BEHAVIOUR AND EFFECTS OF PERIPHERAL OLFACTORY ENSHEATHING CELLS TRANSPLANTED AT THE SITE OF ACUTE SPINAL CORD INJURY

by

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B.Sc.H., The University of Western Ontario, 1999

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES

(Department of Neuroscience)

We accept this thesis as conforming to the required standard:

THE UNIVERISTY OF BRITISH COLUMBIA

October, 2003

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ABSTRACT

Mammalian olfactory neurons are replenished from a progenitor pool in the PNS and extend axons into the CNS throughout adult life. This capacity for neurite outgrowth has been attributed in part to olfactory ensheathing cells (OECs), glia that fasciculate olfactory axons in both the PNS and the CNS. When transplanted at or near the site of spinal cord injury (SCI) in rodents, OECs from the olfactory bulb of rodents and humans have elicited regeneration and remyelination of damaged axons, sometimes with concomitant functional recovery. As a result, OECs have emerged as candidates for autologous transplantation to repair human SCI, and Phase I clinical trials are underway.

OECs from the olfactory bulb have been isolated and examined in culture and after transplantation into animal SCI for more than a decade; however, their behaviour upon transplantation remains ambiguous, as they lack a defining molecular marker, and the mechanism of OEC-mediated repair of SCI remains debatable. More pertinent to the use of OECs in the clinic is the dearth of experiments testing the efficacy of OECs derived from the lamina propria (LP-OECs), the source of OECs for clinical autotransplantation.

Here, LP-OECs were purified from GFP-expressing mice and transplanted at the site of acute dorsolateral funiculus crush in immunosuppressed rats and mice; spinal cords were examined histologically at 48h, 7d, 28d and 60d (rats) and 28 d (mice). LP-OECs had limited survival and migration in both rats and mice: after 28d, LP-OECs were confined to the lesion site in both species, and no LP-OECs were encountered in rat spinal cords after 60d. However, LP-OECs prevented cavitation and altered astrogliosis: after 28d, the lesion sites of LP-OEC transplanted animals contained many axons, as well as Schwann cells and new blood vessels, both of which might provide a scaffold for axonal growth. Serotonergic and noradrenergic

bulbospinal axons exhibited robust sprouting, with a few axons regenerating across the lesion site; anterogradely-labeled rubrospinal axons did not regenerate. Thus, despite a transient presence and limited migration within host spinal tissue, LP-OECs have beneficial effects at the site of acute rodent SCI, and support growth of some supraspinal axons.

(word count = 349)

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LIST OF ABBREVIATIONS

βIIIT: beta-III-tubulin

BBB: Basso, Beattie, Bresnahan open-field locomotion score (see Appendix I)

BDA: biotinylated dextran amine

BDNF: brain-derived neurotrophic factor

BDT: biotinylated dextran tetramethylrhodamine

bFGF: basic fibroblast growth factor

C1: first (most rostral) cervical segment of the spinal cord

CFDA, SE: carboxyfluorescein diacetate, succinimidyl ester

CGRP: calcitonin-gene-related peptide

CNS: central nervous system

CsA: cyclosporine A

CSPG: chondroitin sulfate proteoglycan

CST: corticospinal tract

DβH: dopamine beta-hydroxylase

DH: dorsal horn

DiI: 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (used to trace neurons in fixed tissue)

DLF: dorsolateral funiculus

DREZ: dorsal root entry zone

DRG: dorsal root ganglion

ECM: extracellular matrix

F: Fischer (rat strain)

FB: fast blue (diamidino 253/50, Sigma; a fluorescent, retrograde neuronal tracer)

FG: Fluoro-Gold (Fluorochrome Inc., CO; a fluorescent, retrograde neuronal tracer)

GAP-43: growth-associated protein-43

GFAP: glial fibrillary acidic protein

GFP: green fluorescent protein

IHC: immunohistochemistry

I, II, III… : Rexed's laminae; cytoarchitectonic regions of the grey matter in the spinal cord

L1: first (most rostral) lumbar segment of the spinal cord

LE: Long Evans (rat strain)

LP-OEC: olfactory ensheathing cell residing in or harvested from the lamina propria

MEP: motor-evoked potential

MP: methylprednisolone

NF-200: high molecular weight neurofilament

NGF: nerve growth factor

OB: olfactory bulb

ON: olfactory nerve (fasiculated olfactory fibers intracranial to the cribiform plate)

OB-OEC: olfactory ensheathing cell residing in or harvested from the olfactory bulb

OEC: olfactory ensheathing cell

ORN: olfactory receptor neuron

PNS: peripheral nervous system

P0: protein zero (in peripheral myelin)

p75: low-affinity nerve growth factor receptor

PKH26: Red Fluorescent Cell Linker kit (Sigma) for phagocytic cell labeling

RECA-1: rat endothelial cell antigen
S1: first (most rostral) sacral segment of the spinal cord
SC: Schwann cell
SD: Sprague Dawley (rat strain)
SEP: sensory-evoked potential
T1: first (most rostral) thoracic segment of the spinal cord
TH: tyrosine hydroxylase
WGA-HRP: wheat germ agglutinase-horseradish peroxidase (used for both anterograde and
           retrograde neuronal tracing)
W: Wistar (rat strain)
5HT: serotonin

ACKNOWLEDGEMENTS

For ongoing support and lots of good times, I thank the members of the Tetzlaff, Ramer and Roskams Labs: I was fortunate to work with so many good scientists and good friends.

For wonderful conversations during surgery, for patient teaching, and for unwavering enthusiasm for this and all projects, I thank Jie Liu.

For valuable intellectual contributions and personal support, I thank Drs. John Steeves, Tim O'Connor, and Steve Vincent.

For compelling me to go on when it would have been easier to let me fall by the wayside, I thank Wolfram Tetzlaff.

For constantly helping me make deadlines, without a word about how things could be done before the last possible second, I thank Cheryl Niamath (without whom I would have missed the deadline for graduation).

For fun times, hard work, and for trying to warn me, I thank Erin Flynn.

For being supportive when he could rightfully have been angry, I thank Jason Dyer.

For being with me through seemingly endless emotional crises, for honest input, and for some of the best times of my life, I thank Brodie Smith, Sandra Kolybaba, Dave Malfair, and Chris Murray.

For loving me no matter how crazy they think I am, I thank my family; for loving me because I am crazy, I thank my sister.

For rekindling my passion for life and science, for insisting that I be honest with myself, and for being my best friend, I thank Matt Ramer.

For understanding, I thank Mary Bunge.

STATEMENT OF CONTRIBUTION

(CHAPTER II)

I performed approximately half of the primary culture and maintenance of OECs used in this project, and prepared OECs for approximately half of the transplantation experiments. I performed all immunocytochemistry, microscopy of OECs in vitro (Fig 3), and quantification of OEC antigenicity (2.3.1). I assisted in surgeries (animal preparation, anesthetic, and post-operative care), and performed immunosuppression injections daily for all rats and mice used in this study; I assisted in a similar capacity with anterograde tracing. I performed all necropsy, perfusions, dissections, and histology (but had assistance with some sectioning) for Figures 4,5,8,9,10; I took the pictures required for these figures with some assistance and guidance at the microscope. I performed quantification of OEC migration (Fig. 4). Finally, I co-wrote the first draft of the paper, and participated extensively in its preparation for publication.

Leanne Ramer

I certify that Leanne contributed to this work significantly and as described above.

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DEDICATION

To my mothers and grandmothers, for their examples of class and courage, and for their fine

advice:

Being happy is hard work; work hard at making it look easy.

When you get to the end of your rope, tie a knot and hang on.

Smile. It makes people wonder what you're up to.

Illegitimi non carborundum.

CHAPTER I

OECs: Optimal or Equivocal Candidates for bridging the site of SCI?

The great tragedy of Science - the slaying of a beautiful hypothesis by an ugly fact.
Thomas H. Huxley

1.1 The olfactory system: neurogenesis, not regeneration

In regeneration research, we are inundated with the oversimplified doctrine of failed regeneration of nerves in the central nervous system (CNS) *versus* successful regeneration of nerves peripheral to the brain and spinal cord. The olfactory system is often touted as a singular exception to this dogma. A phylogenetically ancient component of the mammalian nervous system (Ariens Kappers *et al.*, 1960), the olfactory system is established around a primary signal transduction element, the olfactory receptor neuron (ORN), which is unique in several capacities. The soma resides in a neuroepithelium, rather than a sensory ganglion; the ORN arises outside the neural tube, from the olfactory placode; as well, the neuron makes direct physical contact with the external environment, on cilia that project from dendrites in the olfactory epithelium into mucus covering the nasal cavity. More remarkably, the ORN is the only neuron in the adult mammal that is known to be routinely replenished from a well-defined progenitor pool: this turn-over occurs both in normal function throughout life and in response to traumatic or chemical damage of dendrites, soma, axons or synaptic targets in the olfactory bulb (Graziadei and Monti Graziadei, 1977; 1978). Conferred with the ability to replace its environmentally-bared neurons, the olfactory system is often described as the only portion of the adult mammalian CNS where "regeneration" occurs.

However, as neurons were originally described as perennial cellular elements (cells that are never replaced, even when destroyed), the term "regeneration" applied to the nervous system indicates regeneration of parts of the neuron (typically of the axon) and not of the cell body

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(Ramon y Cajal, 1928; Graziadei and Monti Graziadei, 1983). By this classical definition, regeneration has not been reported in the olfactory system: ORNs have not been shown to spontaneously regrow their axons in response to damage, but rather to rapidly degenerate (Graziadei and Monti Graziadei, 1980; Doucette et al., 1983) and die by apoptosis (Deckner et al., 1997) without restoring functional connectivity. Therefore, the olfactory system does not exhibit regeneration but neurogenesis: ORNs are replaced, literally re-generated, throughout life.

Traumatic or chemical damage to ORNs, or removal of their target and associated trophic support via damage to (or removal of) the olfactory bulb, induces rapid cell death through a well-characterized program of apoptosis (reviewed by Cowan and Roskams, 2002). Such damage triggers a concomitant increase in both proliferation of basal cells and differentiation of ORN precursors, resulting in the replacement of some lost neurons (Camara and Harding, 1984; Gordon et al., 1995; reviewed by Calof et al., 1996). The olfactory system's exceptional ability to respond to trauma by reconstituting a portion of its primary neurons was identified by histologists in the 19th century (discussed in Graziadei and Monti Graziadei, 1977) and has been demonstrated since in many models of experimental injury to the mammalian olfactory epithelium (Mulvaney and Heist, 1971; Matulionis, 1976; Burd, 1993; Schwob et al., 1995, 1999; Ducray et al., 2002), nerve (Harding et al., 1977; Graziadei and Monti Graziadei, 1978; Monti Graziadei et al., 1980; Graziadei and Monti Graziadei, 1980; Doucette et al., 1983; Morrison and Costanzo, 1995; Yee and Costanzo, 1995) or bulb (Graziadei et al., 1978; Monti Graziadei, 1983; Butler et al., 1984; Monti Graziadei and Graziadei, 1992; Hendricks et al., 1994). Despite a predictable wave of neurogenesis, however, reinnervation of the olfactory bulb in the wake of traumatic manipulation *does not restore original connectivity to the system*: the degree and accuracy of bulbar reinnervation varies with the location and severity of the lesion (reviewed in Astic and Saucier, 2001). This is reinforced clinically: permanent anosmia is

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relatively common after blunt head injury, when movement of the brain relative to the skull shears ORN axons at the cribiform plate (Costanzo and Zasler, 1991). Permanent dysosmia (distorted sense of smell) can develop in the months after head trauma (Kern et al., 2000), indicative of deviant reinnervation of the olfactory bulb.

There are two exceptional features of the olfactory system that sometimes permit recovery of odour detection and discrimination in spite of meager or aberrant morphological recovery: redundancy in representation of odours and plasticity in processing of odour information. Although the details of these are beyond the scope of this review, and indeed not fully resolved, they are mentioned here in brief to highlight their potential importance in restoring olfaction after trauma.

In adult and neonatal animals, bilateral removal of the olfactory bulb results in permanent olfactory deficits, yet lesions sparing small portions of the olfactory bulb(s) allow animals to detect and discriminate among a wide variety of odours. These data indicate that the olfactory system, required for such vital activities as nipple attachment and suckling, foraging, and detecting predators, is conferred with extensive redundancy. In fact, olfaction is based on a combinatorial receptor coding scheme: most ORNs express a single odourant receptor, but a single odourant can activate several ORNs, and different odours are coded in the activation of different combinations of ORNs (Malnic et al., 1999; Figure 1a). Since ORNs typically project to a few glomeruli in different regions of the olfactory bulb, small portions of the bulb might contain the receptor codes for a wide variety of odours. In mammals, redundant representation of odours is particularly dramatic, as ORNs expressing a single odourant receptor typically project to one or more glomeruli in each olfactory bulb (Vassar et al., 1994; Nagao et al., 2000; Fig. 1b). Further, each olfactory bulb is an approximate duplicate of two mirror-image half bulbs. In

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theory, destruction of one-half of one olfactory bulb, split on the mediolateral axis, or even one whole olfactory bulb, would not reduce the odourant responsiveness of the system (Fig.1b).

If ORNs that participate in coding a particular odour are absent from a remnant of the olfactory bulb, evidence suggests that the olfactory system is capable of significant rearrangement of ORN projection patterns to restore function. After bilateral olfactory nerve transection, the recovery for odour detection and discrimination coincides with the onset of bulbar reinnervation (Yee and Costanzo, 1995). However, the time course of behavioural "recovery" is similar to the time required for initial training, and more recent data suggests that restoration of olfactory function is really relearning of a stimulus during the process of innervation (Yee and Costanzo, 1998). Studies in transgenic mice reveal that ORNs replenished after axotomy converge onto appropriate regions of the olfactory bulb, but not to normal (preoperative) glomeruli, such that topographical representation of odours on the olfactory bulb is altered after nerve injury (Costanzo, 2000). ORNs in transgenic mice engineered to express a rat odourant receptor (r17, responsive to octanal and heptanal) in place of one mouse receptor established functional connections and responded selectively to ocatanal and heptanal in the mouse olfactory bulb (Bozza et al., 2002). These results reveal a remarkable plasticity in the olfactory bulb that is induced by the arrival of novel olfactory sensory neurons (discussed in Vosshall, 2003). This capacity of ORNs to recruit appropriate postsynaptic targets, rather than accurate reinnervation of the olfactory bulb, seems to be the critical feature allowing the olfactory system to recover function after nerve injury.

Although extensive redundancy and astonishing plasticity render the olfactory system incomparable to other known functional circuits of the CNS, these features do not detract from the significance of neuronal replenishment in preserving olfaction throughout life. Neuronal precursors in the olfactory epithelium of the adult retain the ability to differentiate, grow and

navigate from the peripheral nervous system (PNS) to the CNS, and establish functional connections in the mature olfactory bulb. These exceptional capacities of olfactory receptors are differentially attributed to inherent capabilities of ORNs and ORN precursors (Mumm *et al.*, 1996; Calof *et al.*, 1998a,b; Shou *et al.*, 1999), to cues and support from other cells unique to the olfactory system (Weiler and Farbman, 1999) and to the glial environment of the olfactory system (Barber and Lindsay, 1982; Doucette, 1984, 1990; Raisman, 1985; Ramon-Cueto and Nieto-Sampedro, 1992).

Figure 1: Combinatorial coding of odours and redundant odour representation in the olfactory system.

(a) The olfactory system uses combinations of approximately 1000 G-protein-coupled olfactory receptors (GPCRs) to define an enormous range of odours. The olfactory receptors are located on the olfactory cilia, which extend into a layer of mucus covering the olfactory epithelium: the cilia are specializations of ORN apical dendrites, and they are the site of sensory signal transduction. The cilia from each ORN express only one type of GPCR: however, most odourant molecules are recognized by more than one receptor, and each receptor can recognize several odours which share a molecular feature. Therefore, the identification of an odour depends on both which receptors are activated and to what extent. Here, colour and shape represent two molecular features: receptors are maximally activated if bound by molecules that have both appropriate features (for example, the red square odourant at the bottom right), but can still recognize molecules that have only one of the appropriate features (such as the red circle odourant at the bottom right). (b) The combinatorial coding of odours permits redundancy of odour representation within the olfactory system. ORNs expressing a single GPCR project to several different glomeruli within each half olfactory bulb: the glomeruli are arranged so that each olfactory bulb is an approximate mirror-image of the other olfactory bulb, and each bulb is an approximate duplicate of two mirror-image half-bulbs. Thus, even a small portion of one olfactory bulb likely contains a glomerular repertoire sufficient to encode a wide variety of odours. After Firestein (2001).

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a

4. Coded odour information is sent to higher brain structures via the lateral olfactory tract

3. Activated ORNs in different portions of the OE project to the same glomeruli in the OB

2. Olfactory receptors on cilia are activated

1. Odourant molecules enter nasal cavity

LATERAL OLFACTORY TRACT

OLFACTORY BULB

CRIBIFORM PLATE

OLFACTORY EPITHELIUM

MUCUS

b

Medial

Lateral

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1.2 Olfactory ensheathing cells: a new type of glia

OECs were unremarkable to histologists in the time of Ramon y Cajal: after *la reazione nera* (Golgi silver impregnation), OECs appeared as "astrocytes of the olfactory bulb" (Ramon y Cajal, 1995). When the olfactory system was revisited through the electron microscope decades later (Gasser, 1956; de Lorenzo, 1957), an exceptional relationship between axons and glia was revealed: the plasma membrane of the OEC (then termed olfactory Schwann cell) forms a mesaxon for fascicles of extremely small fibres (0.1-0.5 μm in diameter; Figure 2). Fasciculation begins in the olfactory mucosa, where primary olfactory axons exit the basement membrane and enter OECs in groups of 15-40 (Gasser, 1956), and increases by convergence to form unmyelinated fibres (the olfactory nerves) constituting the *fila olfactoria* central to the cribiform plate of the ethmoid bone. The olfactory nerves enter the CNS in the olfactory bulb, where they shed their OEC ensheathement, terminate in glomeruli, and synapse on periglomerular cells (interneurons) and mitral cells (second order neurons of olfaction). Thus, the association between OECs and primary olfactory neurons is distinct from other unmyelinated axons, since OECs ensheathe axons in bundles and never individually. While other unmyelinated axons, such as those of dorsal root ganglion cells, are also "connected in bunches" peripherally by Schwann cells (Gasser, 1955), the numbers of fibres congregated in mesaxons are many times larger in the olfactory system than in other nerves.

Although they ensheathe axons in the periphery, evidence quickly mounted to reinforce the notion that olfactory "Schwann cells" represented an entirely new class of glia. Typically, Schwann cells are individually enclosed by basal laminae and collagen fibres (Glees and Meller, 1968; Carlsen and Behse, 1980) and collagen fibres extend into peripheral nerve fascicles, demarcating Schwann cell-axon units (Bunge *et al.*, 1989). In the olfactory nerve, basal laminae form around nerve fascicles, such that contributing OECs share a common basal lamina and

there is no collagen in the fascicle interior (Frisch, 1967; Barber and Lindsay, 1982). Like astrocytes, OECs express GFAP, terminate in end feet on the basement membrane of blood vessels, and contribute to the glia limitans at the surface of the olfactory bulb (Barber and Lindsay, 1982; Doucette, 1984). The developmental origin of OECs, derived from the olfactory placode (Marin-Padilla and Amieva, 1989; Farbman and Squinto, 1985; Doucette, 1989; Chuah and Au, 1991), distinguishes them from both astrocytes and Schwann cells, derived from precursors in the neural tube and neural crest, respectively (Le Douarin, 1986; Raff, 1989). In addition to the ambivalent nature of OECs, their organization within the olfactory bulb caught the interest of regeneration scientists. In the PNS-CNS transitional zones of spinal afferents and cranial nerves III-VI, the basal laminae of Schwann cells are continuous with those of the glia limitans of astrocytes, such that axons entering the CNS abruptly encounter oligodendrocytes (in the case of myelinated fibres) or astrocytes (Maxwell *et al.*, 1969; Steer, 1971; Fraher, 1988a,b, 2000). Olfactory neurons cross from PNS to CNS where their axons enter the nerve fibre layer in the ventral olfactory bulb: in this "nerve entry zone" OECs alone comprise the glia limitans, while astrocytes are excluded (Doucette, 1991). Thus, primary olfactory axons navigate into the CNS in the company of OECs, and without meeting astrocytes, until they synapse in the glomeruli. The unusual nature of OECs, and their unique arrangement at the PNS-CNS interface of the first cranial nerve, led to the hypothesis that these atypical glia are the critical elements that cause or allow olfactory receptor axons to grow into the olfactory bulb and establish functional synapses there throughout adulthood (Barber and Lindsay, 1982; Raisman, 1985; Doucette, 1984, 1990, 1993).

Figure 2: Location of OECs in the olfactory system.

