differentiation of stem cells into neurons (Wobus, Zschiesche and Grosse, 1990).

When BLBP was first investigated, its spatiotemporal distribution in embryonic radial glia, starting at embryonic day 10 in murine system, suggested that it was involved in either neurogenesis or neuronal migration upon radial glia scaffolding, and the ongoing development of glial cells in more plastic areas of the
adult CNS (Kurtz et al., 1994). It is now known through transgenic fate mapping, that a wave of BLBP expression marks the neurogenic stage in radial glia from which the majority of CNS neurons are generated, and then migrate upon radial glial scaffolding (Anthony et al., 2004). Postnatally, BLBP expression persists in developing astrocytes, before it is restricted to specialized astroglia in areas such as the retina, hippocampus, cerebellum, cerebral cortex and spinal cord and olfactory system (Anthony et al., 2004, Kurtz et al., 1994, Schmid, Yokota and Anton, 2006). In situ expression patterns collected from public database shows that pattern of BLBP expression in the hippocampus, cerebellum and olfactory system (figure 1.3).

BLBP expression does not just correlate with neuronal migration, BLBP expression is reversibly lost when neuronal contact is removed. Postnatal Bergmann glia cultured at the time of peak granule cell migration, when BLBP expression is normally robust, lose that expression in vitro. However, if granule neurons are added to the culture system, transcription of BLBP is rescued through what was first called the radial-glial cell specific element (RGE), and glial BLBP expression is rescued (Feng and Heintz, 1995). Later, BLBP was identified as a direct target of Notch signaling. Through both Notch1 and Notch 3 signaling, BLBP is induced via canonical CBF/RBP-Jκ mediated signaling. Notch 1 and Notch3 receptor knockouts showed decreased BLBP expression (Anthony et al., 2005).

The effect of migrating neurons on BLBP expression is not unidirectional; BLBP has a very specific role in glial-neuronal interactions. Function blocking
Figure 1.3 In situ data from the adult brain shows expression patterns of DNER and BLBP in regions of interest

(A) The olfactory bulb shows cells in the mitral cell layer and glomeruli that express DNER and (B) BLBP expressing cells are OECs in the olfactory nerve layer. (C) Purkinje cells in the cerebellum express DNER beside (D) Bergmann glia expressing BLBP. (E) The hippocampus shows patterns of DNER expression in both the CA1-3 and dentate gyrus regions. (F) BLBP is found in those same areas, but expression is most robust in the dentate gyrus. G: Glomerular layer. M: Mitral cell layer. ONL: Olfactory nerve layer. DG: Dentate gyrus. All images collected from the Allen Brain Atlas; www.brain-map.org.
antibody experiments have shown that BLBP expression in glia is required for neuronal migration upon glial processes (Feng, Hatten and Heintz, 1994, Anton et al., 1997, Miller et al., 2003). When in culture with BLBP blocking antibodies, radial glia decrease the number of radial fibers extended. And though co-cultured neuronal precursors adhere to the remaining radial glial processes, precursor migration down existing radial glial processes does not occur, showing that blocking BLBP expression in glia specifically blocks neuronal migration. This BLBP-dependent extension of radial fibers occurs both in radial glia and neuronal precursors from both the embryonic cortex (Anton et al., 1997), and the developing cerebellum (Feng, Hatten and Heintz, 1994).

BLBP expression is not exclusive to the CNS as was once previously thought. BLBP is expressed in some Schwann cell precursors, but is absent in the normal adult PNS (Kurtz et al., 1994). However, after a sciatic nerve crush, the developmental programme of Schwann cells is recapitulated, with robust BLBP expression induced 14-30 days after crush. Similarly, peripheral Shwannoma show an upregulation in BLBP expression; BLBP antibody blocking in Schwannoma resulted in a change in morphology and axonal interaction, though there was no significant change in either tumor growth or migration (Miller et al., 2003).

In the olfactory system, BLBP is robustly expressed in olfactory ensheathing cells in both central and peripheral olfactory ensheathing cells; OECs maintain the most robust BLBP expression observed in the CNS, both in development and adulthood (Au and Roskams, , Au and Roskams, 2003). However, expression of BLBP in OECs in vitro is lost quickly with passaging, and expression of BLBP is
reduced in those OECs which are not in close apposition to ORNs (Au and Roskams, ). Furthermore, BLBP expression in the olfactory system maintains a similar pattern with Notch 3 expression (Carson, Murdoch and Roskams, 2006).

1.6 Notch expression in the olfactory system and other related areas of the CNS

Notch expression across the developing nervous system is well known to be involved in nervous system fate determination, gliogenesis and neurogenesis; with high levels of Notch expression during development that then drops in adulthood (Yoon and Gaiano, 2005). However, Notch 1 expression remains in certain populations of specialized glia during adulthood, including the hippocampal astroglia (Breunig et al., 2007), Müller glia of the retina (Furukawa et al., 2000), and Bergmann glia of the cerebellum (Tanaka and Marunouchi, 2003). Briefly, Notch signaling in development begins with receptor activation by binding with a ligand from a neighboring cell; this is followed by a cleavage event in which the intracellular domain of the Notch receptor is cleaved by factors such as presenilin and γ-secretase. The intracellular domain then translocates to the nucleus where it complexes with canonical or non-canonical transcriptional factors to activate or repress downstream targets which determine cell fate (Kadesch, 2004) (figure 1.4).

In the olfactory system, a number of Notch ligands are expressed in different cell types. Some OECs express Notch 3 embryonically and postnatally, both in vivo and in vitro (Au and Roskams, 2003, Carson, Murdoch and Roskams, 2006). Notch 3 expression overlaps with other known OEC markers, and those in vivo which
Figure 1.4 Diagram of the potential mechanism of Notch signaling whereby BLBP expression is maintained by ORN contact

ORNs expressing DNER interact with Notch expressed in neighboring OECs. A signaling event via canonical CSL-dependent and non-canonical Deltex dependent pathways then maintains BLBP expression, and OEC phenotype respectively. The end result is a BLBP expressing OEC which maintains a more plastic phenotype capable of supporting neuronal growth and regeneration. Figure adapted from (Saito and Takeshima, 2006).
express the receptor are those in closest contact with ORN axon bundles. Notch ligands Delta-like 1 and 4, as well as Jagged, are found in the basal progenitors (Carson, Murdoch and Roskams, 2006, Schwarting, Gridley and Henion, 2007). Jagged is also found in the olfactory epithelium where it is most likely expressed by ORNs (Lindsell et al., 1996). However, it is not known what specific mechanisms induce BLBP expression or maintain the ongoing robust BLBP expression in OECs in the LP and olfactory bulb, and if this regulation occurs through Notch signaling.

1.7 DNER, a novel Notch ligand

DNER (Delta/Notch-like EGF-related receptor) was originally isolated by two independent groups, one of whom named it BET (brain specific EGF-like transmembrane protein) (Eiraku et al., 2002, Nishizumi et al., 2002). DNER is a 90 kDa protein which was suggested to be part of the DSL family involved in Notch signaling due its structure of a single pass transmembrane domain framed by a short intracellular tail, and a large extracellular domain with multiple EGF repeats homologous to other DSL family proteins (Eiraku et al., 2002). In situ data shows early expression beginning as early as E14.5 in the mouse with expression peaking postnatally and dropping in the adult. DNER expression during these developmental timepoints was shown in Purkinje cells in the cerebellum, the neurons in the pyramidal layer, the neurons in dentate gyrus of the hippocampus, cortical neurons, and mitral cells of the olfactory bulb. The peak of DNER expression during early postnatal development suggests a role in the development of post-mitotic neurons, and later drops in adult tissue (Eiraku et al., 2002,
Nishizumi et al., 2002). In situ expression patterns collected from adult mouse CNS tissue available by public database shows a pattern of interest in the olfactory bulb, hippocampus and cerebellum (figure 1.3). In the CNS, DNER undergoes somatodendritic sorting resulting in dendritic expression (Eiraku et al., 2002).

DNER is a Notch ligand; DNER is expressed in Purkinje cells of the developing and postnatal cerebellum where it acts as a ligand for Notch 1 expressing Bergmann glia, where Notch signaling regulates both the functional and morphological differentiation of these cells (Eiraku et al., 2005, Komine et al., 2007). Interestingly, DNER lacks the DSL Notch-binding motif which is found in all other Notch ligands, it instead binds Notch by the first two EGF-like repeats; it has been suggested that this may indicate a preference for another Notch ligand such as Notch 3, which is expressed in OECs (Carson, Murdoch and Roskams, 2006, Eiraku et al., 2005). When DNER expression is knocked out there is a delay in cerebellar maturation with granule cell migration perturbed, resulting in abnormal morphology and fissure organization of the cerebellum leading to motor discoordination (Tohgo et al., 2006). The Purkinje and granule neurons in the cerebellum maintained electrophysiological integrity, but Bergmann glia maturation was significantly perturbed resulting in reduced GLAST expression, and a decrease in the number of radial fibers extended which are the normal conduits for granule neuron migration (Eiraku et al., 2005, Tohgo et al., 2006).

Notch signaling through DNER in the cerebellum is two pronged; CSL dependent signaling is responsible for the induction of protein expression such as GLAST, whereas non-canonical deltex-dependent signaling results in radial
process extension. This has lead to a model of Bergmann glia maturation through apposed Purkinje cell signaling which is not unlike a scheme proposed for the maturation of radial glia by another group, to be discussed below, and we suggest here that a similar signaling event may occur in OECs (Saito and Takeshima, 2006, Patten et al., 2006) (figure 1.4).

1.8 Neuronal-gliai crosstalk mediates cell morphology and functional ability

Glial-neuronal interactions appear critical for inducing BLBP, which is in turn functionally critical for facilitating neuronal migration upon glia. It has long been observed that neurons require radial glial scaffolding for migration in a crosstalk process, and neuronal contact in turn regulates the morphology and proliferation of glia. Factors such as astrotactin and glial growth factor are secreted by neurons only when they are migrating; providing a neuronal cue to maintain or induce radial glial phenotype. After migration has ended, these signals are lost and radial glia undergo an identity shift to become astrocytes which persist into adulthood (Hatten, 1999, Hatten, 1985).

A two pronged signaling event that very much resembles the model suggested to occur in DNER-Notch signaling (Saito and Takeshima, 2006), has been shown to occur with erbB/neuregulin signaling in development. Briefly, Notch 1 signaling in developing Bergmann glia in the postnatal cerebellum is activated by contact with Jagged expressed in granule neurons. BLBP is induced through canonical CSL-based Notch signaling, and erbB receptor expression is induced by deltex-mediated non-canonical notch signaling respectively. Granule neurons then
express neuregulin which activates glial erbB signaling, causing a change in phenotype. If either pathway is inhibited, there is no glial acquisition of radial phenotype (Anton et al., 1997, Patten et al., 2006, Rio et al., 1997, Patten et al., 2003). Note that this establishment of BLBP signaling in the developing cerebellum is independent of the mechanism of maintenance through DNER-Notch signaling seen in the mature system as described in section 1.7 above.

The idea that a similar neuronal-glial communication process could maintain specialized glia in the adult system is a relatively new one. OEC heterogeneity and \textit{in vivo} and \textit{in vitro} expression variability was extensively discussed in section 1.2; and though much effort has gone into characterizing OEC expression, relatively little has gone into investigating the specific mechanisms which regulate this expression. Furthermore, OEC morphology has been heavily studied \textit{in vitro} and after transplant \textit{vivo}, but there has been little direct investigation of the suggestion that OECs may consist of a single cell type with more than one phenotypic subtype in their native environment or \textit{in vitro} after culturing (Franceschini and Barnett, 1996). A recent hypothesis has outlined the rationale for suggesting axonal contact and glial-axonal crosstalk as a potential regulator of OEC phenotypic identity, development, and maintenance; a process which is already well defined in the most closely related cell type, the Schwann cell (Wewetzer and Brandes, 2006). A modified proposal based on variability of BLBP expression can be developed from this work (figures 1.5 & 1.6).
**Figure 1.5 Neuronal-glial signaling as a mechanism for regulating OEC phenotypic shift**

BLBP expression is hypothesized to be maintained by axonal signaling in BLBP. Cells removed from axonal contact lose their BLBP expression, and this indicates a phenotypic shift, resulting in an OEC which is no longer as capable at promoting neuronal regrowth and regeneration. However, co-culture with axons or specific ligands which are normally axonally expressed may rescue BLBP expression and maintain OEC phenotype in a state which is better able to support axonal regrowth and regeneration, and is similar to the phenotype of OECs *in vivo* which are in direct contact with ORN axons. Figure adapted from (Wewetzer and Brandes, 2006).
Olfactory ensheathing cell (OEC) phenotype

1. Removal from axonal in vivo axonal contact.
2. In vitro contact with ORNs or other ligands expressed by ORNs.
3. Time in culture away from axonal contact.