Schematic representation of the relationship between ORNs and OECs. ORNs reside in the olfactory epithelium; their axons are ensheathed by OECs as they exit the olfactory epithelium and extend toward the OB. Several nerve fascicles share a common mesaxon, formed by a single OEC (nucleus N). The principle difference between this organization and that of other unmyelinated fibres is the large number of fibres in each fascicle: axons in the center of each fascicle are surrounded by other fibres, and do not contact the OEC membrane. Thus, in addition to OEC cytoplasm, a large portion of the mesaxon is occupied by the small ORN axons. ORN axons cross the cribiform plate, and remain ensheathed by OECs until they synapse within glomeruli (knots of neuropil) in the olfactory bulb. Importantly, OEC ensheathement extends into, but not beyond, the glomerulus, where astrocytic ensheathement begins. The reader should note that, although OECs are typically harvested from either the OB or the LP of the olfactory epithelium, OECs are present along the entire length of the ORN axon. After de Lorenzo (1957) and Raisman (2001).

mitral/periglomerular
dendrites

astrocyte process

ORN axon

OEC process

olfactory bulb

glomerulus

cribriform plate

ORNs

lumen

OEC

olfactory nerve axons

mesaxon

N
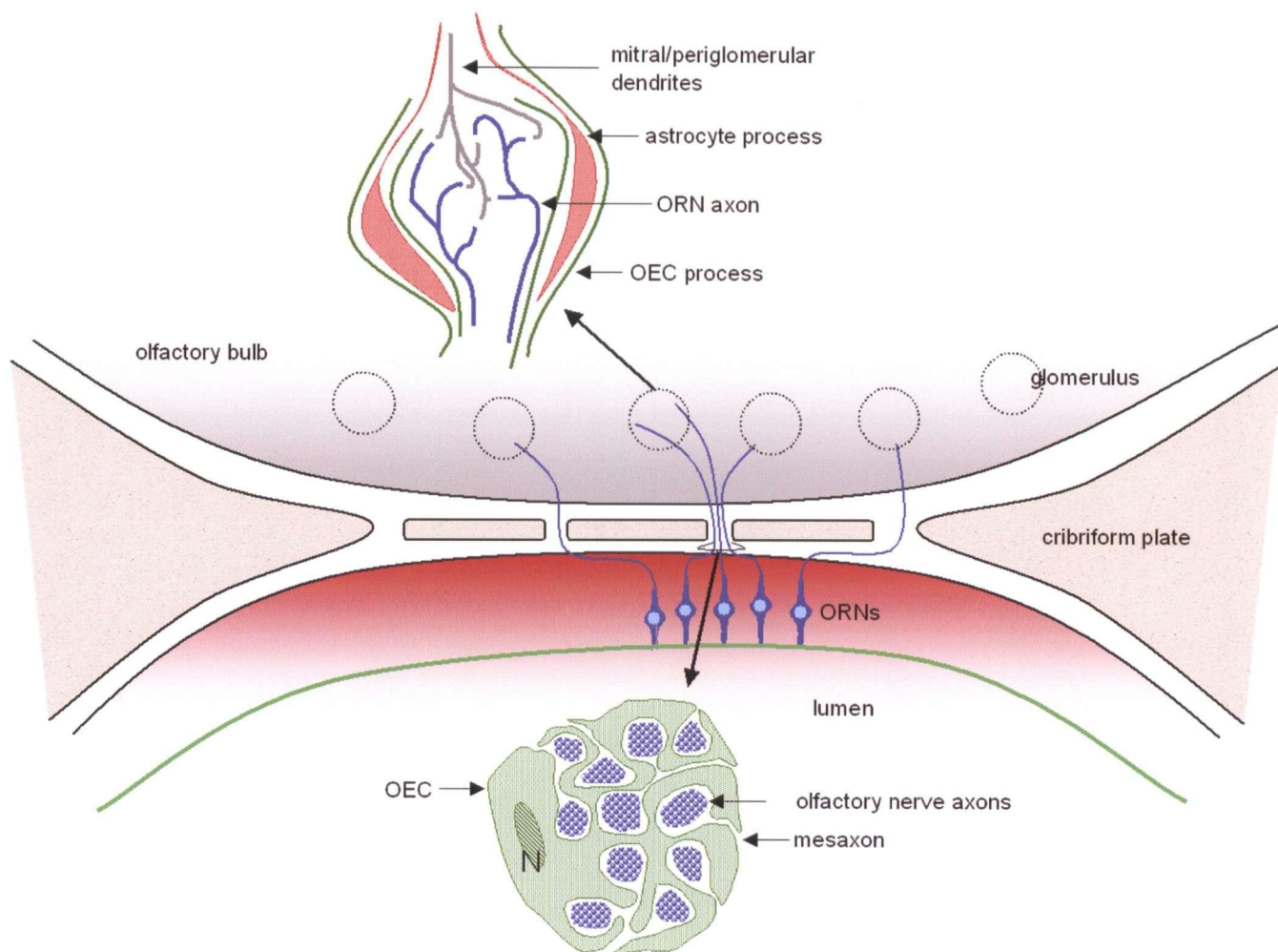
1.3 Obtaining OECs from the olfactory bulb

As a link between axonal growth and OECs was established, the race to develop purified, expandable cultures of these seemingly talented glia began in rodents. Early attempts to examine OECs *in vitro* demonstrated that cultures from the olfactory mucosa contained predominately fibroblasts, while cultures from the olfactory bulb, after removal of the meninges, contained large numbers of OECs (Barber and Lindsay, 1982). When intracranial OECs were found to be restricted to the nerve fiber (outer) layer of the olfactory bulb (Doucette, 1984), the efficiency of obtaining OECs from the olfactory bulb improved dramatically: the first cultures of OECs from the outer layers of the adult olfactory bulb grew readily *in vitro*, and contained mainly OECs after one passage (Ramon-Cueto and Nieto-Sampedro, 1992). The culture protocol relied on accurate dissection of the nerve fiber layer and removal of the pia to obtain cultures free of contaminating cell types, which was problematic due to the presence of interfascicular astrocytes in the adult nerve fiber layer (Doucette, 1993).

By obtaining OECs from the OB nerve fiber layer of neonates, which does not contain astrocytes or astrocyte precursors (Doucette, 1993), and by eliminating contaminating fibroblasts with Thy1.1-mediated complement lysis instead of removing the meninges, one group obtained cultures that were antigenically determined to be almost entirely (96-98%) ensheathing cells (Chuah and Au, 1993). At the same time, another group used the O4 monoclonal antibody, which binds to epitopes on the surface of Schwann cells, oligodendrocytes, and oligodendrocyte-type-2 astrocyte (O-2A) progenitors (Sommer and Schachner, 1981), and fluorescence-activated cell sorting (FACS) to isolate O4-positive, galactocerebroside (GalC)-negative OECs from the neonatal olfactory bulb without physically isolating the nerve fiber layer. FACS sorting for O4-positive cells enriched the proportion of O4-positive OECs in the bulb by nearly 10 times, and generated cultures that were at least 95% OECs (Barnett *et al.*, 1993).

In the search for a neurite growth-promoting factor, one of the most attractive features of OECs is their robust expression of p75, the low-affinity nerve growth factor receptor (Ramon-Cueto and Nieto-Sampedro, 1992; Chuah and Au, 1993; Barnett et al., 1993). Since Schwann cells, which are permissive to regeneration in the periphery, express p75, and this expression increases after nerve injury, this characteristic of OECs was linked with their apparent growth-supporting role in the olfactory system. When a pivotal *in vitro* experiment indicated that p75-expression was a critical, attractive factor in determining the path of neurite outgrowth from an explant of olfactory epithelium (Ramon-Cueto et al., 1993), purification of OB-OECs with immunoaffinity based on p75-expression became a prevalent method of obtaining OECs (Ramon-Cueto and Nieto-Sampedro, 1994; Ramon-Cueto et al., 1998, 2000; Yan et al., 2001; Takami et al., 2002; Plant et al., 2002, 2003). Cell selection based on p75-expression allows OECs to be reliably isolated from the olfactory bulb of adults; however, successful autotransplantation of OB-OECs has not yet been reported. Several characteristics of adult OB-OECs may be rate limiting in achieving experimental autotransplantation: these cells expand slowly, require maintenance *in vitro*, and have a limited life span in culture (Ramon-Cueto et al., 1992).

To circumvent these technical difficulties, and to guarantee a homogenous population of OECs, several groups have produced clonal cell lines of OECs. Using viral infection, nOECs (Goodman et al., 1993), ONOECs (tsT cl 1.6 ONECs; Franceschini and Barnett, 1996), and TEG OECs (Moreno-Flores et al., 2003) have been generated; as well, Rolf B1.T OECs were generated by a spontaneous immortalisation event within dissociated cells from the intracranial portion of the olfactory nerve (Sonigra et al., 1996). Immortalized OECs express many of the same proteins as OB-OECs in primary culture, including those most often associated with a growth-permissive phenotype (Ramon-Cueto, 1998; Boruch et al., 2001; DeLucia et al., 2003).

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Like their nontransformed counterparts (discussed below), the phenotype of immortalized OECs is dependent on culture conditions, such as cell density (Sonigra *et al.*, 1996; Franceschini and Barnett, 1996). Preliminary experimentation with each OEC cell line suggests that they retain their (growth-supporting and myelinating) abilities after immortalisation (discussed below) (Franklin *et al.*, 1996; Sonigra *et al.*, 1999; DeLucia *et al.*, 2003; Moreno-Flores *et al.*, 2003). Although tumour formation has been reported following transplantation of immortalized Schwann cells (Langford *et al.*, 1988), no occurrence of tumours has been reported after transplantation of an OEC line; in fact, recent evidence suggests that nOECs, at least, stop dividing after transplantation (DeLucia et al, 2003).

At present, immunoaffinity-based cell sorting procedures persist as prevalent methods of obtaining OECs (see Table I). Human OECs (hOECs) have been isolated from the adult olfactory bulb by immunopurification and maintained *in vitro* (Barnett *et al.*, 2000), although knowledge of their growth factor requirements remains scant in comparison to our understanding of rodent OECs (Pollock *et al.*, 1999; Chuah and Teague, 1999; Chuah *et al.*, 2000; Yan *et al.*, 2001; Alexander *et al.*, 2002). In the hunt for the proverbial better mousetrap, those in the field continue to pursue OEC isolation protocols designed to be easier, faster and less expensive. For example, OECs have been isolated from the adult rodent olfactory nerve and bulb on the basis of differential rates of attachment between cell types in the primary culture (fibroblasts, astrocytes, and OECs) (Nash *et al.*, 2001). This method produces cultures that are 93% OECs, and may collect a larger proportion of OECs, since it does not select against cells on the basis of antigenic profile. Regardless of the selection criteria, it should be noted that OECs chosen from the central component of the olfactory system for both *in vitro* examination and *in vivo* (transplantation) studies are likely never representative of the entire OEC complement available to ORN axons.

1.4 The elusive and heterogeneous phenotype of the OB-OEC

Over the last decade, the OEC has been subjected to extensive *in vitro* scrutiny by many groups searching to define its mRNA and protein complement. Contributions to the field of OEC molecular biology have been summarized comprehensively in previous reviews (Ramon-Cueto and Avila, 1998; Chuah and West, 2002; DeLucia *et al.*, 2003). The ensuing discussion of the antigenic expression profile of OECs is limited to the principles that confound this issue: an inherent capacity to alter gene and protein expression; an additional variability introduced by differences in experimental methods between research groups; and the incomplete knowledge of OEC behaviour within the olfactory system.

The complexity and plasticity of the OEC phenotype is immediately obvious: OECs vary both antigenically and morphologically during development *in situ* (Miragall *et al.*, 1989, 1992; Miragall and Dermietzel, 1992; Gong *et al.*, 1994; reviewed by Boyd *et al.*, 2003), over time *in vitro* (Franceschini and Barnett, 1996), and in response to different culture conditions (Doucette, 1993; Doucette and Devon, 1995) (discussed in Ramon-Cueto and Avila, 1998). These features hardly render OECs exceptional among glia: it is well established that Schwann cells, for example, vary phenotypically during development and *in vitro* (reviewed in Jessen and Mirsky, 1991). In fact, some of the factors that regulate Schwann cell phenotype *in vitro*, such as availability of extracellular cAMP, also govern OEC behaviour (Morgan *et al.*, 1991; Doucette and Devon, 1994; Doucette and Devon, 1995; Vincent *et al.*, 2003). Similarities between OECs and more familiar glia likely underlie the erroneous description of two distinct populations of OECs, S-type (Schwann cell-like) and A-type (astrocyte-like) OECs, reported to be present both *in situ* and *in vitro* (Franceschini and Barnett, 1996; Li *et al.*, 1998). Recent experiments reveal that OECs exist as a single, homogenous population of malleable cells: OECs rapidly convert between flattened and fusiform cells in response to changes in their *in vitro* environment, such as

addition or withdrawal of cAMP, forskolin, endothelin-1, or serum (Vincent *et al.*, 2003). Although the issue has been resolved, the unfortunate classification of OECs into "S-type" and "A-type" contributed to the confusion surrounding the true nature of these glia, and spawned extensive speculation surrounding the relative contribution of each OEC "type" to axonal repair (Li *et al.*, 1997, 1998; Raisman, 2001).

The description of OECs is further complicated by variation between laboratories, both in methods of obtaining OECs (described above), and in sources of the cells, which vary in age (embryonic, neonatal, or adult) and genera (rodent, canine, porcine, and human). Experience culturing Schwann cells and astrocytes has revealed that glia from animals of different ages and genus respond differently to *in vitro* manipulation (Yong *et al.*, 1992; Morrissey *et al.*, 1995). Much of our knowledge of the OEC phenotype is derived from rodent OECs: now that OECs have been successfully cultured from the human olfactory bulb (Barnett *et al.*, 2000; Kato *et al.*, 2000), it remains to be seen if human OECs will react predictably to *in vitro* manipulation.

In the majority of the nervous system, the glial response to nerve injury has been well-described: Schwann cells are known to respond to axotomy by proliferation and marked upregulation of p75 (Taniuchi *et al.*, 1986); astrocytes upregulate GFAP after peripheral axotomy and amongst degenerating CNS axons (Tetzlaff *et al.*, 1988; Murray *et al.*, 1990). Comparatively little is known about changes in OECs *in situ* after damage to or degeneration of their associated axons. In Schwann cells and astrocytes, p75- and GFAP-expression (respectively) increase with repeated passaging *in vitro* (Yasuda *et al.*, 1987; Passaquin *et al.*, 1994). These reactions may represent an *in vitro* analogue of the effect of axotomy *in vivo* (Yasuda *et al.*, 1987); however, it is not known whether this holds true for OECs. Like astrocytes, OECs upregulate GFAP in response to nerve section (Barber and Dahl, 1987); like Schwann cells, they upregulate p75 after nerve injury (Gong *et al.*, 1994; Turner and Perez-Polo,

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1994, 1998). The reports of *in vitro* expression of GFAP vary from a small proportion to nearly all of OECs obtained (Pixley, 1992; Nash *et al.*, 2001), while their expression of p75 has been reported to increase over time *in vitro* (Alexander *et al.*, 2002). It is not known whether OECs proliferate in response to injury, or indeed if they are ever produced in adult life. At present, it is difficult to assess whether OECs are more phenotypically malleable than other glia, or whether we simply have not learned enough: nonetheless, it is clear that the ability of OECs to alter their molecular complement in response to their environment, asserted as one of their chief advantages as potential regenerative agents, currently precludes concise or efficient description of the proteins they produce or express.

1.5 Do OB-OECs myelinate axons?

Although the CNS has the capacity to replace oligodendrocytes lost in spinal trauma or disease, and remyelination of persisting axons can be spontaneous, spinal remyelination is not reliable, and conduction block due to persistent demyelination likely contributes to poor functional recovery after SCI (Franklin and Hinks, 1999). Soon after their discovery, OECs were identified as candidates for overcoming persistent demyelination of spinal axons. The data on OEC myelination *in vitro* and *in vivo* is summarized here in brief: the efficacy of OECs *versus* other glial transplants in evoking spinal remyelination has been reviewed recently (Franklin, 2002).

1.5.1 *In vitro* demonstrations of OB-OEC myelination

In one of the first experiments using cultured OECs, they were reported to myelinate axons: when embryonic rat OECs were cocultured with embryonic rat dorsal root ganglion (DRG) neurons, myelin sheaths formed that were indistinguishable from those formed by SCs

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under similar conditions (Devon and Doucette, 1992). Recent data, however, suggests that OECs cultured from adult rats do not retain the ability to myelinate embryonic DRG neurons, and that myelin in DRG cultures is formed by Schwann cells that persist despite antimitotic treatment with fluorodeoxyuridine (FUDR) (Plant *et al.*, 2002). This work reveals significant differences in the way OECs and SCs associate with axons, and highlights the importance of SC-free systems in testing the myelinating potential of OECs. For example, while adult rat OECs were reported to enfold individual embryonic rat ORNs *in vitro* (Ramon-Cueto *et al.*, 1993) the possible presence of SCs in the ORN-containing epithelial explant cannot be excluded.

1.5.2 OB-OECs transplanted after focal spinal demyelination

Focal areas of demyelination in the adult spinal cord are created by injections of ethidium bromide or lysolecithin, both of which are toxic to oligodendrocytes. Typically, the demyelinating lesion is created in tissue that has been exposed to 40 Grays of X-irradiation several days previously, which prevents host oligodendrocytes from repopulating the lesion site (Blakemore and Patterson, 1978). Using this model, peripheral-type myelin has been observed in the demyelinated dorsal columns of the adult rat subsequent to transplantation of rat, human, and canine OECs (Franklin *et al.*, 1996; Imaizumi *et al.*, 1998; Barnett *et al.*, 2000; Kato *et al.*, 2000; Smith *et al.*, 2001, 2002; Lakatos *et al.*, 2003). In these experiments, myelination was apparent by two weeks and extensive by three-to-six weeks after transplantation. In at least one study, conduction block at the lesion site was overcome: OEC transplantation reduced conduction latency and increased conduction distance of compound action potentials recorded rostral to the lesion in response to stimulation caudal to the lesion (Imaizumi *et al.*, 1998). Peripheral (SC-type) myelin was identified by expression of P0 and a signet ring appearance under the light

microscope, characteristic of SCs that myelinate demyelinated CNS axons (Blakemore and Crang, 1985; Franklin *et al.*, 1992).

The recent *in vitro* data demonstrating the propensity of SCs to myelinate axons, even in the presence of OECs, has potentially important implications for interpreting the results of these transplantation experiments. X-irradiation destroys the astrocytes of the glia limitans, and thereby permits host SCs to gain access to demyelinated axons: it has long been known that, in the rat, host SCs readily invade the site of spinal demyelination and form peripheral-type myelin (Blakemore, 1975; Blakemore *et al.*, 1995). Based on the data currently available, it is not possible to determine to what extent OECs remyelinate host axons or facilitate influx of host SCs: reliable methods of identifying OECs within the spinal cord are required to conclusively identify the source of peripheral myelin present after OEC transplantation.

1.6 Do OB-OECs promote, or even most effectively permit, regeneration?

In addition to being regarded as a potentially effective remyelinating agent, OB-OECs are being evaluated as a cellular bridge to support axonal growth across the site of SCI (Bunge, 2001, 2002). The SCI field is in agreement that repair of at least some types of SCI will include implantation of a permissive substrate at the site of injury: what remains open for debate is the most suitable bridging agent, and whether it will be biological or synthetic. While early experiments suggested that OB-OECs were so successful in promoting regeneration that they might single-handedly promote repair of spinal axons, it is now generally agreed that a combinatorial approach will be required to effectively treat SCI (Bunge, 2001; DeLucia *et al.*, 2003). The question, then, is whether OECs are the most suitable bridging agent to constitute part of this combination therapy.

1.6.1 *In vitro* experiments testing OB-OECs as regenerative agents

Given the increasingly intense focus on OEC transplantation in the SCI field, one might assume that these cells have been extensively tested in simpler systems in order to demonstrate, optimize, and characterize their role in nerve injury repair. Actually, prior to 1998, only three experiments demonstrated that OB-OECs support the growth of adult ORNs in culture (Goodman *et al.*, 1993; Ramon-Cueto *et al.*, 1993; Chuah and Au, 1994). In the first experiment, ORNs preferred adult, p75-positive OECs over laminin substrate and p75-negative cells; no other glial substrate was used for comparison. The second study established clonal ensheathing cells lines (nOECs) from the olfactory bulb of neonatal and adult rats: both cell lines supported higher levels of neurite outgrowth than astrocytes from the same olfactory bulb. In the third experiment, the extent of neurite outgrowth over a monolayer of OECs was only marginally (but significantly) greater than neurite outgrowth over cortical astrocytes: importantly, adult ORNs did not extend neurites over laminin in response to OEC-conditioned media, suggesting that physical interaction between OECs and axons, and not (just) secreted trophic factor(s), is required for OECs to support neurite outgrowth. This hypothesis is supported by recent data obtained using adult rat OECs in coculture with both adult rat retinal ganglion cells (Sonigra *et al.*, 1999) and embryonic chick sympathetic, ciliary, and Remak's ganglia (Lipson *et al.*, 2003); using all types of neurons, neurite outgrowth was stimulated over OECs, but not in response to OEC-conditioned media.

Conversely, neurite outgrowth of embryonic rat ORNs was facilitated by embryonic rat OECs growing in the same media but spatially separated from neurons (Kafitz and Greer, 1998, 1999). There are several ways to interpret these apparently conflicting data. Greater amounts of trophic factors might be released from immature OECs: alternatively, it has been suggested that OECs produce trophic factors in such small amounts that they only reach efficacious

concentrations when OECs and neurons are in close proximity (Lipson *et al.*, 2003). Finally, only specific populations and/or ages of neurons may be responsive to diffusible trophic cues from OECs. In the midst of this debate, it is of significant interest that OECs do not express trophic factors at any stage in development in normal function of the olfactory system, but are induced to express many trophic factors when processed for culture (Woodhall *et al.*, 2001; Lipson *et al.*, 2003). Whether OECs produce trophic factors in response to injury of ORNs, in a manner analogous to Schwann cells after peripheral nerve injury (Sendtner *et al.*, 1992; Bolin *et al.*, 1995; Kurek *et al.*, 1996) is yet unknown. Neurite outgrowth over OB-OECs and SCs has been directly compared twice, using adult retinal ganglion neurons; however, variation in methods of quantifying neurite outgrowth complicate interpretation of these data. In one experiment, cultures over OECs contained a greater number of neurons bearing neurites (Sonigra *et al.*, 1999); in another study, cultures over OECs contained a greater proportion of neurons with long axons (Moreno-Flores *et al.*, 2003). The difference in total neurite outgrowth between cultures over OECs and cultures over SCs was not reported in either investigation.

It is notable that in a recent experiment, the level of p75 expression in OECs was not correlated with their ability to stimulate/support axonal regeneration (Moreno-Flores *et al.*, 2003). In this study, immortalized (TEG) OECs lost expression of p75, but continued to support outgrowth of retinal ganglion neurons, over time in culture. This report stands in apparent contradiction to data obtained more than a decade ago, in an experiment where olfactory neurites grew preferentially over OECs that expressed p75 (Ramon-Cueto *et al.*, 1993). The resolution of these potentially conflicting data may have important ramifications for immunoaffinity-based purification of OECs (see 1.3).

In addition to cocultures with various populations of neurons, OECs have been studied *in vitro* with other glia and CNS tissue explants. In one of the most influential *in vitro* experiments,

the behaviour of OECs and SCs were directly compared by culturing both types of glia with astrocytes. In this study, SCs and astrocytes in coculture grew in distinct territories separated by clear boundaries, while OECs and astrocytes intermingled without obvious demarcations: however, both SCs and OECs successfully migrated over astrocyte monolayers (Lakatos *et al.*, 2000). Coculture with SCs induced hypertrophy and increased CSPG expression in astrocytes, neither of which occurred in astrocytes cocultured with OECs. Since astrocytic hypertrophy and CSPG expression are consistent with astrogliosis subsequent to CNS trauma (Fawcett and Asher, 1999), the innocuous mingling of OECs with astrocytes rendered OB-OECs particularly attractive as cellular regenerative agents.

1.6.2 *In vivo* experiments: comparing OB-OECs with other bridging strategies

OECs from the olfactory bulb have been recurrently grafted at or near spinal cord injuries in rats in an effort to promote regeneration of both ascending and descending spinal axons (reviewed by Franklin and Barnett, 2000; Ramon-Cueto and Santos-Benito, 2001; Lu and Ashwell, 2002; Santos-Benito and Ramon-Cueto, 2003; see Table I). As implied above, these experiments vary widely in method of obtaining and purifying OECs and in phenotypic criteria for identifying OECs both *in vitro* and *in vivo* (after transplantation). Other sources of variation include timing of OEC grafting after injury, number of OECs implanted and the implantation site(s), and models of SCI. Anatomical and functional evidence of OEC-mediated regeneration reported in these studies is reviewed here; what is immediately obvious is the paucity of experiments directly comparing the effects of OB-OECs to those of other bridging strategies. As different populations of axons have different requirements for regrowth (Bradbury *et al.*, 2000), I attempt to meaningfully compare the relative success of OB-OECs *versus* other bridging strategies by comparing the data available on growth of various axonal populations (primary

afferents, corticospinal, and bulbospinal) after several types of SCI (dorsal rhizotomy, lacerative SCI, and non-penetrating SCI). These animal models of traumatic SCI are differentially appropriate for addressing experimental questions: while penetrating injuries are usually more definitive for assessing anatomical evidence of regeneration, nonpenetrating injuries more accurately mimic the majority of human injuries and are useful for examining pathophysiologic sequelae of SCI (Kwon *et al.*, 2002). The reader should note that while functional recovery ascribed to anatomical regeneration of each population is described, alternate mechanisms, such as collateral or regenerative sprouting, may contribute to the recovery observed in these experiments.

Table I: OB-OEC transplantation after SCI.

All studies were performed in adult rats, strain-matched to the source of OECs; Imaizumi *et al.* (2000) transplanted OECs into adult Wistar rats. Abbreviations listed on page ix.

***Preceding this study, some (6/12) rats were immunosuppressed (CsA, 10mg/kg/d, i.p.) over two weeks following a unilateral dorsal column aspiration and bisbenzimide-labeled OEC transplantation to study OEC survival. The authors reported no overlap between bisbenzimide and OX-42 (immunoreactive macrophages/microglia) in immunosuppressed or non-immunosuppressed rats, and therefore concluded that nOECs survived for two weeks with or without immunosuppression. Immunosuppression was not reported in any of the other studies listed.

Study	Source & Donor	OEC isolation	Antigen or Label	Injury model	OEC injection	Survival & Migration of OECs	Sparing/ sprouting/ growth/ regeneration	Other results reported
Ramon-Cueto and Nieto-Sampedro, 1994	Adult rat (W), OB	p75-positive cell-sorting	Bis-benzimide	Dorsal root transection (T10)	30,000 OECs at root anastomosis	Encountered in I-V of the DH after 21d (<1mm)	CGRP- and GAP-43-positive axons reached I-V of the DH (IHC, DiI at root stump)	N/A
Li et al., 1997	Adult rat (AS), OB, ON	No purification	p75	Electrolytic unilateral CST lesion (C2)	125,000 OECs into lesion site	Encountered 3mm from injection site after 21d	CST axons (BDA at contralateral pyramid)	Improved forepaw reaching in OEC-treated rats (4/7) after 2-3 months
Li et al., 1998	Adult rat (AS), OB	No purification	p75, GFAP, laminin, P0	Electrolytic unilateral CST lesion (C2)	100,000 OECs into lesion site	Encountered 10mm from injection site after 3 months	CST axons (BDA at contralateral pyramid or sensorimotor cortex)	OEC transplant highly angiogenic
Ramon-Cueto et al., 1998	Adult rat (F), OB	p75-positive cell-sorting	Bis-benzimide	Complete transection, (T9)	200,000 OECs at cord stumps, SC-Matrigel tube	Encountered 15mm from injection site after 6 wks	CGRP- and 5HT-positive axons (IHC); ascending propriospinal neurons (WGA-HRP at C7)	Few CGRP- or 5HT-positive axons entered SC-Matrigel tubes without OECs
Navarro et al., 1999	Adult rat (W), OB, ON	p75-positive cell-sorting (frozen)	PKH26	Dorsal root transection (L3-L6)	30,000 OECs at root anastomosis	Encountered in I-V of the DH after 60d (<1mm)	CGRP-positive axons reached I-II of the DH (IHC)	Restitution of H-reflex and withdrawal reflex in OEC-treated rats after 60d
Imaizumi et al., 2000	Adult transgenic pig, OB	No purification	CFDA, SE	Dorsal column transection (T11)	30,000 OECs at 0.5mm above and below lesion	Encountered 8mm from injection site after 5 wks	Ascending axons in the dorsal columns (medial lemniscus) (restored conduction velocity across the lesion site)	SC transplantation also restored conduction velocity across the lesion
Ramon-Cueto et al., 2000	Adult rat (W), OB	p75-positive cell-sorting	Bis-benzimide	Complete transection, (T9)	200,000 OECs at cord stumps	Present after 8 months; no estimate of migration	CST axons (BDA at sensorimotor cortex); 5HT, noradrenergic axons (IHC)	Improved grid climbing, contact placing, proprioception in OEC-treated rats
Pascual et al., 2002	Adult rat (W), OB	p75-positive cell-sorting (frozen)	PKH26	Dorsal root transection (L6, S1, S2)	30,000 OECs at root anastomosis	Survival, migration not reported	DRG axons reached I-II of the DH (WGA-HRP injected into the bladder wall)	Improved bladder function (cystometography)in OEC-treated rats

Study	Source & Donor	OEC isolation	Antigen or Label	Injury model	OEC injection	Survival & Migration of OECs	Sparing/sprouting/ Growth/regeneration	Other results reported
Nash et al., 2002	Adult rat (SD), OB, ON	Differential rate of attachment	Cell Tracker Green	Bilateral dorsal column transection (C3)	100,000 OECs in each cut surface of the dorsal columns	Encountered 19mm from injection site after 7 wks	CST axons (BDT at motor cortex)	Improved forepaw reaching in OEC-treated rats after 6wks: functional recovery potentiated by MP treatment
Takami et al., 2002	Adult rat (F), OB	p75-positive cell-sorting	p75	Moderate contusion (T8)	2,000,000 OECs into contusion site	Present after 3 months; no estimate of migration	CST axons (BDA at motor cortex), bulbospinal axons (FB at T11), CGRP-, 5HT-, DβH-positive axons (IHC)	Reduced cavitation, angiogenesis in OEC- and SC-treated rats
Ruitenberg et al., 2002	Adult rat (F), OB	p75-positive cell-sorting	Bis-benzimide p75, viral eGFP	Unilateral DLF transection (C4)	100,000 OECs, 1mm rostral and caudal to lesion	Encountered only within 0.5mm of lesion center after 4 months	NF-positive axons (IHC)	Redistribution of bisbenzimide to host cells, including astrocytes
Plant et al., 2003	Adult rat (F), OB	p75-positive cell-sorting	p75, S100	Moderate contusion (T10)	2,000,000 OECs into contusion site (DELAYED 30min or 7d)	Present after 8 wks; no estimate of migration	Rubrospinal, other bulbospinal axons (FG 6mm caudal from caudal edge of contusion), 5HT-, GAP-43- and RT97-positive axons (IHC)	Reduced cavitation in OEC-treated rats, improved hindlimb function (BBB 12 vs 10) in 7d delay-treated rats ONLY
DeLucia et al., 2003	Neonatal rat (SD), OB	Transduced with SV40 large T antigen	None	Bilateral dorsal column transection (T8)***	500,000 OECs into the lesion site	Survival, migration not reported	Thionin-stained axons in horizontal section	Reduced cavitation, improved grid-walking (5-20wks after injury) in OEC-treated rats
Verdu et al., 2003	Adult rat (SD), OB	p75-positive cell-sorting (frozen)	None	Photo-chemical lesion (Rose Bengal) (T8)	180,000 OECs into the lesion site (DELAYED 30min)	Survival, migration not reported	No anatomical evidence of regeneration reported	Reduced cavitation, reduction in hypertrophic astrocytes, restitution of MEPs and SEPs and reduced withdrawal latency to heat (10-90d after injury) in OEC-treated rats

Reference	Source	Purification	Label	Lesion	Cell dose	Migration	Anatomical findings	Functional recovery
Li et al., 2003	Adult rat (AS), OB, ON	No purification (50% fibroblasts)	Adeno-viral eGFP	Hemisection (high cervical)	400,000 cells (OECs and fibroblasts) into the lesion site within ECM	Encountered only within 1mm of lesion center after 10d	Thionin- and propridium iodide-stained axons in horizontal section	Recovery of breathing rhythm in ipsilateral phrenic nerve (19/40 rats, 2m after injury) and improved inclined grid climbing (1-7wks)
Ruitenberg et al., 2003	Adult rat (F), OB	p75-positive cell-sorting	p75	Unilateral DLF transection (C4)	100,000 OECs, 1mm rostral and caudal to lesion	Encountered only within 0.5mm of lesion center after 4 months	Rubrospinal axons (BDA at red nucleus) ONLY when OECs expressed BDNF, NF-positive axons (IHC)	N/A
Resnick et al., 2003	Adult rat (LE), OB	p75-positive cell-sorting	Bis-benzimide	Moderate contusion (T8-T9)	250,000 OECs into the contusion site	Encountered several mm from the lesion site after 6 wks	No anatomical evidence of regeneration reported	No improvement in hind limb function (BBB score) in OEC-treated rats

(i) Regrowth of primary afferents after dorsal rhizotomy

OECs have been reported to promote regeneration of primary afferents following dorsal rhizotomy (transection of DRG axons distal to the DREZ) in adult rat (Ramon-Cueto and Nieto-Sampedro, 1994; Navarro *et al.*, 1999; Taylor *et al.*, 2001; Pascual *et al.*, 2002). Dorsal rhizotomy is perhaps the most logical application for OECs, since lesioned DRG afferents regenerate but are stopped at the dorsal root entry zone (DREZ): unlike their sensory counterparts in the olfactory system, they are unable to cross from PNS to CNS (Liuzzi and Lasek, 1987; Carlstedt *et al.*, 1989). When OECs from the adult rat were injected into the spinal cord immediately following single cervical or quadruple lumbar dorsal rhizotomy, CGRP-positive DRG axons were encountered across the DREZ and within the ipsilateral dorsal horn (Ramon-Cueto and Nieto-Sampedro, 1994; Navarro *et al.*, 1999). There is evidence to suggest that these axons established functional synapses in the dorsal horn: spinal reflexes evoked by stimulation of the sciatic nerve reappeared in some OEC-treated adult rats eight weeks after quadruple lumbar rhizotomy (Navarro *et al.*, 1999); thermal stimulation of the ipsilateral forepaw activated c-fos expression in the dorsal horn four weeks after septuple cervical rhizotomy (Taylor *et al.*, 2001); and bladder tone reappeared in most OEC-treated rats 6 weeks after triple, bilateral lumbosacral rhizotomy (Pascual *et al.*, 2002). In contrast to these promising data, a recent study showed that no post-synaptic activity could be recorded from the dorsal horn in response to stimulation of dorsal roots six months after lumbar dorsal rhizotomy plus transplantation of neonatal rat OECs (Riddell *et al.*, 2002).

At present, OEC-mediated repair of the deafferented spinal cord is not evidently superior to ingrowth of primary afferents obtained with other transplant-based interventions. When transected dorsal roots in adult rat were anastomosed to embryonic rat spinal cord and brain grafted central to the DREZ, CGRP-positive axons traversed the DREZ and established

functional synapses with both donor neurons and host neurons within the spinal cord (Itoh and Tessler, 1990a,b; Itoh et al., 1996; Itoh et al., 1999). Comparable results were obtained when E14 spinal cord was cotransplanted with nitrocellulose membranes treated with either NGF (Houle and Johnson, 1989; Houle et al., 1996) or TGFβ (Prewitt et al., 1997). More pertinent to clinical repair of brachial plexus injuries, regeneration of primary sensory axons into the adult rat spinal cord was recently accomplished via a peripheral nerve autograft bridging the rhizotomized lumbar dorsal roots to the ipsilateral dorsal columns (Dam-Hieu et al., 2002).

ii) Regrowth of primary afferents after lacerative SCI

From their cell bodies in the DRG, the axons of primary afferents ascend in the dorsal columns. Regeneration of primary afferents in the fasciculus gracilus was reported when neonatal OECs from pig or rat were grafted rostral and caudal to a bilateral dorsal column transection (Imaizumi et al., 2000a, b). In these experiments, compound action potentials were recorded rostral to the injury site six weeks after injury, in response to electrical stimulation of the dorsal surface of the spinal cord one mm caudal to the injury site. Transganglionic labeling (by biotin injected at the DRG) revealed axons in the dorsal columns up to 20 mm beyond the lesion. In these studies, the extent of regeneration was equivalent for grafts of OECs and SCs.

(iii) Regrowth of corticospinal axons after lacerative SCI

In addition to primary afferents, the dorsal columns contain the corticospinal tract (CST), a large population of descending small-diameter fibres that mediate precise, voluntary movement in higher mammals. As such, the CST is typically regarded as the most critical tract for restoring motor function, and CST regeneration is often considered, perhaps unreasonably, the ultimate goal of regenerative interventions applied to SCI. OECs have been transplanted subsequent to

dorsal column lesions in an effort to evoke regeneration of these reputable efferents. When adult rat OECs were transplanted at the site of an acute electrolytic hemisection targeted on the dorsal-ventral axis to CST axons, anterogradely-labeled CST axons were encountered caudal to the OEC graft, although the authors acknowledge that this partial unilateral lesion often results in sparing of CST axons (Li *et al.*, 1997, 1998) and the morphology of axons shown caudal to the lesion was more typical of spared axons than of regenerating axons (Pallini, 1998). Despite its unusual nature, anatomical evidence of CST regeneration was accompanied by improvement in directed forepaw reaching (DFR) 2-3 months post-injury and OEC transplant. Recently, when cutting replaced electricity to induce a dorsal column hemisection that was confirmed as histologically complete, OEC-transplanted animals recovered respiratory rhythm in the ipsilateral phrenic nerve two months after injury and transplant, and showed significantly improved climbing ability by three weeks after injury and transplant (Li *et al.*, 2003): no anatomical evidence of CST regeneration was reported in this study. In another recent study, adult rat OECs were transplanted at an acute bilateral dorsal column transection, and produced long-distance regeneration of anterogradely-labeled CS axons and significant improvement in DFR 6-7 weeks post-injury and transplant; both of these results were enhanced slightly by combining OEC transplantation with systemic methylprednisolone (Nash *et al.*, 2002). In this experiment, as in its predecessors described above, CST axons encountered caudal to the lesion were straight, unbranched, devoid of end-bulbs, and projected normally within the dorsal columns: this morphology is more consistent with axons that survived the lesioning procedure than axons that regenerated through the lesion (Steward *et al.*, 2003).

In perhaps the most dramatic demonstration of CST regeneration, adult rat OECs were injected into the proximal and distal stumps spanning a complete transection of the spinal cord (Ramon-Cueto *et al.*, 2000). Here, CST axons were encountered in distal host spinal cord eight

months after injury: however, these axons were fine in diameter, tortuous, and projected ectopically (to lamina X). Although this morphology is more suggestive of sprouting/regenerating axons, it is unlikely that CST regeneration mediated recovery of the ability to climb an inclined grid in this study, since the most extensive patterns of CST regeneration within the spinal cord (rather than along the pia mater) were observed in both the best-performing and the worst-performing animals (Ramon-Cueto *et al.*, 2000).

To date, only one group has compared the extent of CST regeneration or sparing after lacerative injury treated with OB-OECs to that obtained with SCs: when OECs and SCs were applied in the same manner, to identical lesions of CST axons, anterogradely-labeled CST axons sprouted into SC grafts but did not exit, while labeled CS-axons exited OEC grafts and entered distal host tissue (Li and Raisman, 1994 and Li *et al.*, 1997, 1998). Regrowth of CST axons has not been achieved with other biogenic bridges, unless they are applied in combination with neurotrophic factors. For example, after a complete thoracic transection (with 5 mm excision), intercostal nerves grafted with FGF promoted regeneration of CST fibres, detected with anterograde WGA-HRP tracing, to the level of the lumbar enlargement (Cheng *et al.*, 1996). In this study, treated animals also recovered hind limb function from three weeks to one year subsequent to injury and treatment, demonstrated by significant improvement in combined behavioural score (Gale *et al.*, 1985), open field walking score (Behrmann *et al.*, 1992), and contact placing (Iwashita *et al.*, 1994). After an injury restricted to the dorsal columns, intercostal nerve grafts transfected to express NT3 also supported CST growth below the lesion with concomitant functional recovery, although in this study, CST fibres grew around, rather than through, the nerve graft (Blits *et al.*, 2000). In addition to SCs, fetal spinal cord tissue (from E14 rat) grafted at the site of complete transection also elicited regrowth of CST axons below the transection site, when applied in conjunction with intrathecal BDNF and NT3 (Coumans *et al.*,

2001). Importantly, this study showed that both regeneration and recovery of weight-supported stepping were enhanced by delaying transplantation by two weeks after injury.

(iv) Regrowth of bulbospinal axons after lacerative SCI

Although typically regarded as essential for coordinated movements of the limbs, the CST is not the only system that might mediate locomotor recovery. Axons descending from brainstem centers, particularly serotonergic axons from the raphe nuclei and noradrenergic axons from the locus coeruleus, have been implicated in rhythmic locomotion in cats (Gerin *et al.*, 1995; Rossignol *et al.*, 2001): these systems are considered modulatory in normal motor function, but may assume a more significant role during recovery from injury (Basso *et al.*, 2002). Evidence for regeneration of serotonergic and noradrenergic axons is both more abundant and more conclusive than analogous reports for CST axons, which are sporadic and unimpressive, often featuring one or two fibres encountered distal to the injury site. This disparity is exemplified in the application of OECs to a complete transection (Ramon-Cueto *et al.*, 2000): while evidence of CST regeneration was provided (see above), growth of serotonergic and noradrenergic (DβH-positive) axons across and distal to the transection site was far more abundant. A similar extent of serotonergic outgrowth was obtained after complete transection when OECs were injected at either end of a SC-seeded Matrigel channel (Ramon-Cueto *et al.*, 1998). Recovery of grid-climbing ability observed in the former study (Ramon-Cueto *et al.*, 2000), and also in a recent study using immortalized OECs from neonatal rat grafted at the site of dorsolateral hemisection (DeLucia *et al.*, 2003), may have been due to growth of these and other axons outside the dorsal columns.

Growth of monoaminergic brainstem-spinal axons has also been achieved with other biogenic grafts, but typically requires concomitant delivery of neurotrophic factors or other

compounds. Schwann cells within PAN/PVC channels grafted after complete thoracic transection at T8 supported ingrowth of propriospinal, but not supraspinal, axons (Xu *et al.*, 1995a). When the same treatment was combined with intrathecal BDNF and NT3, noradrenergic (DβH-positive) axons and serotonergic axons also grew into the graft but did not extend distally into host tissue (Xu et al, 1995b); retrograde tracing indicated that axons in the graft were descended from the raphe, reticular, and most predominately the vestibulospinal nuclei. When SCs secreting BDNF were applied after the same injury, serotonergic fibres regenerated across the distal graft-host interface one month after injury and treatment (Menei *et al.*, 1998). Fetal spinal cord tissue (from E14 rat) grafted at the site of complete transection, in conjunction with intrathecal BDNF and NT3, supported growth of raphe spinal, coeruleospinal, vestibulospinal and reticulospinal axons below the transection site 2 months after injury (Coumans *et al.*, 2001). This regrowth was accompanied by a return of weight-supported stepping on a treadmill and in stair-climbing; interestingly, both anatomical and behavioural recovery were most significant when this transplant was delayed for two weeks after injury. More recently, serotonergic axons grew across the site of a complete thoracic transection through a graft of embryonic spinal tissue, in response to subcutaneous administration of Rolipram, a compound that inhibits phosphodiesterase (PDE), commencing one week after injury (Filbin, 2003).