BLBP +

OECs in vivo actively supporting ORN growth and regeneration.

BLBP -

OECs no longer as proficient at supporting growth or regeneration.
1.9 Working hypothesis and research objective

Working hypothesis: BLBP expression in OECs is maintained by DNER expression in neighboring ORNs.

The aims of this current study were 4-fold:

1) To test that there is in vivo evidence for the regulation of BLBP in OECs by DNER expressed apposing ORNs.

2) To test if other areas of the CNS which are known to have greater potential for growth and regeneration in the adult and developing CNS, express BLBP in close apposition to DNER expressing neurons.

3) To test that in the absence of DNER expressing ORNs, BLBP expression in OECs declines.

4) To test that maintaining DNER expression in close proximity to OECs increases BLBP expression in vitro.
CHAPTER 2 : MATERIALS AND METHODS

2.1 Tissue preparation

**Adult Rats**: Animals were killed with a lethal overdose of choloral hydrate (100mg/kg by IP injection). They were perfused transcardially with PBS, followed by 4% PFA, pH 7.4. Cervical spinal cord was dissected and cryoprotected in successive concentrations of 12, 18 and 24% sucrose in PBS for 24 hours each. Tissue was then frozen on filter paper using OCT in isopentane over dry ice. The spinal cord was then sectioned at 14 um and stored at 20°C.

**Adult and Postnatal Day 21 Mice**: Mice were anaesthetised with Xylaket (25% Ketamine HCL (MTC Pharmaceuticals), 12.5% Xylazine (Bayer Inc.), 15% ethanol, 0.55% NaCl) and rapidly perfused with cold 0.1M phosphate buffered saline (PBS), followed by 4% paraformaldehyde (PFA) in PBS. Brains, olfactory bulbs and olfactory epithelia were dissected out and post-fixed in 4% PFA for 2 hours at 4ºC. Tissue was then equilibrated in 10% sucrose in PBS followed by 30% sucrose for 24 hours each at 4ºC, before proceeding to the embedding step.

**Postnatal Day 7 Mice**: Pups were anaesthetised with AErrane inhalation anaesthetic (Baxter Corporation) and perfused by hand with 3 ml of ice cold PBS followed by 3 ml of ice cold 4% PFA. The mice were decapitated, the skin removed and whole heads were post-fixed for 4 hours in 4% PFA at 4ºC. Heads were then
equilibrated in 10% sucrose followed by 30% sucrose for 24 hours each at 4°C, before proceeding to the embedding step.

**Embryos:** Pregnant dams were anaesthetised as outlined for adults. Embryos were dissected out and immersion-fixed in 4% PFA overnight at 4°C. They were then equilibrated in 10% sucrose in PBS followed by 30% sucrose for 24 hours each at 4°C. For staging of embryos, mid-day after the appearance of a vaginal plug was considered E0.5.

**Embedding and sectioning:** All tissues were equilibrated (under suction, with the exception of embryos) in warm Tissue-Tek embedding medium (Sakura Finetek, Torrance, CA) for 5 minutes, and frozen in liquid nitrogen. Coronal or sagittal sequential sections of 10 – 16 μm were taken on a HM 500 cryostat (Micron), mounted onto charged Superfrost glass slides (Fisher, Edmonton AB) and stored at –20°C for subsequent analysis.

### 2.2 Immunofluorescence

Sections were warmed on a slide warmer at an approximate temperature of 42°C for 10 minutes and post-fixed in 4% PFA for 10 minutes, followed by two 5 minute washes in PBS. Sections were then permeabilized in 0.1% Triton-X-100 in PBS for 30 minutes. Following 2 washes of 5 minutes in PBS, the sections were blocked with 4% normal serum in PBS for 20 minutes, and then incubated at 4°C for 12-20
hours with primary antibodies (see Table 2.1) in 2% normal serum. Sections were washed twice for 5 minutes in PBS and then incubated in secondary antibodies in 2% normal serum for one hour at room temperature. Fluorescently labelled secondary antibodies, used at a dilution of 1:100, were Molecular Probes Alexa 488 and Alexa 594, raised in donkey or goat. Sections were washed twice for 5 minutes in PBS and then nuclei were counter-stained with Diaminopyridine imidazole (DAPI; 1:10 000, Sigma) for 5 minutes at room temperature. Sections were washed twice for 5 minutes in PBS and then coverslips were mounted with Vectashield mounting medium (Vector Laboratories).

Exceptions: Sections probed for NeuN immunofluorescence were subjected to antigen retrieval in 0.01% citric acid, pH 6.0, for 10 minutes in the microwave on high power, washed 5 minutes in PBS between the postfix and triton permeabilization steps. Sections probed for GAP43 were blocked with the Mouse On Mouse kit (Vector Laboratories) for 1 hour in the M.O.M. block followed by 5 minutes in the M.O.M. protein concentrate.

Expression Analysis: For all analyses of gene expression patterns, a minimum of three animals of each developmental stage were used.

Comparative imaging: In all cases were comparative imaging was used, pictures were taken with identical parameters of exposure, gain and offset so as to be comparative.
2.3 Immunohistochemistry

This procedure is identical to immunofluorescence through the primary antibody incubation step. After incubation with primary antibody, the sections were washed twice with PBS and then incubated with 1:200 biotinylated secondary antibody in 2% normal serum for 30 minutes at room temperature. The sections were washed twice in PBS, endogenous peroxidase activity was quenched for 10 minutes in 0.5% hydrogen peroxide (Sigma) in PBS, followed by another PBS wash. They were then conjugated to avidin using the Vectastain ABC kit (Vector Laboratories) for 30 minutes at room temperature. Two more washes with PBS followed and then the sections were developed with VIP (Vector Laboratories). Developing was stopped by a 10 minute wash in ddH2O and coverslips were mounted with Aqua Poly/Mount (Polysciences Inc., Warrington PA).

2.4 Immunocytochemistry

Immunocytochemistry was performed on cells cultured on PLL coated glass or plastic coverslips. The procedure is identical to the Immunohistochemistry method above, with the following exceptions: The coverslips were not pre-warmed, and cells were fixed immediately out of culture with warm 4% PFA, washed twice with PBS, and stored at 4°C in 0.05% Sodium Azide in PBS. The secondary antibodies were used at a concentration of (1:200).
Exceptions: For p75NTR immunofluorescence, cells were not permeabilized. If p75NTR was to be used with another antibody, the cells were first stained for p75NTR in full, and then the coverslips were permeabilized, re-blocked, and then stained for the second antigen.

2.5 Lamina propria OEC cell culture

LP OECs were harvested from the olfactory mucosa of postnatal day 5-7 CD1 mice. The entire olfactory mucosa, including turbinates and septum was dissected from 8-10 pups, was mechanically dissociated, and treated with 0.6mg/ml Collagenase D (Roche), 1.5 U/ml dispase I (Roche), 7μg/ml hyaluronidase(Sigma), 0.5mg/mL bovine serum albumin (ICN Biomedicals) and 100 U/ml DNase I (Sigma) for 30 mins 37°C, prior to centrifugation and plating. Cells were plated at 450,000-700,000 cells/cm2 on poly-L-lysine (Sigma; 50μg/mL in 15mM sodium borate buffer) coated tissue culture plastic (treated 1 hour room temp, washed twice with PBS). Initial plating in MEM-D-Valine, 10% fetal bovine serum (FBS), and 100U/mL of penicillin/streptomycin (Penstrep) was followed 4-5 days later by purification using anti-Thy 1.1-mediated complement lysis as previously described, to remove contaminating fibroblasts (Au and Roskams, 2003). Briefly, after being detached with 0.25% trypsin EDTA, the cell pellet is re-suspended in MDV/10% FCS and 1:5 concentration of Thy1.1 hybridoma supernatant, and 1:10 concentration of Rabbit compliment (sigma) is then added. Cells are incubated at 37°C for 30 mins with agitation before being spun down and resuspended in plating media. Cells were replated in MDV/10% FBS, and 100U/mL PenStrep, allowed to grow for a further 4-
6 days, when they were again subjected to Thy1.1-mediated complement lysis.

After passaging, cells were replated on 1.5X the surface area from which they were removed.

For imaging and immunocytochemical analysis, cells were plated on poly-L-lysine (PLL) coated plastic coverslips.

2.6 Olfactory bulb OEC cell culture

From postnatal day 5-7 CD1 mice, OB OECs were harvested by sagitally bisecting the head, removing the meningeal covering of the olfactory bulb, using a scalpel to partition the rostral-most quarter of the olfactory bulb and extracting the portion of the nerve fiber layer next to the cribriform plate. Nerve fiber layers were dissociated and cultured as described by Ramon-Cueto et al. (1992). Briefly, digestion by 0.1% Trypsin for 15 minutes at 37°C was followed by dissociation by multiple passes through fire-polished pipettes, and the cells were seeded into poly-L-lysine (Sigma; 50μg/mL in 15mM sodium borate buffer) pre-treated flasks (1 hour, room temperature) containing DMEM/F12, supplemented with 2mM L-glutamine, 10% fetal bovine serum (FBS), 50μg/mL gentamycin, and 100U/mL PenStrep. 16-24 hours after primary plating, the media was changed to fresh media as above, supplemented with AraC (Sigma) at a final concentration of 0.01mM. 3-4 Days later, when cells had reached about 80% confluency, the media containing AraC was removed and the cells were subjected to in situ compliment lysis. 500ul of DMEM/10% FCS supplemented with Thy1.1 hybridoma supernatant at a
concentration of 1:5 was added to attached cells in 6 well plates. A 1:10 concentration of Rabbit compliment (sigma) was added, and the cells were incubated for 30 mins at 37°C. Cells were washed twice with warmed DMEM before media was replaced.

For imaging and immunocytochemical analysis, cells were plated on PLL coated plastic coverslips (Nunc).

2.7 Culturing, maintenance and transfection of COS-7 cell line

COS-7 cells (ATCC) were cultured in T75 tissue culture treated plastic flasks in DMEM/10%FCS. At 80-90% confluency, cells were passaged.

For transfection, COS-7 cells were plated on 12mm diameter glass coverslips pretreated with Poly-L-lysine as described above. 24 hours later at a confluency of 60-80%, cells were transfected as follows: 1ug of DNA and 1.5ul of Lipofectamine reagent (Invitrogen) per well were used. 1ug of DNA/well was mixed with serum free DMEM to a total of 25ul/well and incubated for 30 mins room temperature. 1.5ul of Lipofectamine/well was mixed with serum free DMEM to a total of 25ul/well and incubated for 30 mins room temperature. The DNA and lipofectamine mixes were then mixed together and incubated for 30 mins room temperature. Cells were then rinsed with serum free DMEM and incubated in 200ul/well serum free DMEM and 50ul/well DNA-lipofectamine mixture overnight at 37°C. 16-24 hours later the media was removed and replaced with fresh DMEM/10%FCS to allow for plasmid
expression. Cells were transfected with the pDisplay vector carrying the DNER sequence, an empty pDisplay vector, or left untreated for the purposes of co-culture.

After media with serum was replaced and the COS-7 cells were allowed 24 hours for expression of the DNER protein, transfection efficiency was assessed by immunofluorescence. Cells were fixed and stained as described above for DNER, and 4 100X pictures were taken per coverslips. Percentage expression of DNER was determined to be approximately 50% +/- 12%. Staining for DNER was also done without permeabilization to probe specifically for extracellular expression, and though comparable, it was not quantified at this time.

2.8 BLBP expression in culture assays

Cells from a single LP-OEC prep were plated, and cells on coverslips were fixed and stored at primary plating, passage 2 & 3. BLBP or p75NTR expression was then ascertained by immunofluorescence, and pictures across all coverslips were taken at the same exposure, gain and offset settings. Four pictures were taken at 100X magnification from each 12mm diameter coverslip, and the percentage of cells expressing BLBP or p75NTR was counted, using DAPI as the counterstain. All four photos and added together to yield a ‘per coverslip’ count. Data was collected from 3 independent cultures and averages and standard error of the mean were calculated from the ‘per coverslip counts’ normalized to primary plating counts in each experiment, in order to decrease variance when cross-comparing
experiments. This is with the exception of the data generated for passage 2, which came from 2 independent experiments. In order to determine if changes in expression from passage to passage were significant, a standard single tailed heteroscedastic T-test was conducted (P = 0.01).

2.9 LP-OEC-COS-7 co-culture

Two hours before co-culture, transfected or untransfected COS-7 cells plated on coverslips in 24-well plates were treated with the mitotic inhibitor Mitomycin C (Sigma) at 5ug/mL at 37°C 5% CO2. Immediately before co-culture, cells were washed 2X with PBS, and media was replaced with fresh MDV/10%FCS/100 U/mL Penstrep. Primary plated LP-OECs which had grown in culture 5-8 days in vitro, were detached using 0.25% trypsin EDTA, and compliment lysed as described above. LP-OECs were then resuspended in MDV/10%FCS/100U/ml PenStrep and were plated at a density of 35,000-45,000 cells/well. The co-culture was fixed 72 hours later, and cells were assayed by immunocytochemistry for BLBP or p75NTR as described above, as well as for DNER expression in transfected cells.

Pictures of immunofluorescence were taken at the same exposure, offset and gain across coverslips, and processed in the same way as described in section 2.7 above. ‘Per coverslip’ counts were expressed as the total number of cells expressing the protein of interest. Data was collected from multiple coverslips in 3 independent experiments, with the exception of the p75NTR data which was collected from multiple coverslips from 2 independent experiments. In order to be
able to cross-compare experiments without introducing outside variance, cell counts were normalized to the average number of cells expressing BLBP when co-cultured with DNER. A standard single tailed heteroscedastic T-test was conducted to determine if differences between treatment groups were significant (P = 0.01).

2.10 Plasmid construction
A plasmid containing the DNER sequence was gifted to our lab by Dr. Mineko Kengaku. Briefly, the Kengaku lab engineered the plasmid as follows: the 400kb DNER sequence including the plasma membrane insertion sequence, was sub-cloned into the pDisplay plasmid (Invitrogen) inserted between a NotI and BglII site, removing the pDisplay PDGF domain normally used to insert proteins into the plasma membrane. An empty pDisplay vector was used as a control.

2.11 Olfactory bulbectomies
Unilateral bulbectomies were performed on adult (2-4 months of age) CD1 mice. Mice were anaesthetized with inhaled isofluorane (AErrane, Baxter Corporation), and then given an analgesic via a subcutaneous injection of meloxicam (Metacam, Boehringer Ingelheim) at a dose of 1mg/Kg/5% in a 5 mg/mL solution. Then, a 1.4 mm diameter hole was drilled through the skull directly above the right olfactory bulb, 1mm rostral to the bregma and 1mm lateral to the midline, and one lobe of the olfactory bulb was removed by suctioning. The wound was filled with Gelfoam (Pharmacia & Upjohn, Kalamazoo MI) and the skin sealed with 3 simple interrupted stitches (Vicryl 5-0, Ethicon). Mice were sacrificed and perfused or fresh tissue
was collected at 3 days and 11 days post-bulbectomy after ascertaining that the bulbectomy had been complete. The contralateral side of the olfactory system with the olfactory bulb intact was used as control.

2.12 SDS-PAGE and Western blotting

To determine the expression of BLBP after bulbectomy, protein homogenates were prepared from 3 days post bulbectomy and 11 days post bulbectomy olfactory epithelium tissue dissected and snap frozen from a minimum of three mice of each time point. The tissue samples were homogenized in a lysis buffer (50 mM Tris-HCl pH 8, 150 mM NaCl, 1% Triton X-100), containing protease inhibitors (1µg/mL aprotinin, 1 µg/mL leupeptin, 100 µg/mL PMSF), with a Fischer Sciences Powergen 125 and the total protein concentration was determined using the Bradford reagent (Biorad) using the manufacturer’s instructions and the concentration determined by spectrophotometry, using a standard curve from known concentrations of BSA.

10µg of each protein sample was denatured by heating at 70°C for 20 minutes. All protein samples were separated by 7.5% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane (Biorad Transblot transfer medium). Membranes were blocked for 1 hour at room temperature with 5% non-fat milk in Tris-Buffered saline (TBS), incubated 12-20 hours at 4°C in primary antibody in 2% milk/TBS, washed 3 times 10 minutes in 0.05% Tween-20 in TBS and incubated for 1 hour at room temperature in peroxidase-conjugated goat anti-rabbit (molecular probes), rabbit anti-goat (DAKO) or goat anti-mouse (vector) diluted in 2% milk/TBS. Following three washes of 10 minutes in 0.05% Tween-20/TBS, the
membranes were treated with ECL chemiluminescence substrate (Amersham, Pierce) and the signal detected on X-ray film.

2.13 Transgenic mice: DNER null and OMP-cre/ZEG

DNER null: The fixed, anterior portion of the head of 2 litters were sent to us from Dr. Tom Reh, who received the live mice from Dr. H. Takeshima. The tissue was received in 30% sucrose immersion. Tissue was dissected, embedded, and at least three nulls and three wild type littermates were cryosectioned as described above. For a description of the generation of the DNER null, please see Tohgo et. al (2006) Molecular and Cellular Neuroscience. 31; 326-333 (Tohgo et al., 2006).

OMP-cre/ZEG: OMP-cre/ZEG mice were maintained, perfused and sectioned in the Roskams lab. Briefly, OMP-cre transgenic mice were generated by pronuclear injection of an 11kb EcoR1-digested fragment containing the OMP coding as described in Danciger et. al (1989) PNAS. 86(21): 8565-8569 (Danciger et al., 1989). OMP-cre mice were crossed with ZEG reporter mice of C57/BL6 or CD-1 background, which were generated as described in Novak et al. (2000) Genesis. 18(3-4): 147-155. (Novak et al., 2000). Expression and efficiency of Cre-lox recombination was checked by immuno for colocalization of Cre/OMP and GFP/OMP (Murdoch and Roskams, 2008).
2.14 Image analysis and quantification

All images were visualized with an Axioplan 2 Imaging microscope (Zeiss, Jena GER) using a Retiga 1350EX camera (Quantitative Imaging Corporation) with Northern Eclipse software (Empix Imaging Inc., Mississauga, ON) and were compiled using Adobe Photoshop 7.0. For quantification, BLBP, p75NTR and DNER were counted manually; DAPI was quantified using Image J (Wayne Rasband; NIH USA) by first thresholding the picture to render a binary image, then analyzing particles of pixel size 4-infinity and circularity of 0.50-1.0. Confocal images were collected on an Olympus IX81/Fluoview FV1000 confocal microscope. Images were processed with Olympus Fluoview 1.6 software.

Table 2.1 Antibodies used

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CHAPTER 3 : RESULTS; DNER is expressed in neurons and BLBP is expressed in glia in close apposition

3.1 Introduction: DNER and BLBP are expressed in closely apposed neuronal and glial subtypes respectively

BLBP had become of interest as a potential marker for phenotypic shift in OECs. A new Notch ligand, DNER had been recently published as expressed in purkinje cells of the cerebellum, where it regulates BLBP expression in the closely related Bergmann glia of the cerebellum (Eiraku et al., 2005). Previous DNER findings in the lab using immunofluorescent detection had detected DNER expressing axon bundles of the postnatal lamina propria of the olfactory system (MacDonald and Roskams, personal communication), where the axons are in close apposition to OECs. It had been previously observed that BLBP was expressed only in those OECs in direct contact with ORN axons (Au and Roskams, personal communication).

In order to ascertain if BLBP and DNER are in close apposition in the olfactory system, immunofluorescent detection for these proteins was performed on olfactory tissue from embryonic day 10 to adult stages of development.

3.2 DNER and BLBP expression in the embryonic olfactory system

At day E10, when the olfactory placode has invaginated into the olfactory pit, developing ORNs expressing DNER are being ensheathed and guided on their way to the olfactory bulb by BLBP expressing OECs (figure 3.1A); a group of cells
Figure 3.1 DNER is expressed in developing ORNs and BLBP is expressed in OECs in the developing peripheral olfactory system

(A) As early as embryonic day 10, developing ORNs in the mesenchyme outside the olfactory pit can be seen in close apposition to ensheathing cells expressing BLBP. Arrowhead points to a BLBP expressing cell ensheathing a DNER positive cell in the mesenchyme.  (B-C) By embryonic day 17, the peripheral system is well established and two different views of the developing OE show that DNER expressing ORN axons are now completely ensheathed by BLBP expressing OECs.  Arrow in (B) points to a fasciculated ORN axon bundle ensheathed by BLBP expressing OECs.  Arrowheads in (B) & (C) point to DNER expression in ORN axon cell bodies and dendrites.  Blue signal in all pictures is DAPI.  OP: Olfactory placode.  OB: Developing olfactory bulb.  OE: Olfactory epithelium.  M: Mesenchyme. LP: Lamina propria. Scale bars are: 50μm.
outside of the olfactory pit expressing DNER and BLBP are in the mesenchyme between the pit and the area of the telencephalon which will soon be the developing olfactory bulb. By E17, when ORNs have begun to synapse with their central nervous system targets, the peripheral olfactory system is well established; fasciculated axonal bundles, which are known to express typical neuronal markers, in the lamina propria of DNER expressing ORNs are completely ensheathed by BLBP expressing LP-OECs, which also express other OEC markers (figure 3.1 B-C). DNER expression is found not only in the axons of developing ORNs, but also in the dendrites and cell bodies of the developing ORNs found in the olfactory epithelium (arrowheads, figure 3.1 B-C).

3.3 DNER and BLBP expression in the postnatal and adult olfactory system

By postnatal day 5 (figure 3.2A-C) the peripheral olfactory system architecture is more or less established into its adult form. DNER expressing axon bundles of the lamina propria are ensheathed by OECs expressing BLBP. The delineation between OE and LP is marked by BLBP expressing cells which abruptly stop at the basal lamina separating the LP from the OE where OECs do not penetrate. The same pattern of expression exists adult olfactory system (figure 3.1 D-G) where only ORNs maintain a high expression of DNER and the what appears to be OEC processes extend within the ORN bundles as they are known to do.

In the established adult system, DNER is preferentially expressed in more mature ORNS; GAP43 (figure 3.2 H) is a membrane associated protein that is
Figure 3.2 BLBP is expressed in OECs ensheathing DNER expressing ORN axon bundles in the postnatal and adult OE

(A-C) The postnatal olfactory epithelium shows a pattern of BLBP expressing OECs ensheathing DNER expressing ORNs of the lamina propria. (B-G) This pattern is maintained in the mature system which undergoes continuous regeneration. (H-I) The adult system clearly shows a segregation between immature GAP43 expressing ORN cell bodies and DNER expression, though OMP expressing cells do express DNER, suggesting that DNER is expressed preferentially by mature ORNs actively growing. (J-M) At postnatal day 14, and all DNER expression in the axon bundles of the lamina propria overlaps with neuronal marker NCAM. Blue signal in all pictures is DAPI OE: Olfactory epithelium. LP: Lamina propria. Scale bars: 25μm in (M). 50μm in (A-C) & (E-I). 200μm in (D, J-L).
expressed exclusively in immature neurons and involved in neurite formation. It is expressed in those immature ORNs closest to the basal lamina, virtually segregated from the DNER expressing cells closer to the nasal cavity (figure 3.2 H). However, olfactory marker protein (OMP), which is a stage specific protein expressed in those ORNs which are more mature and are leaving the basal lamina to migrate to their targets in the olfactory bulb, appear to abundantly express DNER, as compared to GAP-43 expressing cells (figure 3.2 I). DNER expression in ORN axon bundles overlaps with neural cell adhesion molecule (NCAM), a cell surface adhesion protein which is used as another standard marker of neurons (figure 3.2 J-M).

3.4 DNER and BLBP expression in the central olfactory system

The E10 sagittal brain shows that DNER and BLBP can be found in an apposing pattern of expression throughout the brain (figure 3.3). The area of the telencephalon which is to become the olfactory bulb (boxed in figure 3.3A and expanded in figure 3.3 B-D) shows BLBP expressing cells with a radial glia-like morphology spanning the entire cortex, closely apposed to DNER expressing cells which are most likely neuroepithelial in origin.