(v) Sparing/regeneration and recovery after nonpenetrating SCI

In endeavours to predict the behaviour and efficacy of OECs in clinical application, OECs have been grafted after nonpenetrating injuries that reliably produce post-traumatic spinal cavitation (Noble and Wrathhall, 1985; Gale *et al.*, 1985; Wrathhall *et al.*, 1985; Bunge *et al.*, 1994). These injuries more accurately mimic the clinical presentation of SCI, typified by significant functional deficits despite considerable sparing of spinal tissue (Bunge *et al.*, 1993). Adult rat OECs deposited at the site of photochemical lesion, induced by photosensitizing dye and dorsal spinal irradiation, prevented loss of spinal cord parenchyma and promoted recovery of nociceptive sensitivity (to heat) by 10 days after injury (Verdu *et al.*, 2001, 2003). Although motor-evoked potentials recorded from the tibialis anterior muscles were significantly higher in OEC-treated animals by two weeks after injury, these animals did not recover locomotor function (assessed by open-field movement and inclined-plane walking; Basso *et al.*, 1995; Rivlin and Tator, 1977) within three months after injury. Recently, adult rat OECs were transplanted at the site of moderate contusion injury in adult rat (Takami *et al.*, 2002; Plant *et al.*, 2003). In these studies, OECs promoted tissue sparing, and supported growth/sparing of serotonergic fibres and noradrenergic fibres: these axons grew into and through grafted OECs, but only serotonergic axons were encountered in distal host spinal cord. Behavioural recovery in OEC-grafted animals was observed eight weeks after injury in both experiments: OEC-transplanted rats attained an average BBB score near 11 in the earlier experiment, the same score as animals that received media only; OEC-treated animals scored near 14 in the later experiment, when OEC transplantation was delayed for seven days after injury.

Contusion injuries have been the target of a host of transplanted cells other than OECs, including SCs: in a recent study, SCs (like OECs) supported growth/sparing of serotonergic axons across the lesion site (Takami *et al.*, 2002). Animals that received SC transplants attained

an average BBB score near 12 (compared to 11 in media controls), although functional recovery might improve with delayed SC transplantation (as for OECs, discussed above). Stem cell transplantation has also produced significant functional recovery after contusion injury. For example, neural differentiated mouse embryonic stem cells, transplanted at the site of thoracic contusion nine days after injury, stimulated recovery of open-field locomotion: one month after injury, stem-cell treated animals achieved an average BBB score of 10, compared to an average score of eight in vehicle-treated animals or animals that received adult neocortical cells (McDonald *et al.*, 1999). Similarly modest but significant functional improvements were provoked by transplants of neural progenitor cells (Ogawa *et al.*, 2002), human neuroteratocarcinoma neurons (Saporta *et al.*, 2002), and bone marrow stromal cells (Chopp *et al.*, 2000); locomotor recovery was similarly accelerated by immediate transplantation of fibroblasts modified to express either BDNF or NGF (Kim *et al.*, 1996).

1.7 The clinical reality: OECs from the olfactory mucosa

Despite the considerable variation between laboratories in methods of obtaining OECs (see 1.3), describing OECs (see 1.4), and testing the ability of OECs to repair CNS axons *in vitro* and *in vivo* (see 1.5 and 1.6), these are not the most substantial hurdles to a safe and effective translation of OEC transplantation to the clinic. It is paradoxical in the extreme that all of the data described above pertains to OECs isolated from the olfactory bulb, a structure that can only be obtained using invasive neurosurgical procedures, while OECs residing in the olfactory epithelium remain virtually untested. The olfactory epithelium can be easily biopsied in humans without adverse effects (Lanza *et al.*, 1994; Feron *et al.*, 1998) and at least one protocol has been developed to isolate OECs from human biopsy tissue (Bianco *et al.*, 2003). Although clinical trials that are currently underway presumably involve some portion of the olfactory epithelium,

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there is an alarming dearth of data to verify the efficacy of these peripherally-derived OECs: while they may be identical in nature to their counterparts in the olfactory bulb, our current knowledge of how these glia respond to their local environment suggests that there may be important differences between peripherally- and centrally-derived OECs.

The first attempts to examine OECs *in vitro* showed that cultures from the olfactory mucosa contained large numbers of fibroblasts (Barber and Lindsay, 1982): in fact, this early discovery may have discouraged other groups from pursuing peripheral sources of OECs in favour of less-contaminated glia in the olfactory bulb. It was nearly two decades later that OECs were isolated from the olfactory nerve fascicles (rather than the olfactory neuroepithelium) of the neonatal rat, expanded in culture, and subjected to preliminary characterization (Tisay and Key, 1999). At first glance, these peripheral OECs were comparable to central OECs, as they expressed p75, GFAP, and s100β, and supported outgrowth of olfactory neurites from embryonic epithelial explants. Time-lapse imaging of these cells revealed that, like bulb-derived OECs, peripheral OECs rapidly convert between fusiform and flattened phenotypes *in vitro* (van den Pol and Santarelli, 2003). However, the neonatal olfactory nerve fascicles do not represent a large tissue reserve from which to obtain OECs, which may be the reason that these cells were never tested *in vivo*.

At around the same time, another forward-looking Australian laboratory grafted strips of olfactory epithelium or cultured but unpurified OECs from the epithelium from adult rats at the site of a complete transection of adult rat spinal cord (Lu *et al.*, 2001). Rats that received grafts of either strips of tissue or injections of epithelial cells attained an average BBB score near 5 by 10 weeks after injury; rats that received respiratory epithelial tissue or media injections scored near 2. This was accompanied by regeneration of raphespinal axons caudal to the lesion site, demonstrated by retrograde tracing to the brainstem and growth of serotonergic axons at least 3

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mm caudal to the lesion site. In a subsequent experiment, these scientists showed that strips of olfactory lamina propria could evoke the same degree of functional recovery and anatomical regeneration when transplanted four weeks after a complete transection (Lu *et al.*, 2002). Since rats that received strips of respiratory lamina propria, neighbouring tissue that does not contain OECs, the recovery observed in these experiments was attributed to the presence of OECs in the lamina propria.

Recently, a protocol has been developed to easily and reliably isolate OECs from the lamina propria of the neonatal mouse olfactory epithelium (LP-OECs; Au and Roskams, 2003). In this method, LP-OECs are purified from the entire olfactory epithelium by removing fibroblasts with Thy1.1-mediated complement lysis, used commonly to remove fibroblasts from cultures of Schwann cells (Brockes *et al.*, 1979). LP-OECs, subjected to rigorous *in vitro* characterization, expand very rapidly in culture compared to OECs from the olfactory bulb, and express many of the same proteins reported in bulb-derived OECs, as well as some not yet reported in central OECs. The expansion kinetics of these OECs render them ideal for transplantation experiments, and may finally permit the SCI community to examine the efficacy of OECs that are accessible, numerous, and the true candidates for autotransplantation to repair clinical SCI.

CHAPTER II

Peripheral olfactory ensheathing cells prevent cavitation and support axonal growth when injected at the site of acute spinal cord injury

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1.1 Introduction

In the adult nervous system, the response to traumatic disruption varies with location. In the peripheral nervous system (PNS), damaged axons have the ability to regrow and restore at least partial function (Fawcett and Keynes, 1990). In contrast, axons in the central nervous system (CNS) atrophy and fail to spontaneously regenerate (Kwon et al., 2002). The dire consequences of abortive regeneration in the CNS are exemplified by spinal cord injury (SCI), in which functional deficits are often permanent (Amar and Levy, 1999).

The differential potential for regeneration between adult PNS and CNS is attributed in part to the glia encountered by injured axons. In the PNS, Schwann cell (SC) expression of adhesion molecules and trophic factors has been linked to axonal regrowth (Ide, 1996). Failure of CNS regeneration has been associated with loss of trophic support (Ramer et al., 2000), as well as inhibitory molecules expressed by oligodendrocytes (Caroni and Schwab, 1988; McKerracher et al., 1994) and extracellular matrix molecules deposited by both oligodendrocytes and astrocytes after injury (reviewed by Fitch and Silver, 1997).

Olfactory receptor neurons (ORNs) are unique in the adult as the only neurons that are routinely replaced: ORNs are continually generated by precursors in the olfactory epithelium that differentiate, navigate their axons to the olfactory bulb, and establish functional synapses (Graziadei and Monti Graziadei, 1978; Schwob, 2002). Damaged ORNs degenerate and die but

are replaced by new neurons that grow from PNS to CNS and restore function to the system (Harding and Wright, 1979; Doucette *et al.*, 1983; Yee and Costanzo, 1995; Yee and Costanzo, 1998; Astic and Saucier, 2001). Thus, the olfactory system mounts a unique regenerative response that is typical neither of the PNS nor the CNS.

Neuronal replacement and axon guidance in the olfactory system occurs in the company of a unique population of glia, olfactory ensheathing cells (OECs) (Doucette, 1990). OECs ensheathe ORN axons from their exit point at the basal lamina of the olfactory mucosa (lamina propria OECs; LP-OECs) through the cribiform plate and into the nerve fiber layer of the olfactory bulb (OB-OECs) (Doucette, 1991; Doucette, 1993). These glia migrate from PNS to CNS with ORNs in development (Tennent and Chuah, 1996) and express adhesion molecules, trophic factors, and extracelluar matrix molecules associated with axonal growth (reviewed by Ramon-Cueto and Avila, 1998).

To change the glial environment of CNS axons damaged in SCI, and so to augment the regenerative response of these axons, SCs and OECs have been grafted at the site of spinal lesions (reviewed by Bunge, 2001). SCs and/or OECs promote regeneration of sensory and brainstem axons when transplanted at the site of thoracic spinal transection, hemisection, or moderate contusion in adult rat (Xu *et al.*, 1995, 1997, 1999; Ramon-Cueto *et al.*, 1998; Ramon-Cueto *et al.*, 2000;Takami *et al.*, 2002). OECs have emerged as particularly attractive candidates for CNS transplantation, as they have been reported to support regeneration of corticospinal axons (Ramon-Cueto *et al.*, 2000) and to mitigate astrocytic reactivity *in vitro* (Lakatos *et al.*, 2000).

The behaviour of OECs upon transplantation, and thus the mechanism by which they might support axonal growth, remains poorly understood. These cells lack a defining molecular marker, and have an antigenic and morphological profile that is highly plastic and overlaps with

endogenous cells, particularly with Schwann cells (discussed in Imazumi *et al.*, 1998; Takami *et al.*, 2002). While dye-loading of OECs prior to transplantation has provided some clues, vital dyes may enter other cells *in vivo*, complicating interpretation (Iwashita *et al.*, 2000; Ruitenberg *et al.*, 2002).

The majority of experiments testing the efficacy of OECs in promoting CNS regeneration use OB-OECs; however, if OEC transplantation is to be a viable treatment for clinical SCI, LP-OECs represent a more accessible source for allo- or autotransplantation. Despite experiments demonstrating that grafts of intact (Lu *et al.*, 2001) or dissociated (Lu *et al.*, 2002) olfactory mucosa promote regeneration of brainstem and sensory axons, purified LP-OECs remain untested in animal models of SCI. The importance of characterizing the behaviour of grafted LP-OECs is underscored by the initiation of human trials featuring autotransplantation of LP-OECs into patients with chronic SCI (http://www.ausbioinfo.com/news/articles/2002-07-12_108.php; (http://carecure.rutgers.edu/Spinewire/Articles/SCITrials2002/SCITrials2002.htm).

We have generated purified cultures of LP-OECs from transgenic mice expressing enhanced GFP driven by the β-actin promoter. These cells are similar (but not identical to) OB-OECs (Au and Roskams, 2003). Here, for the first time, we trace the fate of genetically-labeled LP-OECs grafted at the site of acute dorsolateral funiculus crush in rats and mice. With the interaction of transplanted and host cells reliably determined by combining GFP fluorescence with species-specific antibodies, we show that grafted LP-OECs collaborate with host glia to prevent cavitation at the site of injury and support sprouting of sensory and bulbospinal axons.

2.2 Materials and Methods

2.2.1 Preparation of OECs from GFP mice

OECs were harvested and cultured from the olfactory mucosa of homozygous transgenic mice expressing enhanced GFP in all cell types (GFP-U-NAGY; Hadjantonakis *et al.*, 1998; kind gift of Frank Jirik, U. Calgary), driven by a combination of β-actin promoter and CMV-enhancer. OECs were isolated using the procedure described by Au and Roskams (2003).

Between postnatal day five and seven, GFP mice were decapitated and the heads were split sagitally, allowing easy access to the olfactory turbinates. The olfactory mucosa was carefully dissected into ice-cold DMEM/F12 (Life Technologies, Rockville, MD), minced into small pieces, and incubated in DMEM with 1 mg/ml bovine serum albumin (Sigma, St. Louis, MO), 1.2 mg/ml Collagenase D (Roche Diagnostics, Laval, PQ), 3 U/ml dispase I (Roche), 30 µg/ml hyaluronidase and 100 U/ml DNAse I (Roche) for 1 hour at 37°C. After centrifugation (330 g), the dissociated cells were resuspended in 2 ml DMEM, triturated, and filtered: the flow-through was plated in MEM-d-valine with 10% heat-inactivated fetal bovine serum (FBS) and 1% (100 U/ml) penicillin/streptomycin (pen/strep) (all from Life Technologies) on an uncoated plastic flask (Sigma), and maintained at 37°C in 5% CO_2. After 4 days *in vitro* (DIV), the cells were passaged with 0.25% trypsin and 1% EDTA (Life Technologies) and incubated in DMEM/F12 with 100 µl anti-Thy 1.1 hybridoma supernatent (American Type Culture Collection, Manassas, VA) and 125 µl rabbit complement (Sigma) for 30 min at 37°C to remove contaminating fibroblasts. Cells were plated in DMEM/F12 with 10% FBS and 1% pen/strep at 5800 cells/cm^2 and maintained at 37°C in 5% CO_2; Thy1.1 antibody-mediated complement lysis was repeated at 8 DIV, after which OECs grew to confluency (2-4 further DIV) prior to transplantation. The total time from dissection to transplantation ranged from 11-14 DIV.

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2.2.2 Cryopreservation of OECs

For some experiments (3 of 28 d rats, all 60 d rats and all 28 d mice), OECs were cryopreserved after 9 DIV (i.e. 1 day after second Thy1.1 antibody-mediated complement lysis). OECs were passaged with 0.25% trypsin and 1% EDTA, rinsed in DMEM/F12, and resuspended at 1 million cells/ml in 50% FBS, 40% DMEM/F12, and 10% DMSO (Sigma). OECs were cooled to -80°C at a rate of 1°C/min and stored between -172°C ($N_{2(l)}$) and -150°C for 2-6 months. Cryopreserved aliquots of OECs were thawed in tepid (10-15°C) water, resuspended in DMEM/F12 with 10% FBS and 1% pen/strep, plated at 6000-13,000 cells/cm^2, and grown to confluency for 2-4 days until transplantation.

2.2.3 Immunocytochemistry

Each time cells were prepared for transplantation, a small aliquot was reserved for immunocytochemistry to determine the purity of OECs used for grafting. Samples of each culture were plated at 5800 cells/cm^2 in DMEM/F12 with 10% FBS and 1% pen/strep on at least two multi-chambered glass slides (Nunc, Rochester, NY) coated with poly-L-lysine (Sigma) and cultured for 24 h. Cells were fixed with 4% paraformaldehyde, rinsed in PBS, permeabilized in 0.1% TritonX 100 and incubated with primary antibody to S100β (1:500, Sigma), low-affinity nerve growth factor receptor (p75; 1:500, Chemicon, Temecula, CA), and glial fibrillary acidic protein (GFAP; 1:1000, DakoCytomation, Denmark) overnight at 4°C: only one antibody was used in each chamber. Cells were incubated with secondary antibodies (1:200, Jackson ImmunoResearch, Mississauga, ON) raised in goat and conjugated to Alexa 594 for 1 h at room temperature, dipped in bisbenzimide (1 µg/ml, Sigma) for 1 minute and coverslipped in glycerol mounting medium (Sigma). OEC cultures were characterized by randomly selecting a field of view (FOV) from each slide chamber under the UV filter, and counting cells positive for p75,

s100β, and GFAP. The proportion of positive cells was determined in at least 10 fields per antigen, with each FOV containing 25-30 cells.

2.2.4 Crush injury of the dorsolateral funiculus

Male Sprague-Dawley rats (n = 33, 150-200 g, Charles River Breeding Laboratories) and male C57Bl/6 mice (n = 12, 25-30 g, Charles River) were immunosuppressed (CsA, Novartis Pharmaceuticals, Mississauga, ON; 10 mg/kg/d, i.p.) commencing 2 d prior to injury and OEC transplantation, and maintained throughout survival. All animal procedures were performed in accordance with the guidelines of the Canadian Council for Animal Care and approved by the University of British Columbia Animal Care Committee.

Animals were anesthetized with ketamine (70 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.) prior to being secured in a stereotaxic frame (David Kopf Instruments, Tujunga, CA). A midline incision was made in the skin over the cervical vertebrae: the first (most superficial) muscle layer was cut along the midline, the second muscle layer was separated at the midline by blunt dissection, and the deep muscle layers were cut along the midline and deflected with retractors placed over the midline. The left half of the fourth cervical spinal cord segment (C4) was exposed by scraping off attached muscle laterally and using rongeurs to make a narrow window in the C4 vertebra: the medial extent of this window reached the midline, while the lateral extent exposed the edge of the spinal cord. Within this window, the dura was cut with microscissors and lidocaine was applied to the surface of the spinal cord. Fine surgical forceps (#5, Fine Science Tools, Vancouver, BC) were inserted to a depth of 1 mm, with one prong at the lateral border of the grey matter and one prong outside the dorsolateral funiculus (DLF): the forceps were closed and held for 18-20 s to crush the DLF. Upon completion of the injury, animals were randomly designated as treated or control by an investigator other than the surgeon.

2.2.5 OEC transplantation

Immediately prior to transplantation, OECs were harvested in 0.25% trypsin and 1% EDTA, washed in sterile PBS, and resuspended in DMEM at 50,000-100,000 OECs/µl. Directly following the crush injury, 75,000-150,000 OECs (1.5 µl of cell slurry for rats, 1 µl of cell slurry for mice, divided over three injection sites) were stereotaxically microinjected into the lesion site using a pulled glass pipette (tip diameter = 20 µm) glued to a Hamilton syringe. All three injection sites were located at the rostrocaudal level of the DLF crush; i.e., OECs were introduced directly into the site of acute SCI. The first injection site was approximately 300 µm lateral to the medial border of the white matter, at a depth of 1.3 mm (1.0 mm in mice); the second site was at a depth of 0.9 mm (0.7 mm in mice) directly above the first site, such that the pipette remained in the same position; the third site was approximately 400 µm medial from the lateral aspect of the DLF, at a depth of 0.7 mm (0.4 mm in mice) The cell slurry was introduced at a rate of 100 nl/min, and the pipette remained in place for five minutes after each injection. Control animals received identical volumes of DMEM injected at approximately the same rate and sites. Five minutes after the final injection, the pipette was slowly withdrawn and the muscle and skin were closed with interrupted sutures. Survival times were 48 h (n = 6), 7 d (n = 6), 28 d (n = 15) and 60 d (n = 6) for rats and 28 d (n = 12) for mice.

2.2.6 Anterograde labeling of rubrospinal axons

One week before the end of the survival period, rats were anesthetized as described above, and placed in a stereotaxic frame. The skin over the skull was cut along the midline, and the head was adjusted to a level position by taking height measurements at bregma and lamda. A dental drill was used to make a hole in the skull: the stereotaxic coordinates were 5.9 mm caudal to bregma and 0.7 mm lateral of the midline. A pulled glass pipette (tip diameter approximately

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20 μm) attached to a Hamilton syringe was introduced intracranially in the vicinity of the right red nucleus, to a depth of 7.0 mm. Biotinylated dextran amine (BDA) (10,000 kDa MW, 25% in 0.5% DMSO, Molecular Probes, Eugene, OR) was injected at a rate of 50 nl/min, with a total injection volume of 0.5-0.6 μl. The pipette remained in place for five minutes after injection, after which it was slowly withdrawn, and the skin over the skull was closed with interrupted sutures or surgical clips.

2.2.7 Immunohistochemistry

At the end of the survival period, animals were killed with an overdose of chloral hydrate (100 mg/kg, i.p.) and perfused transcardially with PBS followed by phosphate buffered, 4% paraformaldehyde (pH 7.4). The cervical spinal cords were dissected, post-fixed in 4% paraformaldehyde overnight, cryoprotected in 12%, 18%, and 22% sucrose in 0.1 M phosphate buffer over 3 days, and frozen in isopentane over dry ice. Cervical segments from C2 to C6 were cut into 20-micron longitudinal sections in the horizontal plane on a cryostat and stored at -80°C. Frozen sections were thawed on a slide warmer for five minutes, rehydrated in PBS, permeabilized for one hour in 0.1% Triton-X 100, and incubated with 10% goat serum for 20 minutes to prevent non-specific binding. The following primary antibodies were used: rabbit anti-green fluorescent protein (anti-GFP, 1:1000, Abcam, Cambridge, UK), rabbit anti-p75 (1:500, Chemicon), mouse anti-RECA-1 (Serotec), mouse anti-rat p75 (1:50, Boehringer-Mannheim, Mannheim, Germany), rabbit anti-GFAP (1:1000, Dako), mouse anti-neurofilament (anti-NF200, 1:500, Sigma), mouse anti-βIII tubulin (βIIIT;1:500, Sigma), mouse anti-CSPG (1:20, Sigma) rabbit anti-serotonin (anti-5HT, 1:8000, ImmunoStar, Hudson, WI), sheep anti-tyrosine hydroxylase (anti-TH, 1:500, Chemicon) and rabbit anti-calcitonin gene-related peptide (anti-CGRP, 1:4000, Sigma). All primary antibodies were applied overnight at 4°C; primary

antibodies were omitted on some sections to control for non-specific binding. Secondary antibodies (1:200, Jackson) raised in goat or donkey and conjugated to Alexa 594, Alexa 488, Alexa 350, Cy3, or aminomethylcoumarin (AMCA) were applied for 1 h at room temperature. BDA was visualized using Cy3-conjugated streptavidin (1:200, Jackson) applied for 1 hour at room temperature. Sections were coverslipped in glycerol mounting medium (Sigma) or Vectashield (Vector Laboratories, Burlingame, CA).

2.2.8 Image analysis

Digital images were captured with an Axioplan 2 microscope (Zeiss, Jena, Germany), a digital camera (QImaging, Burnaby, BC) and Northern Eclipse software (Empix Imaging Inc., Mississauga, ON) and were processed using Northern Eclipse and SigmaScan Pro (SPSS Inc., Chicago) software. Cavitation was quantified by measuring the area of cavity in 10 serial horizontal sections per animal with a section- to-section interval of 100 μm starting at the dorsal surface of the spinal cord. These 10 measurements were added to produce the cumulative area of each animal as a relative measure of the lesion size. The Student's t-test was used to compare treatment groups. Rostral-caudal migration of OECs was measured as the distance of each GFP-positive object (cell or group of cells) from a designated line drawn perpendicular to the spinal cord, through the lesion epicenter. Cells in the pia mater were excluded. Measurements were taken from 100x images of three-to-five longitudinal sections per animal.

2.3 Results

2.3.1 LP-OECs *in vitro* expressed GFP plus p75, S100β and GFAP

To examine the purity of the mucosa-derived OECs, an aliquot of each cell culture used for transplantation was replated for 24 hours and characterized by immunocytochemistry. The mucosa-derived OECs assumed both fusiform (asterisks) and flattened (arrowheads) phenotypes, as well as intermediate morphologies (Fig. 3). As expected, all cells were GFP-positive without immunological amplification, indicative of reliable and robust expression of GFP driven by CMV:β-Actin promoter. Virtually all cells (97.9±2.2 %) stained positive for p75 (Fig. 3a). Similarly, nearly all cells were immunoreactive for S100β (99.5±1.6 %); this protein was associated with the cytoskeleton and robustly expressed (Fig. 3b). The vast majority of OECs were also GFAP-positive (99.7±0.95%), although expression levels varied considerably (Fig. 3c). Thus, these cells were comparable to those previously described (Au and Roskams, 2003). No morphological or antigenic differences were detected between OECs cultured fresh and passaged 3 times (i.e. after 4 DIV, 8 DIV, and 12 DIV) compared to those frozen after 9 DIV and passaged 4 times: this is consistent with previous reports that one cycle of freezing and thawing does not affect the biological properties of OECs (Smith *et al.*, 2002).

Figure 3. OECs cultured from the lamina propria (LP) of GFP mice expressed GFP plus p75, S100β, and GFAP *in vitro*.

(a-c) LP-OECs reserved from cultures used in transplants and replated for immunocytochemical characterization. Images were captured from sparser edges of cultures to exhibit cellular morphology and do not reflect cell density *in vitro*. LP-OECs assumed both fusiform (asterisks) and flattened (arrowheads) phenotypes, as well as intermediate morphologies. All cells expressed GFP, which was readily visible without immunological amplification. (a) 97.9±2.2% of cells expressed p75, which appeared mainly in the cytoplasm due to permeabilization. (b) 99.5±1.6% of cells expressed S100β, which was associated with the cytoskeleton. (c) 99.7±.95% of cells expressed GFAP, although cytoplasmic expression levels of this protein varied considerably (n = 4 cultures from 2 separate primary cultures). Scale bar: 100 μm (a-c).
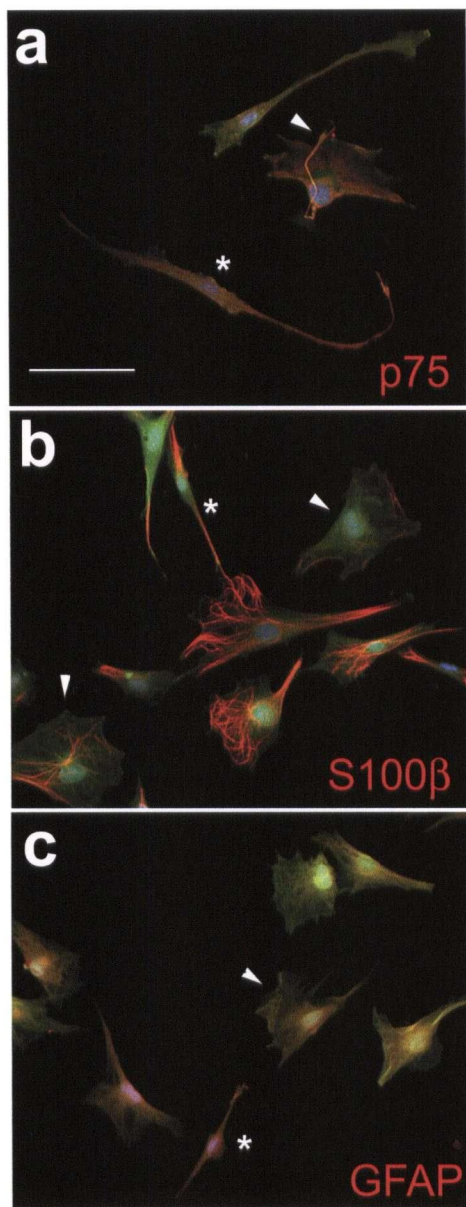
2.3.2 LP-OECs prevented cavitation but showed limited migration and survival

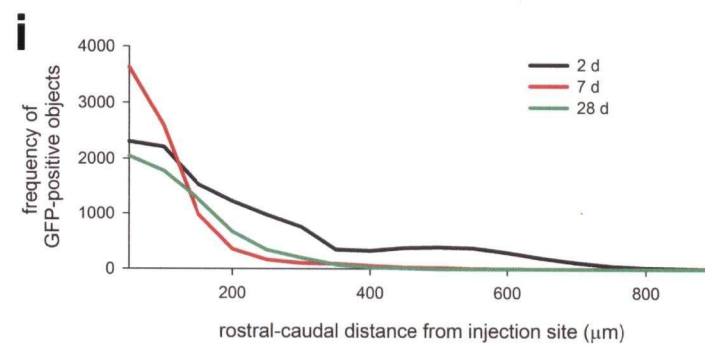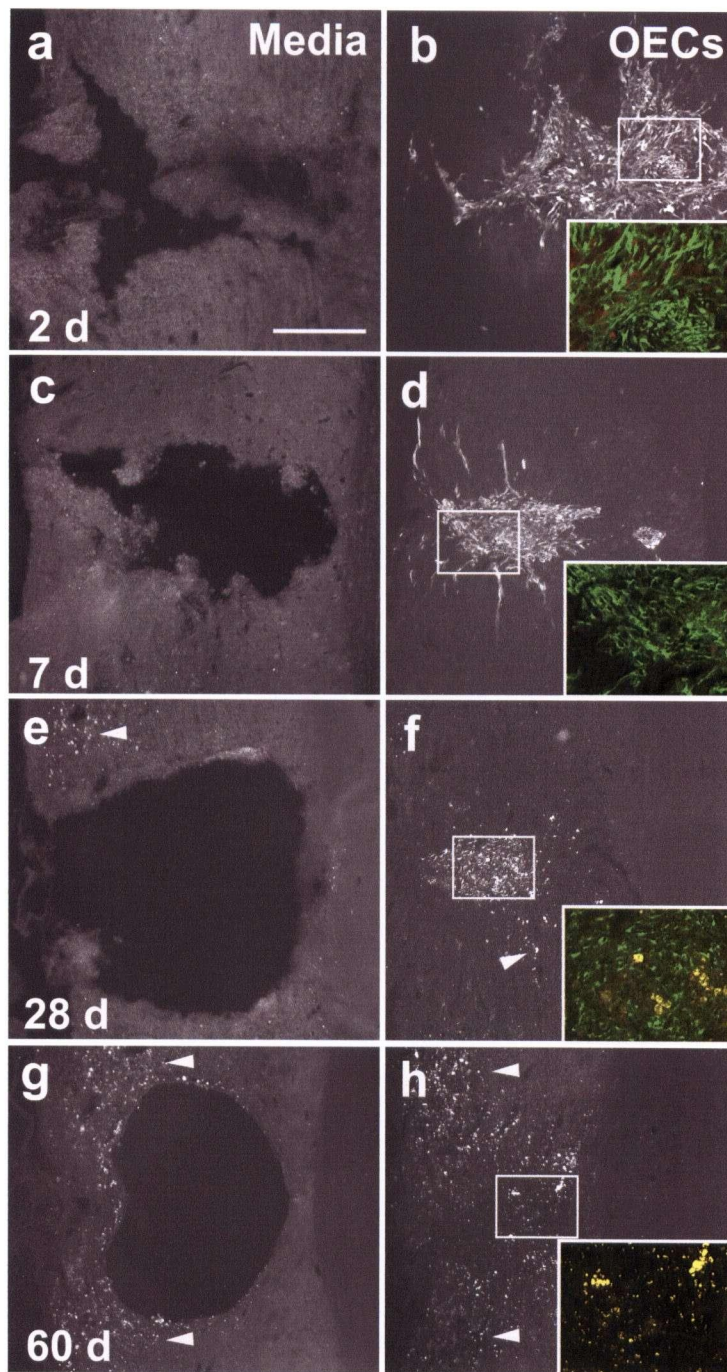In order to assess the behaviour and effects of OECs transplanted at the site of spinal cord injury we injected 75,000-150,000 cells (1.5 μl total volume) into an acute lesion of the dorsolateral funiculus in adult male Sprague Dawley rats. Typically, during the first week, a cavity formed at the site of injury in rats that received control injections of media (1.5 μl) (Fig. 4a,c). The size of the cavity generally increased in size over 28 days, reaching maximal diameters of 0.5 to 1 mm (Fig. 4e,g). In contrast, cavitation was significantly reduced (and usually absent) in rats that received OEC injections (Fig. 4b,d,f,h). Measurement of cavity area in horizontal sections of the spinal cord revealed a more than 99% reduction ($p<0.001$) on day 28 after lesion: the average area of cavitation in media-injected rats was 0.735 ±0.293 mm^2, and in rats that received OECs, 0.01 ± 0.004 mm^2.

The use of GFP as a stable genetic marker allowed us to assess survival and migration of the injected OECs. Immunohistochemistry with an antibody to GFP was used to enhance endogenous GFP expression of transplanted cells: however, this enhancement did not reveal any additional cells, indicating that the OECs expressed sufficient GFP to be detectable without enhancement. Overall, the number of OECs appeared to decline over time after transplantation: GFP-positive cells were encountered in only five of nine of the injected rats surviving 28 days and in none (of three) of the animals surviving 60 days. Without immunosuppression using CsA, GFP-positive cells disappeared within 2 weeks (data not shown). Similar to the regressive changes in survival, the migration of OECs appeared to diminish with time *in vivo* (Fig 4b,d,f). After two days, OECs filled the center of the lesion site, and numerous OECs migrated into the host tissue over distances of up to 700 μm from the lesion core (Fig. 4b). At seven days after injury and transplantation, fewer strands of OECs extended from the lesion center into the host tissue (Fig. 4d) and by 28 days, OECs were largely restricted to the center of the lesion site (Fig.

4f). Twenty-eight days after transplantation, OECs were not encountered farther than 400 μm away from lesion center (Fig. 4i). From seven to sixty days following lesion, strongly auto-fluorescent (GFP-negative) cells characteristic of monocytes had also invaded the lesion site and could be seen along the degenerating DLF several millimeters from the lesion center both in media- and OEC-injected animals (arrowheads).

Figure 4. LP-OECs prevented cavitation but showed limited survival and migration in rat spinal cord.

(a-h) longitudinal sections of rat spinal cord after injections of either 1.5 µl of serum-free media (a,c,e,g) or 75-150, 000 LP-OECs (b,d,f,h) into the site of acute dorsolateral funiculus crush. In animals that received control injections of media only, a cavitation formed at the injury site during the first week after injury: the cavitation reached maximal diameter (0.5-1 mm) by 28 days after injury. Cavitation was almost completely absent in animals that received OECs: at 28 days after injury, OEC-transplanted animals had a significant reduction in total area of cavitation measured in horizontal sections compared to media controls (0.01 ± 0.004 mm^2 *versus* 0.735 ±0.293 mm^2, p<0.001) (n = 6 media-injected, n = 9 OEC-injected rats). Despite immunosuppression, the number of OECs appeared to decline over time after transplantation, as GFP-positive cells were encountered in only 5/9 animals 28 days after transplantation and 0/3 animals 60 days after transplantation. At these time points, autofluorescent macrophages were present in and around the lesion site in both media- and OEC-injected animals, and were found several segments rostral and caudal to the lesion (arrowheads). (i) Migration of GFP-positive cells or groups of cells, measured rostrally and caudally from the injection site, also appeared to diminish with time (2 d: n = 3; 7 d: n = 3; 28 d: n = 3). Scale bar: 400 µm (a-h).

i

frequency of GFP-positive objects

rostral-caudal distance from injection site (μm)

2 d
7 d
28 d

53

2.3.3 LP-OEC transplantation enhanced Schwann cell invasion of the lesion site

The prevention of cavitation by OECs despite their limited survival prompted us to characterize the cellular elements of the lesion site. The availability of a rat specific antibody to p75 allowed us to distinguish endogenous p75-expressing cells, most likely Schwann cells, from the transplanted mouse OECs: importantly, the presence of p75-positivity identified cells as host-derived rather than donor-derived cells with undetectable GFP expression (Fig. 5). In media-injected rats, few host Schwann cells were encountered in the lesion site 28 days after injury (Fig. 5a), while in OEC-injected rats, the lesion site was filled with host Schwann-cells (Fig. 5b): the host Schwann cells persisted in abundance at 60 days after OEC transplantation, when OECs were no longer present at the lesion site (Fig. 5c). At seven days after injury, the transplanted OECs intermingled with host-derived Schwann cells (Fig. 5d). At increasing times following lesion, infiltrating Schwann cells migrated out further from the lesion center: by 28 days following lesion (when remaining transplanted OECs were confined to the center of the lesion site), host Schwann cells were found at the perimeter of the lesion, in close proximity to GFAP-expressing astrocytes (Fig. 5e). At all time points examined, the lesion was encircled by a laminin-rich boundary, bordered by host Schwann cells within (Fig. 5 f,g; arrowhead) and astrocytes without (not shown).

Figure 5. LP-OEC transplantation enhanced Schwann cell invasion of the lesion site.

(a) 28 days after media injection, host Schwann cells, identified by a rat-specific p75 antibody, were found at the lateral aspect of the lesion site (left); a few were present in the spinal cord, around the GFAP-positive border of the cavitation (n = 6). (b,c) 28 days after OEC transplantation, the lesion site was completely filled with host Schwann cells (n = 9); these Schwann cells persisted at 60 days, after the disappearance of OECs (n = 3). (d,e) Higher-power images of lesion site borders, showing the arrangement of host and grafted glia. After 7 days, OECs were intermingled with host Schwann cells in the center of the lesion; Schwann cells were encountered among GFAP-positive astrocytes adjacent to the lesion, while OECs appeared relatively segregated to the lesion site (n = 3). At 28 days, OECs were confined to the center of the lesion, and surrounded by a broad zone of host Schwann cells. (f,g) At all time points examined, the lesion was surrounded by a laminin-rich perimeter, separating Schwann cells on the lesion side (arrowhead) and astrocytes on the spinal cord side. Scale bars: a-c,f: 200 μm; d,e,g: 100 μm.

GFAP/rat p75/GFP

Laminin/rat p75
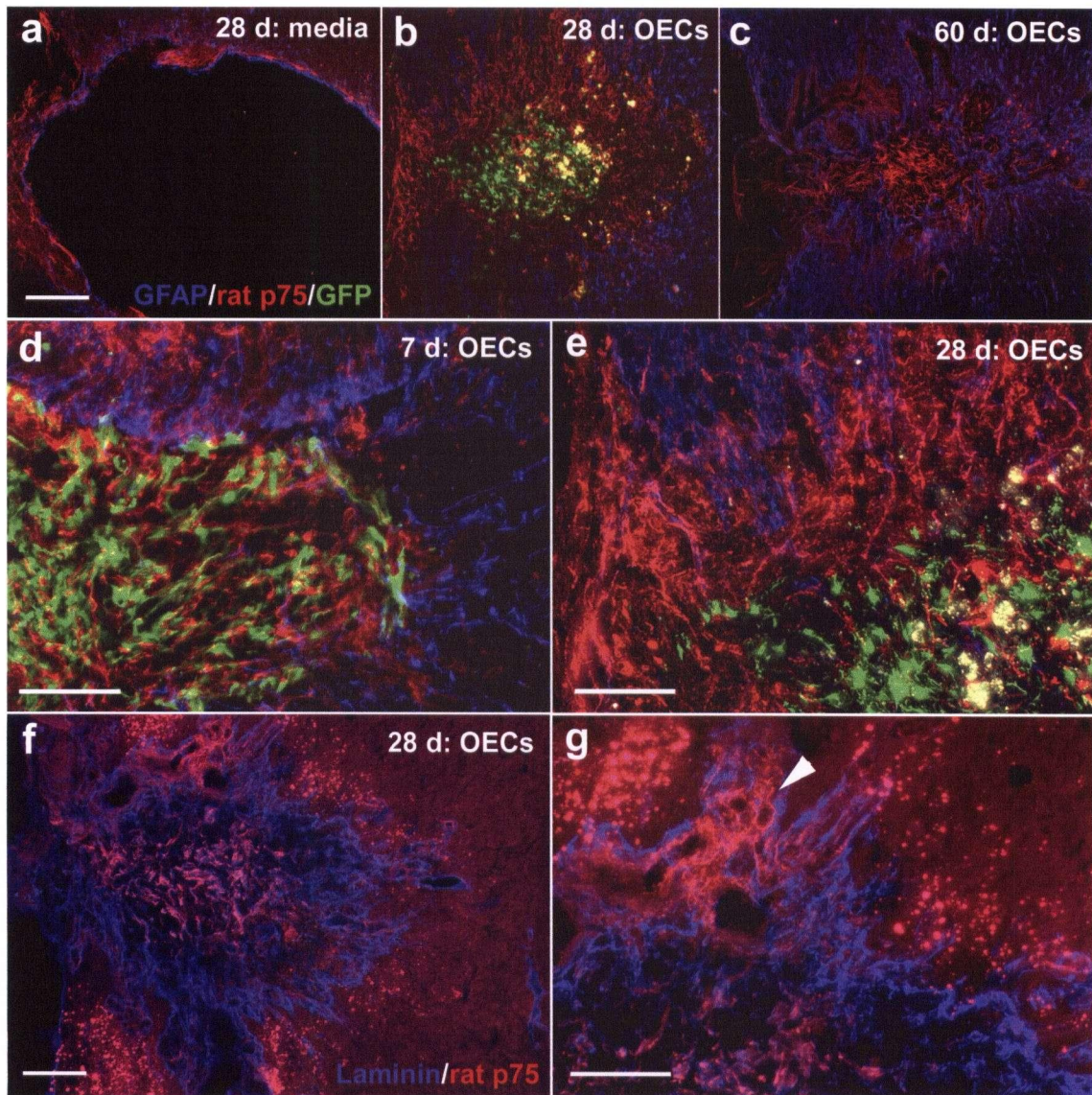
2.3.4 LP-OEC transplantation altered the arrangement of astrocytes and CSPG deposition around the lesion site

OECs coexist alongside astrocytes in the normal adult CNS, in the outer nerve fiber layer of the olfactory bulb; as well, OB-OECs have been reported to mitigate the reactive astrocytic response in a coculture model (Lakatos *et al.*, 2000) and when transplanted at the site of SCI (Li *et al.*, 1998; Verdu *et al.*, 2001; 2003). Therefore, we used immunohistochemistry for GFAP and CSPGs to examine the response of host astrocytes to OEC transplants at the lesion site. At 28 days after injury, the cavitation wall in media-injected rats was lined by a thin, dense zone of GFAP-positive reactive astrocytes (Fig. 6a-c). In contrast, a broader, diffuse zone of GFAP-positive astrocytes surrounded the OEC-treated lesion site: this wide band terminated in irregular finger-like projections, extending towards the lesion center (Fig. 6d-f). These astrocyte-filled projections interdigitated with the channels of Schwann cell-rich projections emanating from the lesion center (seen in Fig. 5), and did not form a continuous GFAP-rich boundary characteristic of media-injected rats. CSPG expression was evident in the tissue adjacent to astrocytes in both media- and OEC-injected rats: however, CSPG expression around the LP-OEC transplanted lesion site occurred mainly along the astroycte-rich projections, with CSPG-poor regions between the astrocyte processes (Fig. 6d, e). Thus, rather than inhibit astrocytic reactivity and CSPG deposition, the presence of donor OECs and host Schwann cells at the lesion site reorganized astrocytes and CSPG deposition into radial streams extending from the edge of the lesion, and prevented the formation of a dense barrier perpendicular to descending spinal axons.