In a sagittal section of the E13.5 olfactory bulb (figure 3.4 A-D), the BLBP expressing OECs are closely apposed to DNER expressing fasciculated axon bundles of ORNs and also to DNER expressing cells in the mitral cell layer. Radial glia extend their processes from the ventricular zone, next to what is most likely
Figure 3.3 Apposing patterns of DNER and BLBP expression begins as early as E10 across the entire brain

A) At E10 closely associated DNER and BLBP expressing cells can be seen across the entire brain. The area of the telencephalon which will develop into the olfactory bulb is boxed in (A) and expanded in (B-D). The arrowhead in (B,D) points to a BLBP expressing radial glial process spanning the developing telencephalon. Blue signal in all pictures is DAPI. NP: Nasal pit. OS: Optic stalk. Scale bar: 400μm in (A) and 100μm in (B-D).
Figure 3.4 DNER and BLBP are expressed in neuronal and glial cell types respectively in the developing and adult olfactory bulb

(A-C) The embryonic olfactory bulb shows BLBP expressing cells both in the OECs of the ONL, and astrocytes of the bulb, with DNER expressed in closely apposed ORNs and nearby developing mitral cells. (D) Magnified field boxed in (A). (E-G) The postnatal bulb shows more distinct expression in the ONL and in individual astrocytes of the medial bulb. The arrow points to mitral cells, the arrowhead points to a glomerulus, both are expressing DNER. (H) Magnified field boxed in E. Arrow points to a mitral cell, arrowhead points to a glomerulus. (I-J) BLBP shows a similar relationship to, but does not overlap with Doublecortin (DCX) expressing neurons in the early postnatal brain, as compared to patterns of DNER and BLBP. (L-N) In the adult bulb, BLBP expression is limited to the ONL and DNER expression is expressed in a clear pattern in the glomeruli and mitral cell layers. Blue signal in all pictures is DAPI. ML: Mitral cell layer. VZ: Ventricular zone. ONL: Olfactory nerve layer. Scale bar: 100 μm in (A-C), (E-G), & (I-K). 50 μm in (D) & (H). 200 μm in (L-M).
DNER expressing migrating neuroblasts, as they share a similar expression pattern to those cells which express doublecortin (DCX; figure 3.4 I-K), which is a microtubule associated protein expressed in migrating neuronal precursors and immature neurons. DCX positive cells are also in close association with BLBP expressing cells at postnatal day one. At postnatal day 5, BLBP expressing astrocytes are scattered through the olfactory bulb, with OECs of the ONL robustly expressing BLBP, apposed against DNER expressing ORNs. Mitral cell bodies, and glomeruli which are made up of mitral cell processes and tufted cells, also robustly express DNER (figure 3.4 E-H)

In the adult system, the major cell layers of the olfactory bulb can be clearly distinguished (figure 3.4 L-N), with mitral cell bodies strongly expressing DNER, and the glomeruli which is made up of dendrites of mitral/tufted cells in the glomerular layer expressing DNER. BLBP expression in the astrocytes of the granular layer is no longer present, but OECs, the only resident glia of the olfactory nerve layer, stop just short of the glomerular layer where ORNs meet their synaptic targets, and continue to maintain the most robust BLBP expression observed in the adult CNS.

3.5 DNER and BLBP outside the olfactory system; Introduction

In the established CNS BLBP expression generally decreases as astrocytes mature, but BLBP is maintained in specialized areas which show enhanced capability for regeneration or repair (Anthony et al., 2004, Kurtz et al., 1994). After having used immunofluorescence to probe for the expression of both proteins
throughout the development of the olfactory system, we hypothesized that this relationship could be found across the CNS, possibly maintaining BLBP expression in specialized glia, both in development and in adulthood in the specialized regions. Of interest to us were the areas in which other Aldynoglia were present; namely the hippocampus, the retina, and the spinal cord (Gudino-Cabrera and Nieto-Sampedro, 1999). Immunofluorescent probing for DNER and BLBP in the cerebellum was done to reproduce that which was published previously, showing DNER expressed in cells which match the morphology and antigenicity known to define Purkinkje cells and BLBP expressed in neighboring cells with a morphology and antigenicity indicating that they are Bergmann glia (Eiraku et al., 2005) (figure 3.5 I-L).

3.6 The hippocampus and cerebellum

The apposing pattern of expression of DNER and BLBP can be found across the CNS as early as E10 (figure 3.3A). In postnatal development, BLBP is widely expressed in astrocytes; but also in discrete areas where it is in closer proximity to DNER expressing cells. In the postnatal hippocampus DNER is expressed in the cell bodies of the granular layer and in processes of the molecular layer, both in the dentate gyrus and the CA1-CA3 regions (figure 3.5 A-D). BLBP is expressed in scattered astrocytes and cells in the dentate gyrus which bear a radial morphology. At postnatal day 5, GFAP expression is not yet established across all astrocytes;
Figure 3.5 The hippocampus and cerebellum express apposing DNER and BLBP in their neuronal-glia cell types

(A-C) the postnatal hippocampus shows DNER expression in both the neurons of the dentate gyrus and the CA1-CA3 regions. BLBP expression at this time is found in cells of the dentate gyrus as well as in scattered astrocytes. (D) BLBP expressing glia of the P5 dentate gyrus also express GFAP. Arrowhead points to a radial astroglia which colabels for BLBP and GFAP. (E) DNER expression in the 50μm thick slices of adult hippocampus is less robust but maintained. BLBP expression is reduced to the dentate gyrus astroglia. (F-H) Magnified view of the dentate gyrus boxed in (E); radial astroglia of the dentate gyrus express BLBP in close apposition to DNER expressing neurons. Arrowhead points to DNER and BLBP expressing cells in close apposition in the edge of the granular layer. Arrow indicates a BLBP expressing cell with radial glia-like morphology in the subgranular zone of the dentate gyrus. (I-K) Immunofluorescent probe in the postnatal cerebellum shows a robust pattern of DNER in purkinje cells and BLBP in neighboring Bergmann glia, and also in scattered astrocytes. (L) The adult cerebellum shows no expression of BLBP in astrocytes, but the Purkinje cell: Bergmann glia pattern remains well maintained. The inset shows the purkinje cell layer in high magnification, arrowhead points to a BBP expressing Bergmann glia, the arrow points to a DNER expressing purkinje cell. Blue signal in all pictures is DAPI. Scale bar: 100μm in (A-C) & (E). 50μm in (D), (F-H) & (I-L).
however, the astroglia in the molecular layer of the dentate gyrus express GFAP in a pattern which overlaps with BLBP expression (figure 3.5 D).

In the adult hippocampus, DNER remains expressed in scattered cells throughout the CA1-CA3 regions which most likely represent hippocampal neurons which have not been further defined here; but BLBP expression is limited to the dentate gyrus (Figure 3.5 E). In the adult dentate gyrus, DNER expressing cell bodies are in close apposition to BLBP expressing cells with a radial morphology, which are most likely radial astroglia (Figure 3.5 F-H).

DNER is very highly expressed in Purkinje cells which are in very close apposition to BLBP expressing Bergmann glia. Bergmann glia extend their radial processes through the molecular layer (figure 3.5 I-L). This pattern of expression is maintained into adulthood, with both Purkinje cells and Bergmann glia robustly expressing DNER and BLBP respectively; but expression of BLBP in the astrocytes of the granular layer is lost (figure 3.5 L).

### 3.7 The retina

Early in development, at E10.5, neither DNER nor BLBP can be detected in the developing retina; retinal ganglion neurogenesis begins at E10.5, so it is possible that DNER expression has not yet begun (figure 3.6A). However, by E17, a pattern of DNER and BLBP expression in neuronal and glial cells can be detected in what is most likely developing retinal ganglion cells and Müller glia respectively. Muller glia can be seen at the periphery of the inner nuclear layer. BLBP
Figure 3.6 The developing retina expresses apposing patterns of DNER and BLBP expression in neurons and glia respectively

(A) At E10, when the generation of the major output neuron, the retinal ganglion cell, is just beginning, no DNER and BLBP expression can be observed. However, by E17, DNER and BLBP expression can be observed in both the superficial (B) and deep (D-F) retina. In the deep retina (D-F) DNER expression is found in developing retinal ganglion cells in the inner plexiform layer, while BLBP expression is found in the cell bodies of Müller glia in the inner nuclear layer (arrowhead) and also at the border of the outer nuclear layer where it is most likely expressed by astrocytes (arrow). (C) Cells expressing DNER at this time point also express NST. (G-I) The P1 retina shows a pattern of expression in both the retina and the optic nerve, where BLBP is expressed in Müller glia of the inner nuclear layer, and astrocytes associated with the olfactory nerve respectively. (J-L) Later in development, robust DNER expression can be seen in the ganglionic cell layer (arrowhead), next to Müller glia expressing BLBP in the inner nerve layer. DNER expression can also be found in the layer of developing photoreceptors (arrow). Blue signal in all pictures is DAPI. NFL: Nerve fiber layer. IPL: Inner plexiform layer. ONL: Outer nerve layer. ON: Optic nerve. GCL: Ganglion cell layer. INL: Inner nuclear layer. Scale bar: 100μm in (A-C). 50μm in (D-I). 25μm in (J-L).
expression closer to the optic nerve fiber layer is more likely astrocytes which are known to interact with the optic nerve at this point. This pattern of expression is found in the more superficial (figure 3.6 B) and deep (figure 3.6 D-F) retina at this time point. In the superficial retina, DNER expression overlaps with the neuron marker NST (figure 3.6 C).

In the deeper layers (figure 3.6 D-F) DNER is expressed in the most medial aspect of the retina, in the axons of the ganglion cell layer making up the nerve fiber layer. More laterally, DNER is expressed in neurons which are most likely retinal ganglion cells with cell bodies in the ganglionic layer, and dendrites projecting through the inner plexiform layer. At the most lateral aspect of the developing retina DNER is expressed in the outer nuclear layer of the retina, in what is most likely developing photoreceptors. Alongside DNER expressing ganglion cells, BLBP is expressed in the inner nuclear layer in the cell bodies of Müller glia, which are the only intrinsic glial cell within the retina; astrocytes are only present at the edge of the optic nerve layer. The Müller glia then extend their processes through the inner plexiform layer, alongside the ganglion cell dendrites. BLBP expression can also be observed in the nerve fiber layer (NFL) where it is most likely expressed in astrocytes which contact the NFL at this time.

By early postnatal development (figure 3.6 G-I), a pattern of DNER and BLBP becomes more refined in both the retina and the optic nerve. Within the retina, cell bodies of the retinal ganglion cells strongly express DNER; their axons which make up the nerve fiber layer and optic nerve also strongly express DNER.
Müller glia in the inner nuclear layer of the retina express BLBP; and what is most likely astrocytes in the optic nerve express BLBP as well.

Later in postnatal development at day 5 (figure 3.6 J-K), the retinal ganglion cell layer is robustly expressing DNER in their cell bodies, and faintly in processes closer to the outer nuclear layer. Another boundary can be distinguished as faint DNER expression in the developing photoreceptors. In close proximity, the inner nuclear layer where Müller glia cell bodies reside robustly express BLBP.

3.8 The spinal cord: BLBP is expressed by a novel glial cell type closely associated with DNER expressing neurons

In the early embryo at embryonic day 10, DNER and BLBP expression in the developing spinal cord is robust, where DNER marks neuroepithelium of the spinal cord, and BLBP is expressed in radial glia which span the spinal cord with their processes (figure 3.7A-C). By E17.5 these large-scale areas resolve themselves more specifically, and a closer association of morphologically defined cell types expressing DNER and BLBP are found throughout the spinal cord, with a layer of BLBP expressing cells with a radial morphology at the borders of the spinal cord (figure 3.7 D). This pattern is very similar to that observed in the early postnatal spinal cord where BLBP expression is robust in astrocytes (figure 3.7 E).

The adult spinal cord reveals two areas of interest with apposing DNER and BLBP patterns. In the longitudinal adult spinal cord the large cell bodies of motor neurons in the ventral horn robustly express DNER; this most likely represents not cell surface expression, but areas in which DNER is being processes within the cell.
Figure 3.7 Apposing patterns of DNER and BLBP are expressed in the developing and adult spinal cord

(A-C) At embryonic day 10, DNER marks the neuroepithelium, with BLBP marking radial glia. Radial glia extend their processes through the cord up to and through the neuroepithelium to the ventral boundary (arrowheads). (D) At E17, this pattern becomes resolved into individual cells which resembles the early postnatal cord (E). (F) The longitudinal adult spinal cord shows DNER and BLBP associations in the grey matter (arrow), particularly along the central canal (arrowhead). The central canal can also be seen in a transverse section (J-L) where DNER appears to be apically expressed in cells line the canal, contacted by BLBP expressing processes (J-L). (G-I) The transverse cord shows a pattern of ‘tract’ like structures (arrowhead) expressing DNER and in close contact with BLBP expressing cells. Blue signal in all pictures is DAPI. V: Ventral. D: Dorsal. L: Lateral. M : Medial. CC: Central canal. Scale bar: 50µm in (A-D), & (G-L). 100µm in (E) & (F).
The processes of the motor neurons express DNER, and these appear to be in close contact with BLBP expressing cells (figure 3.7 F). Both proteins are expressed in cells lining the central canal of the spinal cord. Cells directly lining the canal appear to express DNER are most likely ependymal cells (figure 3.7 J, I). The BLBP expression in this area is most likely from tanyctyes, which extend cell processes into the central canal to contact CSF, also included in the ‘Aldynoglia’ designation.

Looking at the transverse adult spinal cord, we can view the central canal from another perspective (figure 3.7 J-L). The cells directly within the canal appear to be apically expressing DNER; these are apposed to BLBP expressing processes which cannot be traced back to specific cell bodies. The lateral aspect of the transverse spinal cord reveals a pattern of DNER and BLBP expressed in tracts that radiate from the lateral to medial aspect of the spinal cord (figure 3.7 G-I). The pattern of BLBP cells ensheathing DNER expressing neurons in this case greatly resembles that which has been observed in the olfactory system (discussed above). Though it is possible that the patterns of expression observed may represent colocalization, it is unlikely as previous work has shown DNER is exclusively expressed in neurons, and BLBP is exclusively expressed in progenitors and glia (Eiraku et al., 2002, Kurtz et al. 1994).

Interestingly, the glia in these ‘tracts’ which express BLBP cannot be fully defined as astrocytes. The astrocytic marker GLT-1 is a glutamate transporter protein that is expressed by astrocytes both in development and in the established CNS (Swanson et al., 1997). GLT-1 expression in the spinal cord only partially
overlaps with BLBP expression; with minimal overlap where the two markers meet within the same ‘tract’ of glia (figure 3.8 A-C). BLBP expression begins at the most lateral aspect of the spinal cord, and the radial processes show less BLBP expression as the ‘tract’ moves in to the most medial aspect and into the grey matter. GLT-1 expression at the lateral aspect is absent, with the majority of the expression being in the grey matter, radiating outwards towards the lateral aspect only a short distance. BLBP expression appears to be glial as all GFAP expression overlaps with BLBP (figure 3.8 D-F).

DNER expression in the adult spinal cord is neuronal, as in the large DNER expressing motor neuron nuclei in the grey matter that co-express NST (figure 3.8 G-I). However some DNER expressing cells with a smaller, very different morphology may also overlap with NG2 (figure 3.8 J-L). NG2 (Neuron glia antigen 2) is expressed in cells in the oligodendroglial lineage and progenitors (Rivers et al., 2008) and though the staining found here was very weak, it was also persistent and not present in primary only controls. What appear to be NG2/DNER expressing cells, very faintly expressing NG2, are most often found in the white matter and not the grey matter of the adult spinal cord, closer to the periphery of the structure.

3.9 Other areas of interest in the CNS that express DNER and BLBP

The developing telencephalon at embryonic day E10 (figure 3.3A) shows a strong relationship between DNER and BLBP expression in what will become the developing cortex. However, at postnatal day 5, the cortex shows little specific
Figure 3.8 DNER is expressed in spinal cord neurons whereas BLBP expression is in a poorly defined cell type

(A-C) BLBP expression oriented in radial ‘tracts’ in the transverse adult spinal cord shows minimal overlap with astrocytic marker GLT-1, a glutamate transporter protein. The area of overlap can be observed between the arrow and arrowhead.

(D-F) BLBP expressing cells in the adult cord overlap completely with GFAP expression. (G-I) DNER expression in cell bodies of neurons of the grey matter (arrowhead) overlap with NST expression. (J-L) DNER expression also overlaps in some cases with NG2. Blue signal in all pictures is DAPI. L: Lateral. M: Medial. WM: White matter. GM: Grey matter. Scale bar: 100μm in (A-C) & (G-I). 50μm in (D-F) & (J-L).
association between the expression of two proteins; though DNER appears to be expressed in a specific pattern in the cortex, BLBP expression is in scattered astrocytes in an unassociated pattern (figure 3.9 A-C).

However, in areas of neuronal migration or progenitor activity, such as the postnatal rostral migratory stream (RMS; figure 3.9 D-F) or adult lateral ventricle (LV figure 3.9 G-I) DNER and BLBP expression can be found in very close proximity. In the postnatal RMS, neuroblasts from the lateral ventricle are migrating to the olfactory bulb, where they will become the interneurons of the bulb; at this stage of development, the RMS is a relatively wide stream of cells actively migrating in one of the few instances of neuronal cell types migrating along one another, in a process termed ‘chain migration’ that does not require glia. The cells within and on the border of the RMS appear to express DNER in a punctate pattern that is also found in ORN axon cell bodies. There is also a layer of BLBP expressing cells at the edges of the RMS, which may most likely represent astrocytes bordering the stream of migrating neuroblasts (figure 3.9 D-F). The migrating neuroblasts of the RMS are generated in the subventricular zone (SVZ) of the lateral ventricle. In the adult lateral ventricle small DNER expressing cells can be seen next to the extended processes of BLBP expressing cells which are very closely associated with the ventricle (figure 3.9 G-I).
Figure 3.9 Other areas of interest within the CNS which show DNER/BLBP expression patterns

(A-C) Though BLBP expressing Radial glia span the cortex through development, BLBP expression can only be seen in scattered astrocytes in the postnatal cortex. DNER appears to be expressed in specific layers of cortical neurons. (D-F) BLBP expressing astrocytes in the early postnatal rostral migratory stream border DNER expressing neuroblasts migrating towards the olfactory bulb. Arrow indicates the middle of the RMS and the direction of migration. Arrowhead indicates the BLBP-expressing border. (G-I) Radial process bearing cells (arrowhead) of the adult subventricular zone express BLBP in close association to DNER expressing cells (arrow). Blue signal in all pictures is DAPI. L: Lateral. M: Medial. V: Ventricle. Scale bars: 100μm (A-C). 50μm (D-I).
CHAPTER 4 : RESULTS; BLBP expression in OECs is regulated by DNER

4.1 Introduction: DNER regulates BLBP expression in OECs

The aim of this portion of this study was to test if BLBP expression in OECs is regulated by DNER. BLBP expression and morphology of Bergmann glia is reversibly changed by the removal or addition of DNER in organotypic slices of cerebellum (Eiraku et al., 2005). My hypothesis is that ORNs expressing DNER regulate BLBP expression in adjacent OECs. We first sought to remove DNER in the in vivo system and test if BLBP expression changes as a result. We then sought to test a gain of function effect wherein adding DNER to cultured LP-OECs could change BLBP expression.

4.2 Unilateral olfactory bulbectomy shows a decrease in the robustness of BLBP expression

We first tested the effect of removing DNER from the in vivo system by a method previously used in our lab (figure 4.1). A unilateral olfactory bulbectomy involves the removal of one lobe of the olfactory bulb, resulting in olfactory receptor neuron death on that side of the epithelium. The contralateral, unbulbectomized side of the olfactory epithelium of the same animal then serves as an internal control, and with the exception of the septum, remains largely unaffected. OECs do not exhibit significant cell death in this system, but are maintained in tracts and

Figure 4.1 BLBP expression is less robust when DNER expressing neurons are removed from the system by unilateral bulbectomy
(A-F) At 3 days post-bulbectomy a robust drop in DNER expression due to loss of neurons can be seen in the (A-C) bulbectomized epithelium when compared to (D-F) the unbulbectomized control epithelium. However, with regards to BLBP expression, little difference can be seen between the (D-F) bulbectomized and the (A-C) unbulbectomized control epithelium at this time point. (G-L) At 11 days post-bulbectomy the (G-I) unbulbectomized control shows BLBP expression which is much more robust than the (J-L) bulbectomized epithelium, which is still showing a significant reduction in DNER expressing neurons, though there is also ORN regeneration occurring. Diagrams to the left of each micrograph set represents a low-power view of the olfactory epithelium; the black box indicates the location of OE which was sampled from each side at each time point. All images were captured using identical parameters so as to be comparative. NBX: Unbulbectomized internal control. BX: Bulbectomized epithelium. Scale Bar: 200μm
assist ORN regeneration (Williams, Franklin and Barnett, 2004). BLBP expression was ascertained by immunofluorescence; pictures of the bulbectomized side of the epithelium were taken at the same exposure, offset and gain settings as the unbulbectomized side in the same animal, from the same area of epithelium.

Maximal cell death is seen at 3 days post-bulbectomy in the bulbectomized side (Cowan et al., 2001) as can be seen by the decreased DNER expression in the missing ORN cell bodies of the epithelium, and the degenerating axon bundles of the epithelium (figure 4.1 D-E). However, when compared with the unbulbectomized epithelium at this time, no difference in the pattern or robustness of BLBP expression can be observed between the bulbectomized and unbulbectomized epithelium (figure 4.1 A-C).

At 11 days post-bulbectomy when ORN regeneration is at its peak (Cowan et al., 2001), we can see a difference in the robustness of BLBP expression between the bulbectomized and the unbulbectomized olfactory epithelium (figure 4.1 G-L). Though ORN re-growth has already begun, and a significant amount of axonal DNER expression can be detected in the lamina propria, BLBP expression appears to be less robust in the bulbectomized when compared to the unbulbectomized epithelium.

To determine changes in protein expression via another method, a western blot was performed on dissociated fresh frozen tissue from animals at 3 and 11 days post-bulbectomy (figure 4.2). DNER and BLBP antibodies detected single bands at 90 KDa and 15 KDa as predicted. At both 3 days and 11 days post-bulbectomy there is a decrease in DNER expression as a result of the loss of
Figure 4.2 Western detection shows no change in BLBP expression
(A) 3 days postbulbectomy, probing for DNER shows a change in protein 
expression in the bulbectomized (BX) versus unbulbectomized (NBX) side. It 
appears as if there is a change in BLBP, but this coincides with the change in the β-
actin loading control and therefore does not likely represent a change in expression.
(B) 11 days after bulbectomy, the unbulbectomized (BX) side still shows less DNER 
expression as compared to the unbulbectomized (NBX) control side. However, 
there is no difference in BLBP. BX: Bulbectomized. NBX: Unbulbectomized control 
tissue. B-actin: β-actin.
ORNs. But at 3 days and 11 days post-bulbectomy, no change in the level of BLBP expression can be found between the bulbectomized and unbulbectomized sides of the olfactory epithelium using this method. It is likely that another time point between 3 days and 11 days post-bulbectomy would have been required for a difference to be detected with this method.

4.3 The DNER null shows a decrease in BLBP expression in the OECs of the lamina propria.

In the DNER null tissue, no DNER expression can be detected by immunofluorescence (figure 4.3 & 4.4); though the lamina propria shows normal expression of other neuronal markers such as neuron specific tubulin (NST; β-III tubulin; Tuj1) within axon bundles (figure 4.4 G-H), there is absolutely no expression of DNER detected in either the cell bodies of ORNs in the epithelium or in axon bundles of the lamina propria.

When DNER is specifically removed from the system as in the DNER null, changes in most parameters of glial morphology or expression are not immediately visible. Immunofluorescence for other OEC markers, β-Dystroglycan and p75NTR display a similar pattern of expression in the OECs of the lamina propria in both the DNER null and the wild-type litter-mate control (figure 4.3 A-F). p75NTR is a well established marker of OECs; β-Dystroglycan is a newly published marker of OECs, where it is involved in anchoring to ECM components and therefore marks the outermost boundary OECs in each axon bundle (Takatoh et al., 2008). By comparing both markers we can see that the overall density of OECs is not
Figure 4.3 The lamina propria of the DNER null shows no immediate differences in the expression of p75NTR and β-Dystroglycan  
To ascertain if any major differences in glial of the lamina propria were affected in the DNER null, matched sections of (A-C) the wild type littermate and (D-F) the null were probed for p75NTR and β-Dystroglycan protein expression.  (C, F) No immediately obvious difference is the expression of these two antigens can be observed either with p75NTR (arrowheads) which marks OECs all the way through the axon bundles.  (B,E) No immediately obvious difference can be seen with β-Dystroglycan (arrows) which marks the outermost limits of axon bundles where OECs contact basal lamina.  Diagrams to the left of each micrograph set represents a low-power view of the olfactory epithelium; the black box indicates the location of OE which was sampled from each side at each time point.  WT: Wild-type littermate control tissue.  DNER -/-: DNER null tissue.  Blue signal in all pictures is DAPI.
Scale Bar: 100 μm.
**Figure 4.4 BLBP expression in the DNER null is less robust than in the wild-type littermate control**

Matched sections of the (A,B) wild type littermate and (C,D) DNER null were probed for BLBP expression, showing that BLBP expression in the null is much less robust than the BLBP expression in wild type OECs ensheathing axon bundles in the lamina propria. Images were captured using identical parameters so as to be comparative. (E,F) What little BLBP expression remains in the DNER null still associates with axon bundles marked with other neuronal markers such as NST (arrowheads). (G,H) Axon bundles expressing NST express no DNER (arrowheads). Diagram indicates area of OE sampled in all pictures. Blue signal in all pictures is DAPI. Ax: Axon bundle. OE: Olfactory epithelium. Scale Bar: 100μm.
obviously changed, as marked by P75 expression, which labels OECs within the axon bundles (figure 4.3 A,C D &F), though this has yet to be determined specifically by quantification. The basic size and shape of OEC ensheathments around axon bundles has also not been grossly altered by the DNER null, as displayed by β-Dystroglycan around the outer limits of the axon bundles (figure 4.3 A,B D &E); only BLBP expression appears to be reduced (figure 4.4).