Figure 6: LP-OEC transplantation altered the arrangement of astrocytes and CSPG deposition around the lesion site.

28d after lesion, a dense band of GFAP-positive astrocytes surrounded the cavitation at the media-injected lesion (a,b; arrow): the astrocytic border was continuous, and astrocytic processes were frequently tangential to the cavitation (c). Deposition of CSPGs was evident in media-injected rats, and extended radially beyond the astrocytic border (a,b; arrowheads). In rats that received LP-OECs, GFAP-positive astrocytes were found in a broad and diffuse zone around the lesion site. (d,e): the astrocytic zone was interrupted and irregular, and astrocytic processes frequently aligned longitudinally (f). At the OEC-treated lesion, CSPG deposition occurred within astrocytic projections (e, arrowhead), leaving CSPG-poor zones throughout the lesion site (d, asterisk). Scale bars: 200 μm (a,b); 50 μm (d,e); 250 μm (c,f).

control — **CSPG**/**GFAP**
+ OECs — **CSPG**/**GFAP**
GFAP
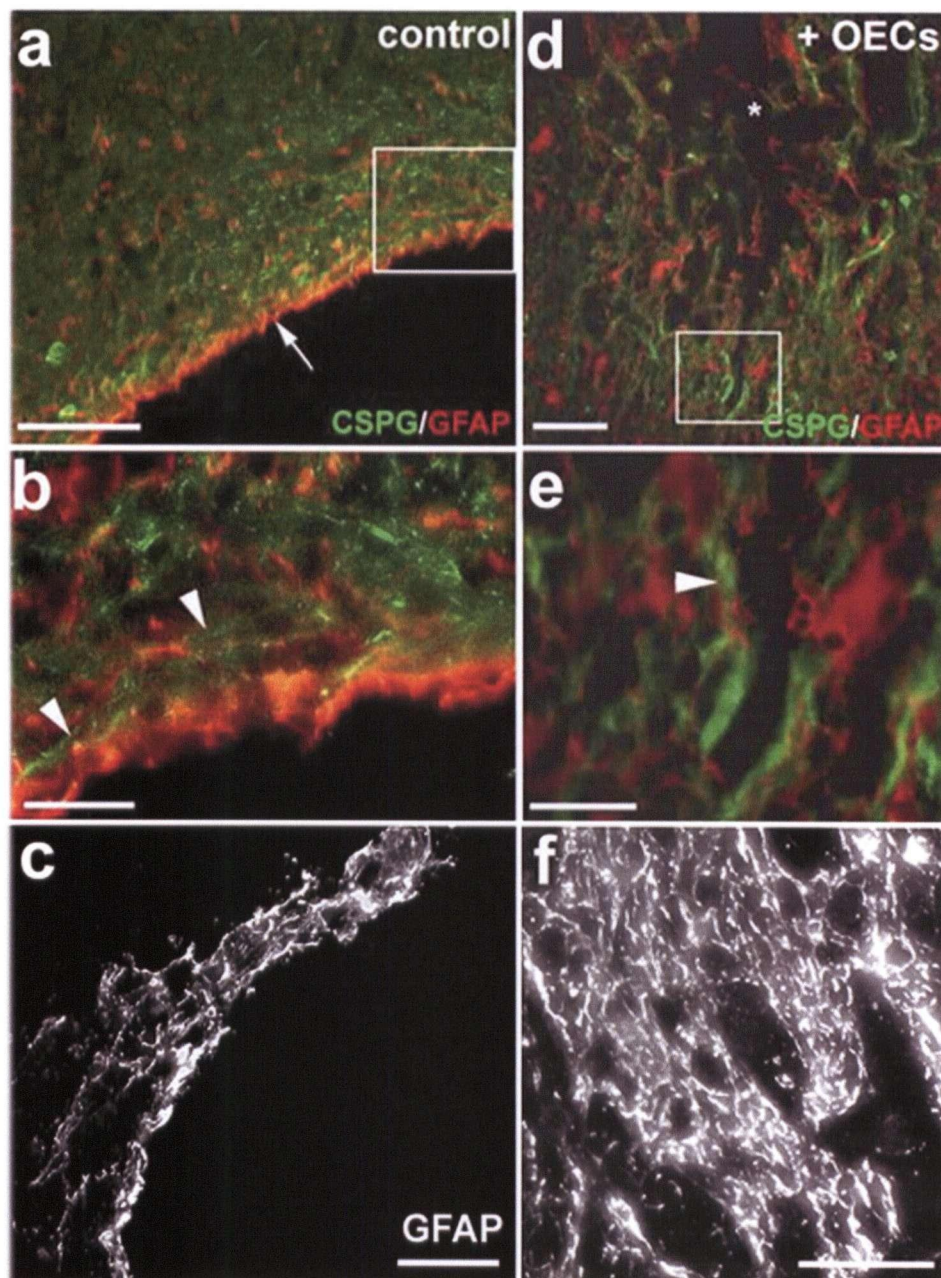
2.3.5 LP-OEC transplantation stimulated angiogenesis

OB-OECs have stimulated angiogenesis when transplanted at the site of acute SCI (Li *et al.*, 1998). It was morphologically apparent in this study that LP-OEC-treated lesions were also perforated by blood vessels, visible in cross-section (as holes) and cut obliquely (as tubes). Microvessels extended out from the lesion site in OEC-treated rats only; in media-injected controls, the spinal tissue surrounding the cavitation was not evidently vascularized. To more thoroughly examine the blood vessels in OEC-transplanted lesions, we used an antibody recognizing the rat endothelial cell antigen (RECA-1). RECA-1 immunostaining has been described during angiogenesis in brain explants exposed to vascular endothelial growth factor, demonstrating its ability to identify immature blood vessel endothelium (Silverman *et al.*, 1999). As well, RECA-1 may be a more reliable indicator of blood vessel viability than, for example, laminin, since basement membranes have been reported to persist in the spinal cord long after the onset of significant endothelial cell pathology induced by SCI (Loy *et al.*, 2002). At 28 days following lesion and LP-OEC transplantation, RECA-1-positive blood vessels (asterisk) surrounded the lesion site (Fig. 7a, **), pierced through the laminin-rich lesion-cord interface, and were found in the lesion center. In the lesion site, blood vessels were colocalized with OECs (Fig. 7a, b; arrow). RECA-1-positive vessels were typically also immunopositive for laminin (arrowhead). We found an apparently preferential association between NF-200-positive axons and RECA-1-positive blood vessels, as axons extending into the lesion center were often found near RECA-1-positive endothelium (Fig. 7c, d; arrowheads; asterisk marks blood vessel core).

Figure 7: LP-OEC transplantation stimulated angiogenesis.

(a,b) 28d after OEC transplantation, the lesion site was surrounded by and filled with the profiles of RECA-1-positive blood vessels: these were found both within the lesion center and beyond the laminin-rich lesion:cord interface (**). Inside the lesion site, blood vessels were closely-associated with OECs (arrow). RECA-1-positive vessels were typically also immunopositive for laminin (arrowhead). (c,d) NF200-positive axons extended along the RECA-1-positive vasculature (arrowheads). The asterisk marks a blood vessel in cross-section. Scale bars: 200 µm (a,c); 50 µm (b,d).

GFP/RECA-1/Laminin

GFP/RECA-1/NF200
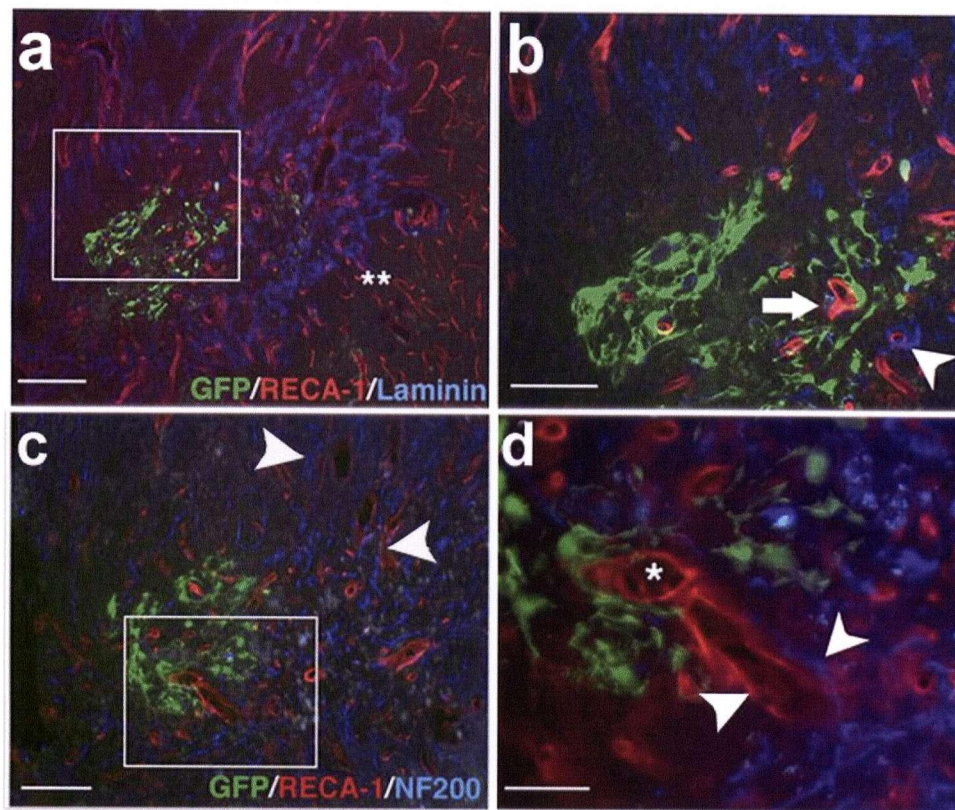
2.3.6 Central and peripheral axons grew into LP-OEC-treated lesions

OB-OEC transplantation facilitates growth and regeneration of axons after spinal cord injury (Ramon-Cueto and Santos-Benito, 2001). We used immunohistochemistry for neurofilament-200 (NF200), βIII-tubulin (βIIIT), serotonin (5HT), tyrosine hydroxylase (TH) and calcitonin gene-related peptide (CGRP) to characterize the axonal populations present at the lesion site 60 days after injury and transplantation of LP-OECs (Fig. 8). In animals that had received injections of media only, axons approached or grew along the wall of the cavitation, and were particularly evident at the lateral aspect of the lesion (Fig. 8a). Although no OECs remained at 60 days, animals that had received OEC injections showed massive growth of axons into (and in the case of some populations, through) the lesion site (Fig. 8b-k). Axons occupying the lesion site were both large- and small-diameter and stained for NF-200 and βIIIT (Fig. 8b-e): these axons were of both central and peripheral origin, indicated by expression of 5HT, TH, and CGRP (Fig. 8f-k). The orientation of axons in the lesion site was generally random, with some bias to an axis perpendicular to the dorsal columns. Projections from the brainstem staining positive for 5HT and TH displayed the typical beaded axonal staining pattern and grew into the lesion site in large numbers. In numerous instances these axons were encountered traversing the caudal lesion-host interface (Fig. 8g,i), some extending several millimeters distally. Sensory CGRP-positive axons grew into the OEC-treated lesion site but typically failed to enter the intact host spinal cord (Fig. 8k).

Figure 8: Central and peripheral axons grew into LP-OEC-treated lesions.

Multiple populations of axons grew into and across OEC-treated, but not media-injected, lesions. (a) 60 days after media injection, axons approached or grew along the wall of the cavitation, particularly at the lateral aspect of the lesion. (b-k) 60 days after OEC injection, although no OECs remained, there was massive growth of axons into and in some instances across the lesion site. (f-i) Projections from the brainstem expressing serotonin (5HT) and tyrosine hydroxylase (TH) showed the most robust regenerative response, and grew into the lesion in large numbers: in numerous instances, these axons crossed the caudal lesion-host interface, sometimes extending several millimeters distally. (j,k) Sensory afferents expressing calcitonin-gene-related-peptide (CGRP) grew into the OEC-treated lesion site but typically failed to enter intact host spinal cord. Scale bar: 250 μm.

βIIIT

NF200

5HT

TH

CGRP
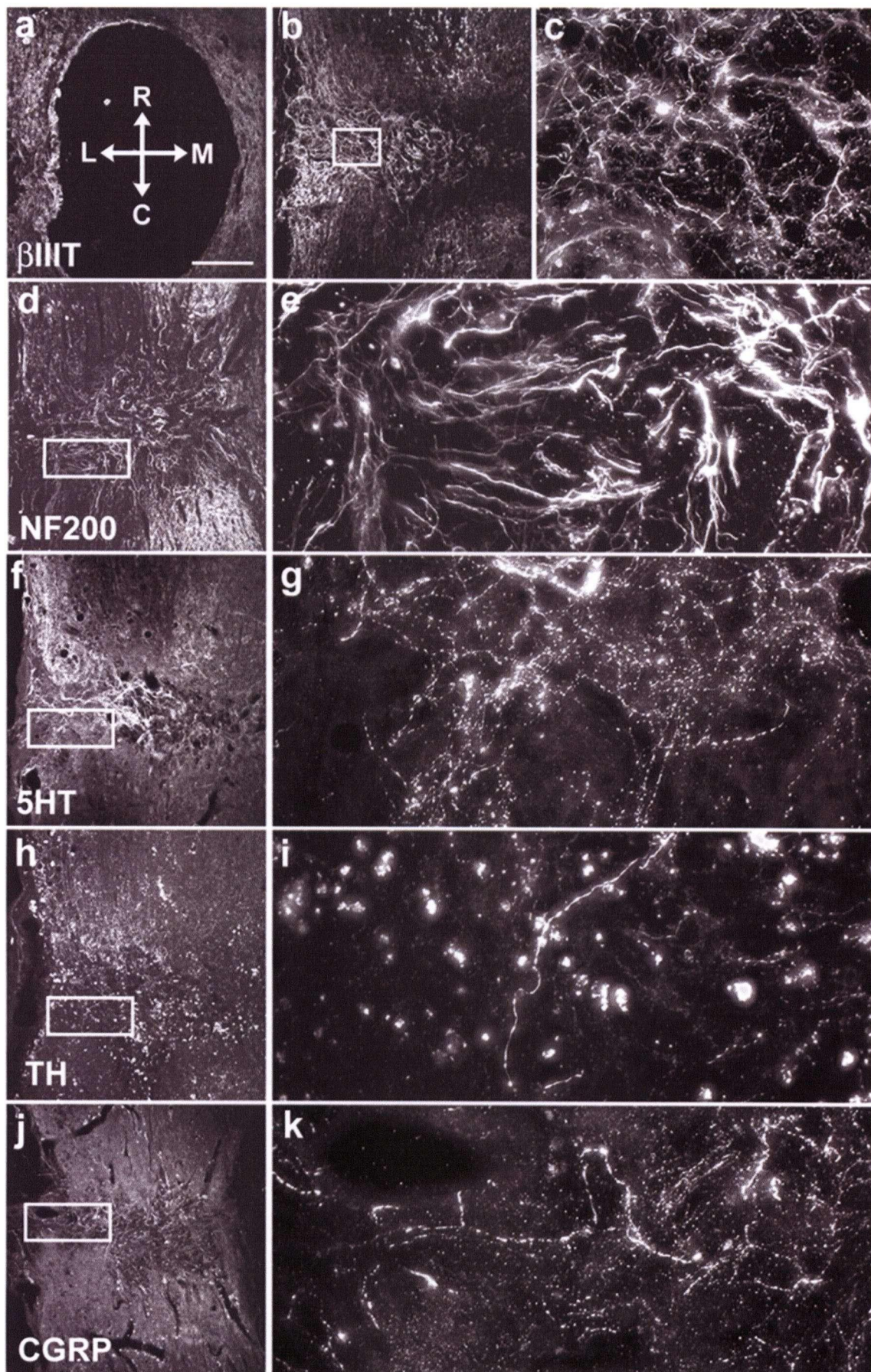
2.3.7 Rubrospinal axons sprouted into, but did not grow beyond LP-OEC-treated lesions

To assess regeneration of rubrospinal axons in response to OEC transplantation, we injected BDA (0.5-0.8 μl) in the vicinity of the right red nucleus (Fig. 9). This produced robust anterograde labeling of the lesioned rubrospinal axons in nearly all rats (Fig. 9a); one rat was omitted from assessment of rubrospinal regeneration due to incomplete labeling (not shown). There was no evidence of spared rubrospinal axons – axons that were large, straight, and growing without branching through the lesion site - in any animal. In rats that received injections of media only, rubrospinal axons were encountered at the rostral border of the cavitation seven days after injury (Fig. 9b). Near the rostral edge of the cavitation, some of these axons assumed a tortuous path characteristic of growing axonal sprouts while most axons displayed morphologic characteristics of end-bulbs (Fig. 9c). Surprisingly, in OEC-treated animals, very few axons were in close vicinity of the rostral edge of the injury site and none were found sprouting into the OEC deposit at this early seven day time point (Fig. 9d). The bulk of the rubrospinal tract was seen to end within 300 μm of the lesion center in media injected animals and within 500 μm of the lesion center with OEC treatment. On days 28 and 60, most BDA-filled rubrospinal axons in control animals were still in close proximity to the rostral edge of the cavitation, but were never seen to grow around the lesion (Fig.9e,h). In OEC treated animals, the majority of the rubrospinal axons ended several hundred microns rostral to lesion (e.g. Fig. 9f); however, a small number of rubrospinal axons had grown into the center of the lesion occupied by OECs (Fig. 9g) and SCs (not shown). Similar rubrospinal axon sprouts were still encountered in the center of the lesion at 60 days, after the disappearance of OECs (Fig. 9i,j). Rubrospinal axons were not seen to cross the lesion site or traverse the caudal lesion-host interface at any time point examined, neither in media-injected nor in OEC-injected animals.

Figure 9: Rubrospinal axons sprouted into, but did not grow beyond LP-OEC-treated lesions.

(a) BDA injection near the right red nucleus produced robust labeling of rubrospinal axons in nearly all rats, shown here in cross-section (at C2) 28 days after DLF crush at C4. (b,c) 7 days after media injection, rubrospinal axons were encountered at the rostral border of the cavitation; while some of these axons assumed a tortuous path characteristic of axonal sprouts, most displayed morphologic characteristics of end-bulbs. (d) 7 days after OEC transplantation, few rubrospinal axons approached the lesion site compared to media controls, and none were observed sprouting into the OEC deposit. (e) At 28 days after media injection, rubrospinal axons were still in close proximity to the rostral edge of the lesion, but were never seen to grow around the lesion . (f,g) 28 days after OEC transplantation, the majority of axons still terminated more rostrally than in media controls; however, a few axons were encountered in the OEC-filled lesion site. (h) 60 days after media injection, rubrospinal axons approached the cavitation among autofluorescent macrophages (yellow); (i,j) 60 days after OEC-transplantation, after the disappearance of OECs, rubrospinal sprouts were found in the lesion site, but never seen to cross the caudal lesion-host interface. Asterisks mark the center of the lesion site in each panel. Scale bars: 800 μm (a); 200 μm (b,d,e,f,h,i); 50 μm (j); 100 μm (a,c,g).
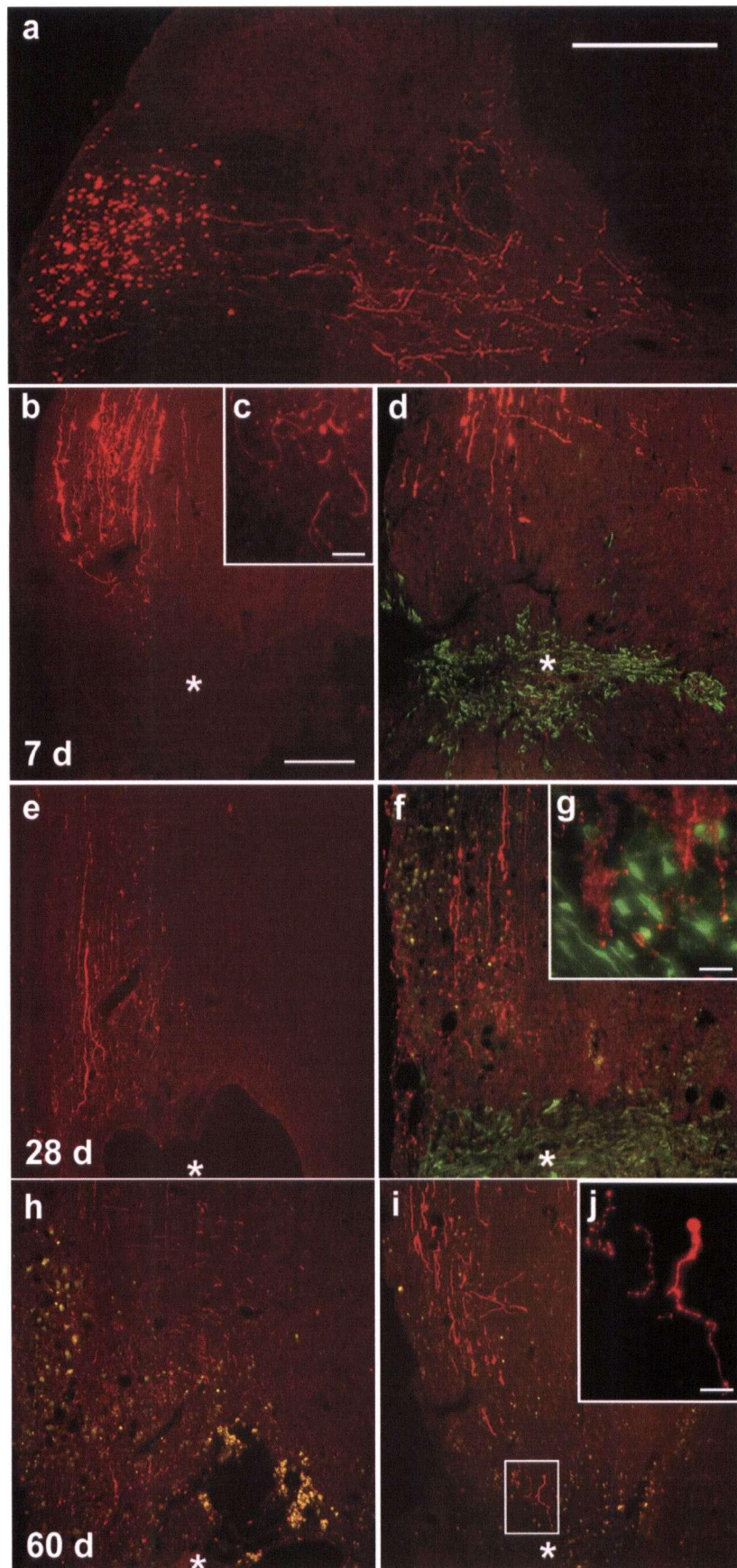
2.3.8 Allotransplantion of LP-OECs enhanced SC invasion, altered astrocyte alignment, and supported axonal growth

The limited survival and migration of OECs reported here could be due to a severe immunological reaction characteristic of xenotransplantation: the host response to xenografts is complex, and certain phases of rejection may not be preventable using standard immunosuppression agents, such as CsA (Cascalho and Platt, 2001). To examine the behaviour and effects of allotransplanted LP-OECs, we injected LP-OECs into a DLF crush site in non-transgenic C57/Bl6 mice (the strain used to generate the GFP-positive LP-OECs). Host mice received cyclosporine A, at the same dose used for the rats (10 mg/kg/day, starting 48 hours prior to surgery and maintained throughout survival) (Fig. 10).