BLBP expression in the lamina propria of the null is still present, displays a trend toward lower level of expression as compared to the wild-type littermate control (figure 4.4 A-D). In order to ascertain if BLBP expression in DNER null tissue differs from the wildtype littermate controls, sections from similar areas of the olfactory epithelium were probed for DNER and BLBP immunofluorescence, and pictures of similar areas within the OE were taken at the same exposure, gain and offset settings. Those OECs which do express a lower level of BLBP associate normally with axon bundles, as indicated by the overlapping expression of NST and BLBP (figure 4.4 E-F).

4.4 BLBP expression is progressively lost when OECs are cultured in vitro

Here, we show a quantifiable drop in BLBP expression as over time in culture as LP-OECs are passaged (figure 4.5). Cells from a single LP-OEC prep were plated, and cells on coverslips were fixed and stored at primary plating, passage 2 & 3. BLBP or p75NTR expression was then ascertained by immunofluorescence, and pictures across all coverslips were taken at the same exposure, gain and offset
Figure 4.5 BLBP expression decreases in cultured LP-OECs over time
(A-C) BLBP expression in cultured LP-OECs decreases in the robustness of expression over time between primary plating and passage 3. Images were captured using identical parameters. (A) Not only does the robustness of expression decrease, but the number of cells as normalized to the number expressing BLBP at primary plating also decreases very quickly and significantly. (B) P75 expression is far more variable, and does not decrease significantly from passage to passage. Though there is an overall trend toward decreasing expression, it is not nearly as quickly as BLBP. ** indicates a significant change between that passage and the other two (P<0.01). Blue signal in pictures is DAPI. Scale bar: 50μm in all pictures.
settings. Four 100X pictures were taken from each 12mm diameter coverslip, and the percentage of cells expressing BLBP or p75NTR was counted, using DAPI as the counterstain. All four photos were added together to yield a ‘per coverslip’ count. Data was collected from 3 independent cultures and averages and standard error of the mean were calculated from the ‘per coverslip counts’ normalized to primary plating BLBP counts in each experiment, in order to decrease variance when cross-comparing experiments. This is with the exception of the data generated for passage 2, which came from 2 independent experiments. In order to determine if changes in expression from passage to passage were significant, a standard single tailed heteroscedastic T-test was conducted (P < 0.01).

From primary plating to passage 2 and 3, the robustness of BLBP expression in cultured LP-OECs drops quickly (figure 4.5 A-C). Not only does the intensity of expression appear to drop within individual OECs, but the number of cells expressing BLBP decreases to less than half of the number of LP-OECs that were expressing at primary plating (figure 4.5 D). These changes are significant from passage to passage. p75NTR immunofluorescence OECs does show a trend toward dropping, but does not change significantly (figure 4.5 E), therefore BLBP expression drops at a much faster rate. At passage 3, it is a vast minority of LP-OECs in culture which express BLBP, and these are mainly those cells which are actively dividing.
4.5 LP-OEC co-culture with DNER expressing COS-7 cells increases BLBP expression

In order to test if BLBP expression in cultured OECs could be increased by addition of DNER to the culture system, a co-culturing paradigm wherein OECs are co-cultured onto a COS-7 monolayer transfected with the pDisplay DNER vector. Cells were transfected for 24 hours with lipofectamine based transfection methods. The media was then changed to media with serum and the cells allowed 24 hours for DNER expression. After COS-7 cells have been given 24 hours for DNER expression, LP-OECs are added to the culture system. This is also the time point at which transfection efficiency was assayed. Transfected monolayers were probed for DNER expression and the number of cells showing immunofluorescence for DNER. On average, a 50% transfection rate was achieved at 24 hours after transfection (figure 4.6 A-B). 96 hours after transfection, when cells are fixed in the co-culture paradigm, DNER expression is markedly less due to either the downregulation of expression or the loss of mitotically inactivated cells. However, staining in both the permeabilized (figure 4.6 C-D) and unpermeabilized (figure 4.6 E-F) is comparable, showing that DNER is most likely expressed on the cell surface at a rate similar to that detected by immunofluorescence on permeabilized cells.

After COS-7 cells are transfected and allowed 24 hours in serum for DNER expression, the monolayer is mitotically inactivated with mitomycin-C. LP-OECs at primary plating are then trypsinized, put through complement lysis to cull excess fibroblasts, and plated onto the COS-7 monolayer which is either transfected with DNER or untreated. These cells therefore are early passage cells at passage 1.
LP-OECs are then left in co-culture for 72 hours before being fixed and assayed for BLBP or p75NTR expression by immunofluorescence. Pictures of
Figure 4.6 COS-7 transfection with the DNER-pDisplay yields a transfection rate of approximately 50%.

(A-B) COS-7 cells at 70-90\% confluency were transfected with the DNER-pDisplay vector using lipofectamine based methods. Average transfection was 52\% +/- 12\% at 24 hours after transfection media was removed and replaced with regular media with serum to allow for expression. (C-F) Immunofluorescent detection at 96 hours after transfection shows a similar pattern between (C-D) permeabilized and (E-F) unpermeabilized cells. Scale Bar: 400 \( \mu \)m in all pictures.
immunofluorescence were taken at the same exposure, offset and gain across coverslips, and processed in the same way as described in section 4.4 above. ‘Per coverslip’ counts were expressed as the total number of cells expressing the protein of interest, because DAPI stains the nuclei of both LP-OECs and COS-7 cells, and no other counterstain could be used to differentiate between the two. Data was collected from multiple coverslips in 3 independent experiments, with the exception of the p75NTR data which was collected from multiple coverslips from 2 independent experiments. In order to be able to cross-compare experiments without introducing outside variance, cell counts were normalized to the average number of cells expressing BLBP when co-cultured with DNER. A standard single tailed heteroscedastic T-test was conducted to determine if differences between treatment groups were significant (P < 0.01).

Co-culture with DNER expressing cells significantly increases the number of BLBP expressing cells nearly twofold (figure 4.7E). p75NTR does not change significantly as a result of co-culture (figure 4.7F). Cells expressing BLBP when co-cultured with DNER expressing COS cells were often in direct contact with DNER expressing cells (figure 4.7A), but not always. Cells assayed for p75NTR expression did not significantly differ in morphology from the BLBP expressing cells observed (Figure 4.7 A-D).
Figure 4.7 LP-OEC co-culture with DNER expressing cells increases the number of LP-OECs expressing BLBP by twofold.

(A) Cells co-cultured with DNER expressing COS-7 cells increase in the total number of cells expressing BLBP. Arrowhead indicates a BLBP expressing LP-OEC, arrow indicates a DNER expressing COS-7 cells. (B) LP-OECs co-cultured with untransfected COS-7 cells still show some BLBP expression. (C-D) p75NTR expression in LP-OECs does not change between co-cultured conditions with (C) DNER transfected and (D) untransfected cells. (E) When quantified, a significant increase in the number of co-cultured LP-OECs express BLBP when co-cultured with COS-7 cells expressing DNER as compared to co-culture with untreated COS-7 cells. (F) Quantification for p75NTR expression shows no significant difference in the number of LP-OECs expressing p75NTR when co-cultured with DNER expressing COS-7 cells as compared to co-culture with untransfected COS-7 cells. Asterisk indicates a significant difference (P<0.01). Scale bar: 100μm in all pictures.
4.6 LP-OECs may lose their ability to respond to DNER expression in culture as passage number increases.

A preliminary experiment cross-comparing the ability of early and later passage LP-OECs to respond to DNER was conducted once. The experiment was conducted and quantified exactly as described in section 4.5 above, with the exception that both passage 1 and passage 2 LP-OECs were assayed. To assess significance between culture conditions in each passage, a single tailed heteroscedastic T-test was performed (P < 0.05). Cells at passage 1 showed a significant drop in the number of cells expressing BLBP when LP-OECs were co-cultured on DNER expressing monolayers, as compared to co-culture on untreated monolayers. However, the passage 2 cells did show an overall trend which is similar, but was not significant (figure 4.8A). p75NTR expression in both cases did not show any significant changes between culture conditions (figure 4.8B).
Figure 4.8 The ability of OECs to respond to co-culture conditions may decrease with increased passaging

(A) cells co-cultured at passage 1 show significant change in the number of LP-OECs expressing BLBP when co-cultured with DNER expressing COS-7 cells as compared to co-culture untransfected COS-7 cells. At passage 2, though there is a trend, this effect is no longer significant. (B) P75 expression as a result of co-culture conditions does not vary significantly in passage 1 or passage 2 cells. Asterisk indicates a (Williams, Franklin and Barnett, 2004) significant difference (P<0.05).
CHAPTER 5: DISCUSSION

The role of olfactory ensheathing cells in their natural environment invites due speculation into the considerable potential of OECs as a therapeutic after CNS injury. OECs dynamically maintain not only a constantly regenerating environment, but one that is open to the outside world and subject to frequent damage from environmental insult and infection. OECs in their native environment must support the growth, migration, targeting and even death of their neighboring olfactory receptor neurons as they cross the peripheral to central nervous system boundary; it is at this boundary where OECs have the additional role of protecting the integrity of the central nervous system by becoming part of the glial limitans (Farbman, 1992).

After CNS injury, the established environment of the brain or spinal cord lacks the growth-permissive environment we see in the olfactory system, or requires a peripheral to central nervous system bridging that is not possible with the endogenous cell types, such as astrocytes and Schwann cells (Kwon and Tetzlaff, 2001). Early investigations into the transplant potential of OECs yielded very positive results, showing functional regeneration, axon growth across the PNS:CNS boundary, and even myelination (Franklin, 2003, Ramon-Cueto et al., 2000). However, issues with replicating these data within the field has prompted speculation as to the specific ability of OECs to promote regeneration. Some have suggested that the observed regeneration is due to factors such as culture contaminants or sprouting of preserved axons outside of the lesion (Ramer et al., 2004a, Barnett and Chang, 2004). Still others show compelling evidence for OEC
potential, including several human transplantations (Feron et al., 2005, Ibrahim et al., 2006).

The above issues have pointed towards the lack of specific knowledge concerning OEC mechanisms of supporting growth and regeneration. OECs are a dynamic cell type; with an antigenicity and morphology that varies in vivo and in vitro. In the latter case, such variations are reversibly changeable on a very short time scale within a matter of hours, with minimal changes to culturing conditions (Vincent, West and Chuah, 2003). My thesis project seeks to investigate mechanisms of maintenance which could support an OEC phenotype which could be more permissive to axonal growth and regeneration. If culture conditions can vary OEC phenotype so drastically, it is possible that culturing conditions have selected for a population of OECs which differ significantly from that observed in their natural environment; this is a process we have termed 'phenotypic shift'. Therefore OECs which have been cultured and transplanted may no longer bear the characteristics which they were sought out for, as they may have lost them over time in culture. Only by investigating the specific pathways by which OEC identity is changed in vivo will we understand how to properly maintain them in vitro; we can then avoid losing the OEC characteristics we wish to foster for the purpose of transplantation.

Almost all defining markers of OECs are variable in their expression depending on the specific cell context either in vivo, or in culture depending on conditions. In order to begin investigating the mechanisms underlying phenotypic shift in OECs, we needed to choose a protein which is expressed by OECs, and
which could potentially be related to OEC shifts in state. Previous work in our lab had found that OECs in vivo do not express BLBP unless they are in direct contact with ORNs; those which are not directly contacting the axon bundles, particularly those close to the peripheral side of the cribriform plate do not express BLBP (Au and Roskams, ). BLBP was of interest because it has a strong significance in development where it is expressed in neurogenic radial glia, and is responsible for the functional modulation of radial glial phenotype leading to their ability to extend radial processes required for neuronal migration upon radial glial scaffolding (Anthony et al., 2004, Feng and Heintz, 1995). BLBP protein expression is downregulated in the adult, and only appears to be expressed in those areas which are prone to enhanced regeneration or repair, and contain specialized glial subtypes for this purpose collectively termed ‘Aldynoglia’ (Gudino-Cabrera and Nieto-Sampedro, 1999). BLBP is also lost quickly in OEC cultures in the absence of neuronal input (Au and Roskams, personal communication).