Despite immunosuppression, there was a pronounced infiltration of autofluorescent phagocytic cells into both media- and OEC-injected lesions (Fig. 10, c-h). Still, survival of allografted OECs appeared to be more successful than survival of xenografted OECs: 28 days after injection into a spinal cord lesion site, GFP-positive LP-OECs were encountered in all mice examined (7/7) (*versus* 5/9 rats examined). As in the rat, however, migration of OECs within the mouse spinal cord was limited: after 28 days, OECs were only encountered within 500 μm of the lesion center (Fig. 10d,h). Since cavitation in this strain of mouse is known to be minimal (Steward *et al.*, 1999), the differences in lesion volume were not assessed. As in the rat, the infiltration of p75-positive Schwann cells into the mouse spinal cord was enhanced following transplantation of LP-OECs: Schwann cells were encountered in host spinal cord beyond the OEC graft-host interface (Fig. 10b; arrows).The zone of GFAP-positive reactive astrocytes surrounding the lesion was broader and more diffuse in mice that had received GFP-positive LP-OEC transplants compared to media-injected controls (Fig. 10c, d). NF200-positive axons entered and crossed the center of the lesion in OEC-treated mice, while axons ventured to the

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lesion boundary, but were unable to cross the lesion center, in mice that received injections of media alone (Fig. 10e, f); similarly, 5HT-positive axons grew robustly into and around the OEC-treated lesion (arrow), but only grew along the lateral aspect of media-injected lesion sites (arrowhead) (Fig. 10g, h). In short, the effects of LP-OEC allotransplantation at the site of acute SCI (in mouse) appeared to be comparable to the effects of LP-OEC xenotransplantation at the site of acute rat SCI.

Figure 10: Allotransplantion of LP-OECs enhanced SC invasion, altered astrocyte alignment, and supported axonal growth.

(a-h) 28 days after injury, OECs persisted at the lesion site of all (7/7) immunosuppressed mice examined; however, migration was limited, and OECs were only encountered within 500 μm of the lesion center. (a,b) Host SCs invaded both media- and OEC-treated mice, but were far more abundant in OEC-treated mice, where projections of Schwann cells extended beyond the lesion into host spinal cord (arrows); no OECs were visible in the section shown in b (green channel is not shown in a or b). (c,d) 28 days after media injection, astrocytes were found close to the center of the lesion: 28 days after OEC injection, hypertrophic astrocytes were loosely arranged in a broad zone surrounding the lesion, without dense clustering. (e-h) In media injected mice 28 days post lesion, some NF-200-positive axons extended into the lesion center, while 5HT-postive axons were encountered only at the lateral aspect of media-injected lesion (arrowhead). Conversely, NF-200- and particularly 5HT-expressing axons grew robustly into and around the OEC-treated lesion site; serotonergic axons were encountered at the caudal aspect of the lesion (arrow). Scale bar: 200 μm.
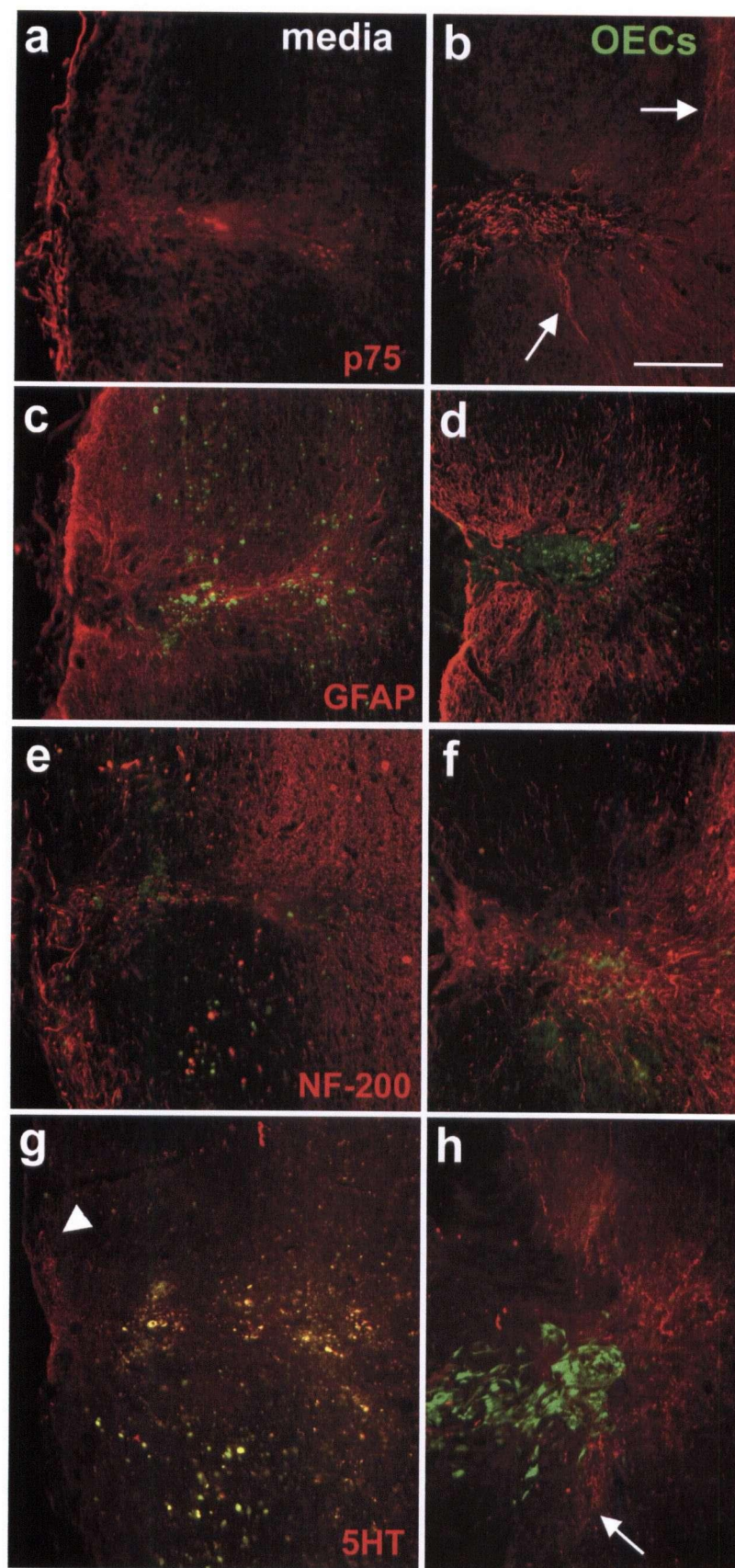
2.4 Discussion

In this study we report for the first time the fate of purified LP-OECs after transplantation into acute spinal cord injury sites in rats and mice. We overcame the problem of reliably tracking transplanted OECs by using a GFP-expressing mouse as a donor. In rats, LP-OECs prevented the formation of a cavitation, but showed limited migration in the host spinal cord and failed to survive to 60 days. Despite their rather limited integration in the rat spinal cord, transplantation of LP-OECs enhanced the invasion of host Schwann cells which ultimately filled the lesion site, and stimulated angiogenesis within and around the lesion site. The dense astrocytic barrier corralling media-injected lesions was absent in OEC-treated animals, where a broad but diffuse zone of astrogliosis surrounded the lesion site. Multiple axonal populations grew into OEC-transplanted lesions, and 5HT- and TH-positive axons regenerated successfully into the distal host spinal cord. Rubrospinal axons entered the center of the lesions but were never seen to cross the caudal lesion-host interface. In mice, LP-OEC transplantation conferred many of the same beneficial effects: OEC-treated lesions exhibited increased angiogenesis, axonal growth, and host Schwann cell invasion, , and altered astrogliosis.

2.4.1 Survival and migration of transplanted OECs

The limited survival and migration of LP-OECs in this study was surprising in light of previous transplantation experiments reporting survival and migration of OB-OECs in host spinal cord for 2 to 8 months (Li *et al.*, 1998; Ramon-Cueto, 2000; Takami *et al.*, 2002). These differences could be due to inherent problems in the use of dye labeling to track cells or to intrinsic differences in the adaptability of bulb-derived *versus* mucosa-derived OECs. In many transplant experiments performed to date, OECs, lacking a defining antigen (Ramon-Cueto and Avila, 1998), have been labeled with dye or bisbenzimide prior to transplantation (Ramon-Cueto

et al., 1994; Ramon-Cueto *et al.*, 1998; Imaizumi *et al.*, 2000a; Imaizumi *et al.*, 2000b; Ramon-Cueto *et al.*, 2000; Lu *et al.*, 2001; Boruch *et al.*, 2001; Lu *et al.*, 2002; Nash *et al.*, 2002). The limitation of these dyes is twofold: they can diffuse out of labeled cells, rendering transplanted cells false negative; more ominously, they can be taken up by host cells, yielding false positive interpretations (Iwashita *et al.*, 2000; Ruitenberg *et al.*, 2002). Alternatively, p75 immunostaining has been used to identify transplanted OECs *in vivo* (Li *et al.*, 1997; Li *et al.*, 1998; Takami *et al.*, 2002). However, our discovery of a significant population of endogenous p75-positive Schwann cells that enter and persist within the OEC-treated lesion makes the interpretation of p75 immunoreactivity somewhat ambiguous. While it is possible that LP-OECs do not adapt as well to migrating through a CNS environment as OB-OECs, recent data suggests that this is unlikely: virally-labeled (GFP-expressing) rat OB-OECs transplanted into an acute rat spinal lesion did not migrate for long distances, and remained clustered at the lesion site at 10 days after transplantation (Li *et al.*, 2003).

2.4.2 The environment of the OEC-treated lesion

Despite their limited survival and migration in host spinal cord, transplantation of LP-OECs significantly modified the site of acute SCI. One of the most dramatic results of OEC transplantation was the reduction/prevention of cavitation at the OEC-treated lesion site. Cavitations, such as those that occurred in media-injected rats, represent a major obstacle to regeneration after SCI; considerable experimentation has been dedicated to identifying the best growth substrate to support elongation of propriospinal and supraspinal axons (Bunge, 2001; Geller and Fawcett, 2002). Since cavities in rats appear similar to those that develop in patients with SCI, reducing or preventing their formation is a significant step toward successful treatment of human SCI (Schwab, 2002).

OEC transplantation also enhanced Schwann cell invasion of the lesioned spinal cord: as early as seven days after LP-OEC transplantation, p75-positive host Schwann cells invaded the core of the OEC-treated lesion, and migrated to the marginal zone of the OEC transplant. As OECs became confined to the lesion core, Schwann cells entered laminin-rich channels radiating into the host spinal cord, similar to those outlined by the early migrating OECs. Schwann cells typically migrate into lesioned spinal cord (Brook *et al.*, 1998); however, in this study, massive numbers of Schwann cells persisted in OEC-treated lesions at all time points examined, while comparatively few Schwann cells were found in media-injected lesions. It cannot be determined from the present work whether OECs produce an attractive signal for host Schwann cells or whether the transient presence of OECs in the lesion site simply creates a permissive scaffold for integration of infiltrating Schwann cells: such details have important implications for optimizing cellular transplantation strategies for repair of SCI.

Schwann cells are the cellular substrate for successful regeneration in the PNS, and when aligned in mini-channels, promote regeneration even more effectively (Martini, 1994; Fu and Gordon, 1997). Schwann cell and peripheral nerve transplantation have shown considerable promise in a variety of SCI models, where they can support ingrowth of sensory, propriospinal and bulbospinal neurons (Martin *et al.*, 1991; Martin *et al.*, 1996; Xu *et al.*, 1997), in addition to being effective in remyelination spinal axons (reviewed in Kocsis *et al.*, 2002). However, in these experiments, the re-entry of regenerating axons from the Schwann cells grafts into the host spinal cord has been limited. Schwann cell transplantation has thus been combined with OB-OEC grafts at the graft-cord interface, evoking the regeneration of bulbospinal serotonergic axons distal to Schwann cell cables and beyond the caudal graft-host interface, after a complete thoracic transection (Ramon-Cueto *et al.*, 1998). Our results indicate that, in injury models where host Schwann cells can access the lesion site, OECs interact with them to provide an

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environment that is conducive to both axonal growth and modification of the graft-host glial interface. The relative contributions of SCs and OECs to axonal growth observed in this and other studies remains to be determined.

Akin to spinal cavitation, the dense astrocytic boundary or glial scar that typically surrounds a spinal cord lesion is considered a major barrier to regeneration and recovery (Shearer and Fawcett, 2001). In our experiments, GFAP-positive astrocytes formed a wide, permeabilized matrix around the LP-OECs, aligning along radial channels formed by invading LP-OECs. This astrocytic network terminated in finger-like projections extending into the lesion centre that interdigitated with invading Schwann cells. The astrocytic arrangement around OEC-treated lesions was distinctly different from the dense, compact boundary of astrocytes surrounding the media-injected lesion sites. Glial scar permeability was evident despite the expression of CSPGs, which are repulsive/inhibitory to neurite outgrowth, around OEC-treated lesions (McKeon *et al.*, 1995; Anderson *et al.*, 1998; Bradbury *et al.*, 2002). The extent of CSPG production did not appear to be markedly altered by LP-OEC transplantation; rather, the pattern of CSPG deposition around OEC-treated lesions, which contained CSPG-poor zones, may be important in allowing axons to grow across the spinal cord-lesion interface.

In addition to minimizing cavitation, enhancing SC invasion, and altering scar formation, transplantation of LP-OECs also stimulated angiogenesis at the site of acute SCI; however, we can only speculate on the mechanism(s) underlying the increase in vasculature that we observed in OEC-treated lesions. Neovascularization may have been stimulated by LP-OECs themselves, which produce VEGF, (Au and Roskams, 2003); infiltrating host SCs may have stimulated angiogenesis, as cultured SCs stimulate blood vessel formation when injected into the rat corticospinal tract (Li and Raisman, 1997). Alternatively or additionally, angiogenesis in OEC-treated lesions may have been induced by macrophages that entered the lesion in response to the

presence of foreign OECs: macrophages secrete angiogenic factors, such as tumour necrosis factor-alpha (TNF-alpha) (Leibovich et al., 1987), and stimulate angiogenesis when transplanted at the site of acute SCI (Franzen et al., 1998). Finally, LP-OEC transplantation and/or ensuing SC infiltration may simply preserve spinal cord parenchyma and with it vasculature that develops at the lesion center spontaneously in the subacute phase of SCI, only to be destroyed as cavitation develops: this pattern of vascular remodeling has been reported after contusion injury in rat (Casella et al., 2002; Loy et al., 2002).

If the mechanism of angiogenesis in the OEC-treated lesion is ambiguous, so too is the implication for angiogenesis in repair of SCI. Reperfusion is an essential component of wound healing in tissues outside the CNS (Clark, 1988), and regeneration after SCI has been suggested to be limited by the lack of an adequate blood supply (Goldsmith et al., 1985; de la Torre and Goldsmith, 1988; Zhang and Guth, 1997). It is generally accepted that the ischemic component of SCI contributes to neuronal death (Amar and Levy, 1999); however, recent data suggests that both antiangiogenic and angiogenic therapies can improve behavioral recovery following contusion SCI (Wamil et al., 1998; Widenfalk et al., 2003). In the present study, sustained neovascularization of the OEC-treated lesion was correlated with dramatically increased axonal growth into the lesion site. The basal lamina of blood vessels at the lesion may serve as a source of laminin, which supports neurite extension both in vitro and in vivo (Rogers et al., 1983; Zhou and Azmitia, 1988), and thus may form a permissive scaffold for growing axons. Additionally (or alternatively), blood vessels may create the spaces in the glial scar (discussed above), and may facilitate axonal ingrowth simply by morphological rearrangement of lesion site.

2.4.3 The effects of OEC-transplantation on lesioned spinal axons

The limited survival and migration of LP-OECs transplanted at the site of acute SCI in the adult rat did not compromise growth of serotonergic and noradrenergic axons, which grew into OEC-treated lesion sites, and were encountered distal to the graft-cord interface 60 days after injury. The growth of 5HT-positive axons in and through the lesion site observed in this study, i.e. with transplantation of purified cultures of LP-OECs, is comparable to that reported after transplantation of heterogeneous strips of olfactory mucosa (Lu *et al.*, 2001). Serotonin and other biogenic amines have been demonstrated to facilitate rhythmic locomotor activity, presumably via a central pattern generator, in cats and rodents (Barbeau and Rossignol, 1991; Jacobs and Fornal, 1993; Gerin *et al.*, 1995). Thus, regeneration of 5-HT positive axons could primarily be responsible for the functional recovery stimulated by transplantation of heterogeneous cells from the olfactory mucosa (Lu *et al.*, 2002). We show here that long-term survival of OECs is not required for massive ingrowth of potentially critical serotonergic axons. While we cannot report conclusively that the growth we observe in this partial lesion model is due to regenerative sprouting (of cut axons) rather than collateral sprouting (of intact axons), the appearance of the OEC-treated lesion sites suggested that regenerative sprouting occurred: in many sections, a thick bundle of serotonergic axons was visible approaching the rostral border of the lesion, and from this, a few, tortuous axons could be followed across the lesion site and into distal spinal cord. Since many axons traveled out of the plane of the horizontal sections, the axons we encountered traversing the lesion and the caudal lesion-host interface likely represent only a small proportion of axons that grew across the lesion after OEC-transplantation.

In addition to serotonergic and noradrenergic bulbospinal axons, CGRP-positive sensory axons also sprouted into the OEC-treated lesion site. Ingrowth of CGRP-expressing afferents has been reported after transplantation of OB-OECs (Ramon-Cueto *et al.*, 1998), Schwann cells

(Martin *et al.*, 1996) and macrophages (Franzen *et al.*, 1998). While axonal growth is generally regarded as a positive result, ectopic or excessive growth of sensory axons into or within the spinal cord can result in hyperalgesia (Romero *et al.*, 2000): this finding underlines the importance of targeting regeneration strategies to specific tracts and regions of the spinal cord.

In contrast to the robust growth of noradrenergic and serotonergic axons into OEC-treated lesions, rubrospinal axons entered but never crossed DLF crush site. Rubrospinal neurons normally undergo massive atrophy 2 weeks after axotomy, and down-regulate the expression of regeneration associated genes (Kobayashi *et al.*, 1997). We observed an initial apparent withdrawal of rubrospinal axons from the OEC-treated lesion during the first week following surgery: this would most likely limit their access to presumed OEC-derived trophic factors. The subsequent massive invasion of Schwann cells may have improved the local availability of trophic factors, encouraging rubrospinal axons to finally sprout into the lesion site. The inability of rubrospinal axons to cross the distal lesion-host interface is most likely due to the failure of the parent cell bodies to sustain a regenerative response for several weeks after injury. This transient preparedness of rubrospinal neurons to regenerate was also seen when BDNF-secreting fibroblasts were transplanted at the site of DLF lesion: such a graft was reported to promote rubrospinal regeneration after acute (Liu *et al.*, 1999) but not chronic (four-week-old) lesion (Jin *et al.*, 2002). Similarly, rubrospinal axons failed to regenerate into peripheral nerve transplants when these were offered 4 weeks after lesion (Houle, 1991); thus far, this lack of regeneration has only been overcome when trophic support was given at the cell body (Plunet *et al.*, 2002). The rubrospinal tract has a large, discrete nucleus, and thus is particularly appropriate for anterograde labeling; however, the poor regenerative response of rubrospinal axons did not allow us to provide unequivocal evidence of regeneration in response to LP-OEC-transplantation from this work.