Of growing interest in the field of both OECs and the closely related Schwann cells has been the role of axonal-glial crosstalk in maintaining or modulating these cell types (Wewetzer and Brandes, 2006). Though most of the emphasis of this work has been focused on axonal cues regulating OEC myelination, we felt that this could be a more global mechanistic input into the regulation of OEC phenotype. It is possible that a neuronal-glial crosstalk bidirectionally regulates the growth and plasticity between ORN axons and OECs. BLBP is a direct target of Notch signaling, and we had previously published data showing OECs in direct contact with ORN axon bundles expressing Notch 3; these
are the same cells which also express BLBP. Furthermore, Notch is highly variable, but expressed in OECs in vitro (Au and Roskams, 2003, Carson, Murdoch and Roskams, 2006).

A recent paper shows that a closely related cell type to OECs, the Bergmann glia, expresses BLBP, and this expression is regulated by the Notch ligand DNER which is expressed in neighboring Purkinje cells (Eiraku et al., 2005). Loss of DNER results decreased expression of BLBP in Bergmann glia, and a change in morphology leading to a lack of neuronal migration upon Bergmann glial fibers and therefore delayed cerebellar development. We therefore hypothesized that BLBP expression in OECs is maintained by DNER expression in ORNs; this is the hypothesis which is investigated by my project.

This hypothesis lead to the following proposed model whereby DNER regulation of BLBP expression in OECs is a Notch dependent interaction. Maintenance of BLBP expression may be a key factor in defining the identity and functional potential of specialized glia throughout the brain. My project has shown that DNER and BLBP are closely apposed in these areas which are considered to harbor specialized glia in the adult; namely the hippocampus, retina and spinal cord. These areas also show Notch expression in development and in the adult systems (Breunig et al., 2007, Furukawa et al., 2000, Chen, Leong and Schachner, 2005). In the cerebellum, it has been proposed that DNER regulation of Bergmann glia morphology and antigenicity occurs by a two pronged signaling event. Notch signaling through the canonical CSL-based transcription factors is posited to be responsible for inducing protein expression, and deltex-dependent non-canonical
signaling results in morphology changes such as radial process extension. Both are required for the full acquisition of Bergmann glia phenotype and functional maturity (Saito and Takeshima, 2006).

A similar two pronged Notch signaling event occurs in radial glia (Patten et al., 2003); in both Bergmann glia and radial glia the deltex dependent signaling event was downstream of the canonical CSL-based event, showing again that BLBP expression may be functionally required for acquisition of glial phenotype (Eiraku et al., 2005, Patten et al., 2006). It is possible that this mechanism of signaling in radial glia and specialized glia such as Bergmann glia, is common to all specialized glia including OECs. In situ database information shows that OECs do express all the components required for this two pronged signaling cascade including Deltex 4, and RBP-Jκ (Carson, Murdoch and Roskams, 2006, Allen institute for brain science); it remains to be experimentally determined that both canonical and non-canonical signaling are required to maintain an OEC phenotype permissive to neuronal growth and regeneration.

Notch expression was initially viewed in developmental neurobiology as a signaling cascade which inhibited neurogenesis and thus may prompt a fate-choice towards glial lineage (Kadesch, 2004). However, more recently, Notch signaling has been shown to play an active role in instructing gliogenesis in development (Wang and Barres, 2000, Gaiano, Nye and Fishell, 2000). More specifically, the role of Notch signaling in stimulating the differentiation of several glial cell types in development or adulthood including radial glia, Müller glia, hippocampal astroglia, Schwann cells, and progenitors in the spinal cord has been established (Furukawa
et al., 2000, Gaiano, Nye and Fishell, 2000, Tanigaki et al., 2001, Taylor, Yeager and Morrison, 2007, Morrison et al., 2000). In areas such as the retina, Notch signaling is specific, and overexpression of signaling components such as Hes transcription factors causes resident stem cells to specifically produce the specialized Müller glia; conceivably increasing the ability of this structure to respond to injury (Furukawa et al., 2000). Similarly, Notch signaling causes the differentiation of hippocampal astroglia in the dentate gyrus, where perturbation of Notch signaling causes changes hippocampal morphogenesis and plasticity through the regulation of glial function and morphology (Breunig et al., 2007, Tanigaki et al., 2001). This leads to the conclusion that Notch is not only an active signaling component for the regulation of gliogenesis, but is also important to the plasticity and neuronal development of the structures in which it acts to determine the fate of specialized glia.

Notch signaling may not stand alone as the signal for glial development or maintenance; it has been suggested that Notch signaling in the above specialized areas maintains or enhances the ability of Notch expressing cells to respond to other instructive signals in their immediate milieu (Wang and Barres, 2000). Notch signaling in radial glia is not only the signal for their developmental induction, but also for the maintenance of their morphological and functional phenotype. Notch 1 expression in the developing forebrain maintains radial glial phenotype by inhibiting radial glial differentiation into less plastic astrocytes (Li et al., 2008). As Aldynoglia are posited to be specialized glia in their resident systems that have not moved fully along the developmental continuum (Gudino-Cabrera and Nieto-Sampedro, 1999),
it is probable that Notch signaling in the systems containing Aldynoglia described above are maintained in an enhanced plastic state by Notch signaling. DNER, or other Notch ligands in these areas, expressed in the resident neuronal cell types may contribute to the neuronal-glial crosstalk which maintains neuronal growth and plasticity in these regions.

My results consistently support the above model and hypotheses. This study commenced with the investigation of the olfactory system for evidence of apposing patterns of DNER and BLBP, which could support the hypothesis that DNER expression in ORNs regulates BLBP expression in neighboring OECs. In the peripheral olfactory system, a closely apposed pattern of DNER and BLBP was detected as early as E10 (figure 3.1) DNER expressing neurons are ensheathed by OECs expressing BLBP in the mesenchyme between the olfactory bulb and the olfactory pit. Though we did not investigate any earlier timepoints, another group has recently probed the E9 brain and found DNER to be expressed in GnRH positive and negative cells in the olfactory placode and mesenchyme, in what is most likely pioneering axons of the olfactory neuraxis. They did not however observe any BLBP expression at this time, though they did reproduce the same pattern at E10 we observed, with BLBP expressing cells ensheathing DNER expressing cells in the mesenchyme (Greer and Miller, personal communication). We had initially chosen to investigate DNER as a mechanism of BLBP maintenance and not induction, as there are other Notch ligands in the olfactory system which are likely to initially induce BLBP expression (Schwarting, Gridley and Henion, 2007). However, if DNER expression is present in the developing olfactory system
before BLBP, it is possible that it may function not only as the signal for BLBP maintenance, but for BLBP induction as well.

The apposing pattern of DNER and BLBP is resolved into fasciculated axon bundles completely ensheathed by OECs later in postnatal development (figure 3.1-3.2). This pattern is as robust in the adult system as it is in development, where DNER expression in ORNs is preferentially expressed in more mature ORNs which are actively migrating and targeting and express OMP (figure 3.2). Those cells which express the immature marker GAP-43 co-express DNER less robustly. This supports the above idea that DNER and BLBP could be involved in an active neuronal-glial crosstalk wherein both cell types support the migration of axons, by providing signaling resulting in the support and dynamic growth of one another.

In the olfactory bulb there are two apposing patterns of DNER and BLBP (figure 3.3 & 3.4). DNER and BLBP are closely apposed both in the ORNs and the OECs of the olfactory nerve layer, but also in radial glia which are supporting neuroblast migration from the RMS. There has been some discussion in the literature as to where OEC processes end within the olfactory bulb. Some maintain that OEC processes do not infiltrate into the glomerular layer where ORNs make their synaptic connections with their target mitral/tufted cells (Au, Treloar and Greer, 2002). However, the expression pattern shows BLBP expressing processes within the glomeruli of the adult olfactory bulb (figure 3.4). This is long after most other astrocytes found within the olfactory bulb have ceased to express BLBP; only OECs appear to maintain robust expression. There exists two possibilities here, either
astrocytes among the glomeruli express BLBP, or OEC processes do extend further into the glomerular layer than was once thought.

Though my project focuses mostly on the olfactory system, immunofluorescence of the entire sagittal brain at embryonic day 10 (figure 3.3) shows a widespread robust pattern of DNER and BLBP expression in adjacent regions which lead to a more thorough investigation of the developing brain in order to determine if DNER maintenance of BLBP expression could be a more widespread mechanism in related areas of the CNS which also show specialized glia associated with regeneration or enhanced potential for repair. Patterns of apposing DNER in neuronal subtypes and BLBP in glial subtypes are found in the developing and adult hippocampus, cerebellum and retina; these are all areas which are known to have Notch signaling (discussed above). This work also briefly investigates DNER and BLBP expression in the cortex, rostral migratory stream and the subventricular zone (figure 3.5, 3.6 & 3.9). The apposing pattern of DNER and BLBP has been previously published in the detail only in the cerebellum (Eiraku et al., 2005). DNER has also been published as being expressed in the pyramidal cells of the cortex, Purkinje cells of the cerebellum, and the pyramidal cells of the hippocampus and dentate gyrus (Eiraku et al., 2002). The DNER expression pattern in the other areas, including in the retinal ganglion cells and photoreceptors of the retina, is novel.

BLBP expression has been well documented in developing radial glia, as well as being expressed in specialized areas of the brain in the mature CNS, including astroglia of the dentate gyrus, and subventricular zone, and Bergmann
glia of the cerebellum (Anthony et al., 2004, Kurtz et al., 1994). In the hippocampus and SVZ in particular, BLBP is expressed in astroglia which are suggested to be neurogenic; astroglia are the resident progenitors of their respective systems, but they maintain their radial glial like morphology when dividing. This morphology then serves to guide and often ensheathed nascent neuroblasts as they migrate away from the neurogenic zone (Seri et al., 2006, Seri et al., 2004, Steiner et al., 2006, Ihrie and Alvarez-Buylla, 2008).

Within the spinal cord, the DNER expression shown here is novel (figure 3.7 and 3.8). Though in some cases it appears that DNER and BLBP expression overlap in the spinal cord, it would be unlikely that this represents co-expression; it more likely represents a very close apposition of cells, as previous work strongly suggests that DNER is exclusively expressed in neuronal cell types, and BLBP expression is expressed exclusively in unidifferentiated progenitors and glial types (Kurtz et al. 1994, Eiraku et al., 2002). The cell bodies which co-express DNER and NST in the ventral horn of the spinal cord are motor neurons. This is consistent with expression patterns observed in other areas where it is primarily the major output neurons which stain for DNER (Eiraku et al., 2002). However, a minority of DNER expression distributed around the periphery of the white matter may also overlap with NG2 expression. NG2 is primarily expressed in cells which are in the oligodendroglial lineage, however some have suggested that NG2 positive cells do give rise to neurons in certain areas of the CNS (Rivers et al., 2008). The cells which appear to be co-expressing NG2 and DNER were a minority, and it remains a
possibility that these could be adult progenitors which give rise to neuronal subtypes.

BLBP has been previously well characterized in the spinal cord through development (Kurtz et al., 1994), but the patterns of BLBP expression in the adult spinal cord, particularly in the transverse sections described here, are novel (figure 3.7 & 3.8). Because the BLBP expressing cells only overlap partially with GLT-1, another established marker of astrocytes, it is possible that the BLB expressing cells represent a cell type more closely related to radial glia than to astrocytes. The cells directly contacting the central canal are most likely tanycytes, which are known to share characteristics with other specialized glia discussed above, and extend processes to contact the cerebrospinal fluid (Gudino-Cabrera and Nieto-Sampedro, 1999). Whereas the DNER expressing cell are most likely ependymal cells which line the apical aspect of the central canal (Meletis et al., 2008).

Recent work from another group has shown a similar pattern of BLBP expression across the transverse spinal cord, with BLBP high in the periphery and low in the medial aspect. They further suggest that BLBP expression in spinal cord glia may be upregulated after injury (White, McTigue and Jakeman, personal communication). Subpopulations of the spinal cord may as yet be defined as similar to those in other areas with an increased potential for repair, such as the retina or cerebellum. The cues for maintaining BLBP expression and thus a more permissive phenotype in transplanted OECs may be abundant in the injured spinal cord, or regulating BLBP expression in the glia of the spinal cord itself may increase autologous repair after injury.
We can conclude that DNER and BLBP appear to be closely associated in neuronal and glial cells respectively, both in the developing and adult olfactory system (figures 3.1-3.4). Similarly, this pattern is maintained in the developing and mature CNS, particularly in areas which are known to be regenerative, or potentially capable of enhanced repair and are known to express Notch (figure 3.5-3.9). This pattern of expression is particularly robust in areas which contain ‘Aldynoglia’, the cerebellum, hippocampus, retina, subventricular zone, and olfactory system. In all cases, either by observation of morphology or by co-labeling with other known markers, BLBP appears to be expressed in glial cell types, with DNER expressed in neuronal subtypes; these are more often than not in intimate association with one another in all areas.

Removing DNER from the in vivo olfactory system decreases the robustness of BLBP expression. This was first investigated with the method of olfactory bulbectomy, wherein unilateral removal of one lobe of the olfactory bulb causes widespread cell death in the epithelium of that same side (figure 4.1). What is being shown in this experiment is not a DNER-specific effect, as ORN death causes the loss of any axonally expressed proteins that may affect OEC phenotype or BLBP expression. Mice were given unilateral bulbectomies, and the olfactory tissue was collected at 3 days or 11 days post-bullectomy. 3 days post-bullectomy marks the peak of ORN cell death, whereas 11 days post-bullectomy marks the peak of ORN regeneration (Cowan et al., 2001). Surprisingly, there is no visible change in the robustness of BLBP expression at 3 days, indicating that BLBP expression in OECs may be slow to change. However, at 11 days after
bulbectomy, even when ORN regeneration is peaking, BLBP expression in the
bulbectomized tissue appears to be less robust than the unbulbectomized control
(figure 4.1). This is most likely due to a specific decrease in BLBP expression and
not the death of OECs, as OEC death after bulbectomy has been shown to be
minimal (Williams, Franklin and Barnett, 2004).

A western blot of bulbectomy tissue shows no significant difference between
the bulbectomized and unbulbectomized side at 11 days (figure 4.2). It is possible
that the method was not sensitive enough not detect the shift; the antibody was also
not used at the minimum possible concentration. It is also possible that the
considerable regrowth of ORNs which can be seen in the axon bundles at this time
can be upregulating BLBP expression above baseline in those OECs that are re-
expressing it. It is possible that another time point between 3 and 11 days may
have yielded a more significant difference.

A DNER specific effect on BLBP expression can be seen in the DNER null
tissue (figure 4.3-4.4). Data from the DNER null line has been published previously
in the cerebellum, where DNER null in purkinje cells causes decreased BLBP
expression and altered morphology in neighboring Bergmann glia (Eiraku et al.,
2005). The expression in the lamina propria here resembles that which has been
published previously in the cerebellum, with BLBP expression in OECs appearing
much less robust in the DNER +/- than in the wild type littermate. This analysis is
ongoing at this time, and it is likely that there are quantifiable differences in OEC
morphology within the axon bundles which can not yet be elucidated by the data
shown here. A decrease in the expression of BLBP changes Bergmann glial
morphology, specifically their ability to extend radial processes for subsequent neuronal migration (Eiraku et al., 2005); therefore it follows that a similar effect should occur when OECs no longer express BLBP.

No other large scale changes in axon bundle morphology can be immediately observed when the DNER null tissue is stained with other common markers within the olfactory system (figure 4.3-4.4). The expression of neuron specific tubulin (NST; β-III tubulin; Tuj-1) shows that OECs associate with axon bundles, but that DNER is completely absent. Furthermore, no changes can be detected with other OEC markers β-Dystroglycan or p75NTR between the DNER null and the wildtype littermate.

Because of the morphological deficits in Bergmann glia, the DNER null shows a delay in cerebellum development due to a lack of granule cell migration along Bergmann glial fibers (Eiraku et al., 2005). We were unable to ascertain if a similar delay in development or olfactory function occurs in the olfactory system because we only have access to 5 week old fixed tissue. No immediately obvious differences in brain size or olfactory bulb size was observed between the wildtype litter-mate controls and DNER null brains.

Though BLBP expression is greatly reduced, it is not completely absent from the OECs of the olfactory system; what appears to be a lower level of BLBP expression persists in the lamina propria. BLBP expression is a direct target of Notch signaling, therefore the persistence of BLBP expression in the absence of DNER input could indicate two possibilities. Either BLBP inductive signaling via Notch receptors in the olfactory epithelium occurs through a different Notch ligand,
and the main function of DNER is the maintenance of a robust level of expression in response to actively growing neurons. The other possibility is that other Notch ligands expressed by ORNs may compensate for the loss of DNER; there is evidence that ORNs express jagged (Schwarting, Gridley and Henion, 2007, Lindsell et al., 1996). BLBP expression in the cerebellum of the DNER null is also not completely absent, and resembles the expression level we find in the olfactory system. The authors of that work similarly suggest that Jagged1 expression in Purkinje cells of the cerebellum may be compensating for the loss of DNER and maintaining a lower expression of BLBP than what is seen in the wild type (Eiraku et al., 2005). It has also been suggested that CSL, normally in the canonical pathway of Notch signaling, may have independent effects when Notch signaling is no longer occurring; this could also be happening in the OECs of the DNER null (Anthony et al., 2005).

BLBP expression is also lost in the absence of axonal input in vitro (figure 4.5). In LP-OEC cultures, the rate of drop in BLBP expression is very quick across passages. As compared to primary plating, less than half of the LP-OECs in culture are expressing BLBP by passage two. Previous work on cultured OECs has demonstrated OEC sensitivity to changing culture conditions in terms reversible changes in morphology and extracellular matrix deposition (Vincent, West and Chuah, 2003, Chuah et al., 2000). Changes in antigenicity have been observed in response to co-culture with neurons or after transplantation into the CNS, where OECs are hypothesized to reversibly switch from a p75NTR-positive phenotype, to a p75NTR-negative phenotype. The p75NTR-negative phenotype expresses
markers of myelination when in contact with specific axonal types (Wewetzer and Brandes, 2006). We show here that the idea of OEC plasticity and response to immediate contextual cues can be extended to rapid, and possibly reversible, changes in BLBP expression in response to a loss of in vivo signaling inputs in the culture system.

BLBP expression and Bergmann glial morphology is rescued in organotypic slices of the cerebellum of the DNER null when they are treated in vitro with DNER or constitutively active Notch. Here, we show a related effect of DNER on cultured OECs (figure 4.7). BLBP expression can be increased in vitro with the addition of DNER to the system via transfection of a COS-7 monolayer with a pDisplay vector carrying the DNER sequence (figure 4.6). The number of cells expressing BLBP after 72 hours in culture with DNER expressing COS-7 cells was on average approximately twice those cultured on untransfected COS-7 cells. At 24 or 48 hours after co-culture, little to no increase in BLBP expression could be detected (data not shown).

Though the number of BLBP expressing cells changes according to co-culture conditions, p75NTR expression is similar when the cells are co-cultured on empty COS cells, COS cells transfected with DNER, or when the OECs are cultured alone. This suggests that DNER has a specific effect on OECs, upregulating BLBP. Though p75NTR expression is known to change with axon contact (Ramon-Cueto, Perez and Nieto-Sampedro, 1993, Turner and Perez-Polo, 1998), DNER most likely does not play a role in this regulation, as the numbers of p75NTR-positive cells did not significantly change in response to DNER.
We had predicted that we would see a similar change in OEC morphology in response to DNER as had been previously published in the Bergmann glia in the cerebellum (Eiraku et al., 2005). However, OECs stained with P75 and BLBP across culture conditions morphologically resembled one another. No strong differences in morphology could be immediately observed, and therefore no quantification of morphological shift was undertaken. Other methods to quantify changes in OEC expression were considered, but OECs lack a stable marker which does not readily change based on context, therefore western blotting changes in protein expression could not be normalized with a protein loading control. FACS analysis was attempted, but the results were too variable to use here, and therefore FACS analysis has not been shown here.

Later passage cells showed a decreased ability to respond to DNER in culture (figure 4.8). This could possibly suggest that BLBP expression must be consistently maintained in culture from the point of primary plating. It is possible that without axonal input, Notch receptor metabolism is perturbed, and lower levels of Notch receptor are expressed leading to an inability of cultured OECs to respond to the addition of Notch ligands such as DNER to the system. Notch endocytosis and recycling is a dynamic process often regulated by factors related to the canonical and non-canonical signaling pathways, and it is possible that loss of Notch ligands causes the downregulation of Notch insertion or Notch receptor translation, or conversely increases degradation or endocytosis (Le Borgne, Bardin and Schweisguth, 2005).
In conclusion, BLBP expression in OECs is decreased by the removal of DNER from the OEC native environment. Both by the wholesale removal of ORN axons which express DNER, and the specific removal of DNER \textit{in vivo} by knockout; or by dissection and culturing of OECs alone, OECs show a decrease in the robustness of BLBP expression as well as a decrease in the number of cells expressing BLBP. BLBP expression can potentially be rescued by maintaining DNER input in the culture system, but it is possible that this must be done consistently from early passage onwards.

Over time, the current method of culturing OECs most likely selects for a specific population of OECs. OECs that are actively ensheathing ORNs of the olfactory system express BLBP and also Notch 3 (Carson, Murdoch and Roskams, 2006), therefore the loss of BLBP could indicate the loss of the innate ability of OECs to support axon growth. Previous work in the lab was the first to make it clear that OECs cultured from different anatomical sources, the olfactory bulb and lamina propria respectively, possessed different characteristics \textit{in vitro}, including migration, expansion and levels of OEC marker expression. These differences in characteristics translated to significant differences in the behavior and ability of olfactory bulb derived versus lamina propria derived OEC respectively after they were transplanted into a lesion (Richter et al., 2005). It is therefore possible that within one subtype of OECs, BLBP-high or -low expressing cells show a differential ability to support outgrowth and regeneration. (Tanaka and Marunouchi, 2003) The relative ability of one phenotypic population versus another to sustain their in situ roles of supporting growth and regeneration has not yet been directly investigated,
but it is possible that maintaining BLBP expression in culture leads to a maintenance of a phenotype which will display enhanced support of growth and regeneration as compared to those OECs which are 'un-maintained', and no longer express BLBP (see figure 1.7).

Elucidating this mechanism in OECs may give insight into the regulation and maintenance of specialized glial cells across the CNS, particularly in the spinal cord wherein there may be similar mechanisms for the regulation of the BLBP expressing glial cells. It is possible that with the right cues, OECs may be shifted to a state more permissive of repair after injury or disease, or may be induced to work cooperatively with transplanted cells such as OECs.
CHAPTER 6: CONCLUDING REMARKS

This thesis has established that DNER expressed in ORNs regulates BLBP expression in neighboring OECs. BLBP and DNER are also expressed in glial and neuronal cell types in close apposition in other specialized areas of the central nervous system including the hippocampus, retina and spinal cord. Future directions for this work are threefold. (1) Establishing the pathway by which BLBP is induced by DNER would give more insight into the specific regulation of OEC phenotype. Furthermore, (2) a difference in the functional consequences of BLBP expression, and (3) the direct role of the BLBP protein itself in the functional ability of OECs should be investigated.

1) BLBP is a direct target of Notch signaling (Anthony et al., 2005), and OECs do express Notch 3 in vivo and in vitro (Carson, Murdoch and Roskams, 2006). Furthermore, DNER is a known Notch ligand in the cerebellum (Eiraku et al., 2005). Therefore, it is likely that BLBP induction in OECs occurs through Notch signaling as a result of interactions with DNER. Investigating the role of canonical and non-canonical Notch signaling in OEC BLBP expression and regulation of other parameters such as morphology, will help us to understand how to maintain or prime OECs before or during their use as a transplant into CNS injury.

2) Once the mechanism of regulation of BLBP expression in OECs has been established, the functional consequences of BLBP expression in OECs must be directly tested. High-BLBP expressing OECs should be directly tested against low-BLBP expressing OECs for their relative rates of migration, support of axon outgrowth, interaction with astrocytes, and myelination. It must be established that
BLBP-expressing OECs represent a subpopulation of OECs which present a differential functional phenotype.

3) BLBP itself is directly implicated in the regulation of cell morphology leading to changes in functional ability; this has been tested in radial glia and Bergmann glia using function blocking BLBP antibodies (Feng, Hatten and Heintz, 1994, Anton et al., 1997). The specific role of the BLBP protein in postnatal glia is still not totally understood, elucidating its functional role as an intracellular protein will help us to understand why certain cells of the CNS are able to better sustain neuronal migration than others which are closely related. It remains to be seen if BLBP participates directly in the regulation of OEC functional phenotype, or if it will serve primarily as a marker of a shift in OEC state.

Though these cells represent a possible candidate for auto-transplantation after CNS injury, they are a heterogeneous population which can vary phenotypically based on their context. Understanding the specific pathways by which OECs and axons dynamically regulate one another is the next step in assessing the ability of cells such as OECs to positively affect their milieu after transplantation. Once we have elucidated the specific mechanisms by which OECs shift in state, and what these shifts in state will mean on a functional level, transplantation efforts can be better controlled and more targeted, reducing variability in the outcome of such paradigms.
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