2.4.4 LP-OEC allotransplantation

Transplantation of GFP LP-OECs into strain-matched C57/Bl6 mice suggested that OECs may persist longer when allotransplanted than when xenotransplanted; however, OEC transplantation appeared to exert similar effects on the site of acute SCI in both rat and mouse. Although cavitation does not occur in C57Bl6 mice, the glial scar around OEC-treated lesions was once again diffuse and permeabilized relative to astrogliosis surrounding media-injected lesions. Schwann cell infiltration was more abundant in OEC-treated lesions than in media-injected lesions. In mice that received media only, few NF- or 5HT-positive axons grew into the lesion, while in OEC-treated mice, NF- and 5HT-expressing axons entered the lesion in large numbers, and 5HT-positive axons were encountered distal to the caudal graft-cord interface.

Although more experiments have been performed in rats and cats, mice are emerging as a new and important animal model for SCI research, particularly since they are the organism of choice for manipulation of the mammalian genome (Steward *et al.*, 1999). The protocol we have developed for isolating LP-OECs from GFP mice holds significant potential for further genetic studies, involving transgenic mice as both donors and hosts of OECs. In applying our knowledge of rat SCI to the mouse model, we must be conscious of important differences between species; for example, cavitation after SCI tends to be absent in most strains of mice, and recent data suggests that the inflammatory response to SCI is different between rats and mice (Sroga *et al.*, 2003). Due to the immunogenic nature of cell transplants, interspecies differences in neuroinflammation are important in studying the regenerative potential of OECs: although the immune response to OEC transplantation was not fully characterized in this study, this area merits further investigation.

2.5 Conclusion

Here we report that transplantation of purified LP-OECs significantly modified acute spinal cord lesion, despite limited survival and migration in host spinal cord. OEC transplantation prevented cavitation, modified scar formation, stimulated angiogenesis and created a permissive environment at the lesion site to support growth of multiple axonal populations. These data thus endorse LP-OECs as candidates for autologous transplantation in SCI, but also raise significant questions surrounding the relative contributions of donor and host cells to SCI repair.

CHAPTER III

What is the future of OEC transplantation into the injured spinal cord?

One thing is certain, however; Scientific research always raises more questions than it answers.
Garland Allen & Jeffrey Baker, 2001

3.1 What have we learned about the potential for LP-OECs in regeneration of spinal axons?

We have harvested OECs from the lamina propria (LP-OECs) of mice expressing GFP in all cells, and purified these cells through immunolytic removal of fibroblasts during *in vitro* expansion. We transplanted these OECs into acute lesions of the dorsolateral funiculus (DLF) in immunosuppressed rats and mice, and examined OEC migration, morphological changes at the lesion site, cellular and extracellular components of the lesion site, and the axonal components present at the lesion using immunohistochemistry and anterograde neuronal labeling.

Although LP-OECs were only ever encountered within 1 mm of their site of injection, they dramatically modified the DLF lesion. OEC-treated lesions exhibited significantly attenuated cavitation, dramatically increased host Schwann cell invasion, altered astrocyte alignment and angiogenesis relative to media-injected lesions. Some or all of these modifications rendered the OEC-treated lesion more permissive to axon growth, as growth of all axonal populations examined was massive at the OEC-treated lesion site compared to the media-injected lesion. Sprouting axons were of both large- and small-diameter and of both central and peripheral origin: the most robust growth was observed in serotonergic and noradrenergic axons, presumably descending from the nucleus raphe and coeruleus, respectively. Despite massive ingrowth of many axons into and in some instances through OEC-treated lesions, conclusive evidence of rubrospinal regeneration was not obtained with anterograde tracing, since rubrospinal axons sprouted but were not seen to traverse the lesion site.

The axonal responses to LP-OEC transplantation observed here were generally comparable to those obtained using OB-OECs (see Table I): serotonergic and noradrenergic axons regenerated/sprouted in response to OB-OEC transplantation (Ramon-Cueto et al., 1998, 2000; Plant et al., 2003); sensory axons expressing calcitonin gene-related peptide (CGRP) were also found in lesions injected with OB-OECs (Ramon-Cueto et al., 1998). OEC transplantation may not prove sufficient to stimulate regeneration of all axonal phenotypes, as rubrospinal axons failed to grow beyond a DLF transection when OB-OECs were deposited immediately following lesion (Plant et al., 2003; Ruitenberg et al., 2003).

The growth of axons in response to our acute transplantation of purified LP-OECs was also comparable to growth provoked by both acute and delayed transplantation of dissociated strips of olfactory mucosa (Lu et al., 2001, 2002). In these experiments, serotonin-positive fibres were encountered in the spinal cord distal to the site of complete transection, and axons caudal to the lesion site were retrogradely traced to the raphe spinal nucleus. These studies were unusual, and as a result are subject to some controversy, since no attempt was made to increase the proportion of OECs relative to other cells present in the grafted material. In fact, these experiments used perhaps the most appropriate control graft, which is respiratory epithelium, a neighbouring tissue containing most cell types present in the lamina propria except OECs. While great pains have been taken to purify OB-OECs (see 1.3), several investigations have demonstrated that OB-OECs containing large proportions of contaminating cells such as fibroblasts are capable of stimulating regeneration and functional recovery (Li et al., 1997, 1998, 2003); these cellular grafts were only recently correctly described as 50% OB-OECs/50% fibroblasts (Li et al., 2003). Theoretically, if we are trying to recreate the local environment encountered by olfactory axons at the site of SCI, strips of olfactory mucosa contain a better complement of cellular elements than OECs purified on the basis of protein expression; this is

particularly true since OECs have a demonstrated capacity to vary their antigenic presentation. The requirement for pure transplants of OECs becomes even more questionable when we consider the role(s) of host glia/microglia in modifying the OEC-treated lesion (discussed below).

3.2 What have we yet to learn about the role of LP-OECs in growth of spinal axons?

While our analysis of axonal growth into OEC-treated lesions is clearly comparable to the work of others, we cannot speak to the effects of purified LP-OECs on the functional outcome of SCI. Raphespinal, rubrospinal, and reticulospinal axons in the DLF are all involved in motor function and coordination, although their precise contributions remain ill-defined. While this injury model does not lend itself to assessing recovery using open-field locomotion, other simple tests, such as horizontal rope walking, grid walking, or directed forepaw reaching, might reveal significant differences between OEC- and media-treated animals. Since monoamines are known to stimulate rhythmic stepping (Gerin *et al.*, 1995), and since a strong (and intuitive) correlation between sparing of spinal tissue and functional recovery has emerged (Ruitenberg *et al.*, 2003), I speculate that behavioural assessment of LP-OEC-treated-rats would reveal significant improvements in return of function, and/or reduced loss of function, after injury.

Certainly, functional recovery should be an important component of future experiments testing the efficacy of LP-OECs after SCI: the most important addition to experimental procedures preceding such a study would be *to culture another population of cells from the GFP mouse* to serve as a control group against which to test LP-OECs. This is not an onerous addition; protocols for culturing (for example) fibroblasts from the skin or SCs from the sciatic nerve are well-established. We followed the field (and simplified our experiments) by using serum-free media as a control; however, media takes up little physical space in the spinal cord

(indeed, it often leaks out when injected). There is a trend that is apparent in the transplantation literature, and I have dubbed it the "non-dairy creamer theory of regeneration": putting something into the site of SCI (even non-dairy creamer) would improve the anatomical and functional outcome of the lesion.

Media injection also (presumably) stimulates little immune response compared to grafted cells, and another pressing question raised by this work lies in whether the axonal growth observed (and indeed, any of the effects reported) are OEC-specific, or simply induced by an immune response to the presence of grafted material. Serotonergic and noradrenergic axons also grow in response to acute transplantation of macrophages activated by exposure to degenerating peripheral nerve (Rapalino *et al.*, 1998). Interestingly, increased Schwann cell invasion, enhanced angiogenesis, and altered astrocytic alignment also occur within grafts of macrophages (Franzen *et al.*, 1998; Rapalino *et al.*, 1998). In order to determine which (if any) of these effects are due to the presence of OECs, other cells isolated from the GFP mouse must be transplanted into similar lesions: the most obvious candidates for cellular transplantation are fibroblasts from the skin, since these can be easily cultured and expanded, and since they are classically considered an innocuous cell in regeneration studies, although (as implied above) they do support growth of some axons (Tuszynski *et al.*, 2002).

While LP-OECs evoked sprouting/regeneration of bulbospinal axons when transplanted at the site of a cervical DLF crush, we cannot confidently predict that they would be equally beneficial if introduced into a similar lesion at the thoracic level. The regenerative response of spinal axons decreases as distance between soma and trauma increases (Richardson *et al.*, 1984), which is likely due to the response of the cell body (Fernandes *et al.*, 1999). Therefore, our cervical lesion model precludes critical comparison between these data and those obtained in experiments where OB-OECs were grafted at the site of a thoracic injury (see Table I). Testing

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LP-OECs in a thoracic lesion is essential to demonstrate their ability to promote robust regeneration.

Of course, the delivery of LP-OECs has not been optimized in this or in any other study, and further experimentation is required to determine the most effective timing and location of OEC injections, the degree of immunosuppression required, and the impact of other treatments applied in combination with LP-OECs. We have examined the effects of LP-OECs injected directly into the site of acute SCI; however, in many experiments reporting regeneration after OB-OEC transplantation, the OECs are injected approximately 1 mm rostral and caudal to the visible extent of acute damage (see Table I). Depositing OECs outside the lesion site might protect grafted OECs from an immune response at the lesion, and permit them to migrate rostrocaudally to reach intact portions of spinal axons, thus forming a permissive scaffold for regeneration across the lesion site (A. Ramon-Cueto, p. comm.). However, OEC injection rostral to the lesion could damage intact axons, effectively increasing the level of SCI; thus, delivering OECs to undamaged regions of spinal cord presents a risk in terms of clinical application, since the procedure could easily worsen the functional outcome. Recent studies suggest that a more clinically relevant method of delivering OECs, direct injection into the subacute lesion, delayed seven days after injury, may be more effective than immediate injection (Plant *et al.*, 2003). Although it is difficult to compare rodent and human lesions in terms of progression and to establish relevant time points for classifying lesions as "acute", "subacute", or "chronic", this work suggests that OECs could be effective if introduced after the initial functional outcome of the human lesion appears to have stabilized.

Immunosuppression of the OEC-treated host is potentially confounding, since immunosuppressant drugs themselves may provoke axonal growth after SCI (Palladini *et al.*, 1996; Bavetta *et al.*, 1999). More significantly, immunosuppression in the clinical setting is

undesirable, since it must be continually administered, it is accompanied by adverse side effects, and it increases morbidity and mortality due to opportunistic infections (Schroeder *et al.*, 2003). Nearly all transplantation experiments using OB-OECs have been performed without immunosuppression (see Table I). In our preliminary experiments, rats were immunosuppressed for two weeks following injury and transplantation; in these experiments, no OECs were encountered in the spinal cord 28 days after injury and transplantation. Despite a very transient presence of OECs, these lesions were filled with both host Schwann cells and NF-200-positive axons (W.T., unpublished observations). Whether immunosuppression is required at all for these beneficial effects, or whether another immunosuppression agent is more effective than cyclosporine A, has yet to be determined.

Since clinical trials involving LP-OECs are ongoing, it makes sense to apply the clinically-accepted treatments for SCI in combination with these cells to determine whether beneficial effects of the treatments are additive. A recent study using OB-OECs showed that functional recovery was potentiated by concurrent injection of methylprednisolone (MP), the cortical steroid routinely administered within eight hours of human SCI (Nash *et al.*, 2002). LP-OEC transplantation might be tested with MP, as well as other compounds in clinical trial, such as monosialotetrahexosylganglioside (GM1), 4-aminopyridine (4-AP), and leteprinim potassium (AIT-082) (http://carecure.rutgers.edu/spinewire/Articles/SCITrials2002/SCITrials2002.htm); in addition to augmenting the effects of LP-OECs, such compounds might clarify the mechanism of OEC-mediated regeneration. For example, minocycline, a compound that suppresses the activation of microglia and inhibits matrix metalloproteinases, may enter clinical trials as a treatment for SCI (http://biomed.brown.edu/Courses/BI108/BI108_2003_Groups/Spinal_Cord/drugs.htm): minocycline might be combined with OEC transplantation to examine the relative contribution

of microglia to OEC-mediated recovery. Surgical stabilization of the vertebrae surrounding SCI is frequently performed in the clinic to prevent further damage to spinal axons caused by vertebral pressure/displacement (Lee and Green, 2002). Preliminary experiments suggest that when the vertebral processes surrounding LP-OEC-treated (rat) lesions are stabilized, OEC survival is improved (M.W.R., unpublished observations). Whether stabilization mediates increased axonal growth into LP-OEC-treated lesions is yet to be determined.

3.3 What are the implications of our data for interpreting OEC transplantation data amassed to date?

In both xeno- and allotransplantation experiments, we found that migration of transplanted OECs with host tissue was extremely limited: OECs were never encountered beyond one millimeter from their injection site, and both survival and migration of xenotransplanted OECs diminished over time. These results are in keeping with the most recent data on OB-OECs: when GFP-labeled by viral infection, OB-OECs are similarly restricted in their migration within the host spinal cord (Li *et al.*, 2003; Ruitenberg *et al.*, 2003). These data generated with GFP-positive OECs indicate that previous reports of both survival and migration of dye-labeled OECs are overestimated. The beneficial properties of OECs as candidates for cellular transplantation do not include extensive migration in the host spinal cord.

Perhaps the most significant contribution of this work is the conclusive report of extensive migration of host Schwann cells into OEC-treated lesions. The distinction between transplanted OECs and host Schwann cells was confirmed by using a species-specific antibodies to p75. Since no cells were encountered that were immunopositive using the polyclonal p75 antibody (recognizing both rat and mouse p75) but immunonegative for the monoclonal (rat specific) p75 antibody, we concluded that there were no p75-positive/GFP-negative mouse-derived-cells present at 60 days. Thus, the transplanted OECs had not lost their GFP expression

but had disappeared. The discovery of massive and persistent host Schwann cell infiltration indicates that reports of OEC migration and survival based on antigens common to both Schwann cells and OECs are also inflated. More importantly, this discovery raises significant questions regarding the relative contributions of transplanted OECs and host Schwann cells to the regrowth and remyelination of spinal axons. We have already reviewed the similarities between transplants of OECs and Schwann cells; a recent study compared OB-OECs and SCs directly, and the functional outcomes of their transplantation were similar, although reported as significantly different (11 *versus* 12; Takami *et al.*, 2002). A companion paper demonstrated the OECs were not capable of forming myelin under *in vitro* conditions that promote myelin formation by SCs, and suggested that Schwann cell contamination might be the source of peripheral myelin in previous cocultures of OECs and neurons (Plant *et al.*, 2002; see 1.5.1). Peripheral myelin formed by Schwann cells and perhaps by OECs is indistinguishable using conventional electron microscopy, and is immunohistochemically identical, characterized by expression of P0. Transplanting GFP-positive mouse OECs into rat SCI is a useful model for resolving the origin of peripheral myelin using immuno-electron microscopy. Antibodies against GFP, as well as antibodies specific for mouse P0, could be used to identify the source of peripheral myelin so frequently encountered in OB-OEC treated spinal lesions.

Finally, the prominent invasion of host Schwann cells that accompanies LP-OEC transplantation calls to question the importance of removing Schwann cells from cultures of LP-OECs. As the first attempts to harvest OB-OECs were plagued by contamination of astrocytes (see 1.3), the use of LP-OECs has met with some resistance due to potential contamination by Schwann cells from the nasal cavity. Unlike astrocytes, however, Schwann cells have an antigenic profile that overlaps with OECs almost completely, and they are not always morphologically distinguishable from OECs *in vitro*. The cultures of OECs used here are

unlikely to contain a significant proportion of Schwann cells, since Schwann cells survive but proliferate very slowly relative to OECs under our (forskolin-free) culture conditions (L.M.R., unpublished observations); as an aside, the proportion of Schwann cells in these cultures could be determined by adding db-cAMP, which induces Schwann cells but not OECs to express P0 (Doucette and Devon, 1995). Since a huge number of host-derived Schwann cells are present in LP-OEC-treated lesions (and likely in OB-OEC-treated lesions, although this remains to be determined), the removal of donor Schwann cells may be unwarranted. Alternatively, the time course of glia infiltration (acute introduction of OECs, permitting a gradual infiltration of SCs) may be critical in modifying the lesion site to permit axonal growth. There is the important caveat that Schwann cell invasion may not occur to the same extent in every lesion model; our penetrative lesion may permit more SC invasion than a non-penetrating lesion.

3.4 OECs in SCI repair: are we sticking our nose where it doesn't belong?

The rationale underlying OEC transplantation as a strategy to regenerate/repair spinal axons centers around the striking regenerative response of the olfactory system. Here, primary neurons (ORNs) are routinely turned over and replenished through differentiation of local precursors. This process is accelerated after damage to the ORN soma, axon, or target: damaged cells rapidly degenerate and new ORNs establish functional connections with second-order neurons in the olfactory bulb. OECs do not degenerate after nerve injury, but persist in a permissive conduit between the olfactory epithelium and the olfactory bulb (Burd, 1993); thus, OECs support axonal growth of immature neurons, but have not been demonstrated to support regeneration of mature axons in the olfactory system. It is the capacity for highly-regulated neuronal replacement, combined with redundant organization and inherent plasticity, and not (just) the presence of OECs, that permits the spontaneous functional recovery of the olfactory system after nerve injury, (see 1.1).

The successful growth of ORNs that occurs both in normal function and after nerve injury may result simply from the absence of CNS glia along the route from olfactory epithelium to olfactory bulb. When ORNs reach their glomerular targets in the outer layers of the olfactory bulb, they contact astrocytes, and do not penetrate further into the bulb. Since there are not astrocytes in the vicinity of the cribiform plate, axotomy of the olfactory nerve at the level of the cribiform plate does not result in the formation of a glial scar. However, when the scar resulting from an optic nerve transection was transplanted at the site of olfactory nerve transection, HRP-labeled replacement ORN axons did not penetrate the scar or reach the olfactory bulb (Anders and Hurlock, 1996). The authors suggested that the presence of myelin-derived factors in the optic nerve scar may be responsible for the failure of ORN growth, and previous studies support this: when the entire olfactory bulb is removed in adult rodents, ORN axons grow across the cribiform plate, but terminate in the forebrain in large neuromas (Monti Graziadei, 1983; Butler *et al.*, 1984); in contrast, after neonatal bulbectomy, ORN axons innervate the ipsilateral forebrain and form synapses (Graziadei *et al.*, 1979; Hendricks *et al.*, 1994). Olfactory nerve penetration into the forebrain is age-dependent, and is blocked from postnatal day 13, coincident with the appearance of mature myelin in the forebrain (Jacobson, 1963; Evers *et al.*, 1996). In the olfactory system, at least, the permissive nature of OECs does not appear to overcome inhibitory effects of CNS myelin.

3.5 Can OEC transplantation be a clinical reality?

Even if both the dubious rationale for grafting OECs at the site of CNS injury and the incomplete data regarding the relative contribution of OECs to CNS repair are overlooked, the safe and effective use of OECs in the clinic faces substantial technical obstacles. Inserting any substance into the spinal cord carries risks, since scar tissue is typically resected rostrally and

caudally from the lesion to allow axons to penetrate the graft (Houle and Jin, 2001). Rostral resection could effectively increase the level of SCI, and worsen the functional outcome of an otherwise stable lesion. Patients with unstable lesions, increasing in size due to progressive syringomyelia, may seem to be ideal candidates for such a procedure; however, the immune response to OEC transplantation must be considered. The acute immune response to SCI is likely one of the principal causes of spinal cavitation and cavity progression (Popovich and Jones, 2003), although this is controversial (Hauben and Schwartz, 2003). By transplanting immunogenic material into a lesion site, we might inadvertently enhance secondary damage.

In addition to issues that plague all biogenic grafts, there are barriers to clinical translation that are OEC-specific. Both the olfactory bulb and the olfactory mucosa represent extremely small sources of tissue for transplantation. OB-OECs are only accessible through an extremely invasive procedure, essentially prohibiting autologous transplantation. Further, OB-OECs proliferate slowly *in vitro*, although their expansion kinetics have been improved with several mitogens (Chuah and Teague, 1999; Yan *et al.*, 2001; Alexander *et al.*, 2002). In contrast, LP-OECs can be readily harvested through a safe and simple biopsy (Perry *et al.*, 2002), and expand readily in culture (Au and Roskams, 2003). Both populations of OECs have a limited life-span in culture, unless they are immortalized *via* viral infection.

3.6 Concluding remarks

While the exceptional generation of ORNs in the adult mammal makes the olfactory system particularly suitable for studying neurogenesis, neuronal differentiation, mechanisms of axon guidance, and regulated cell death, this system has not been shown to exhibit axonal regeneration. The predominant glial component of the olfactory system, the OEC, routinely supports the growth of immature axons, but may not support the regrowth of mature axons.

Although the OEC has generated a great deal of interest over the last two decades due to its apparent ability to promote repair after SCI, 20 years of data generated in many laboratories around the world have not shown OB-OECs to be superior to other bridging treatments that are both more developed and more amenable to clinical translation. The OB-OEC has been recurrently transplanted at the site of SCI, but attempts to demonstrate its evident superiority to other bridging agents have not been conclusive, since they typically involve no alternative bridges as controls. We must now turn our attention to the LP-OEC: if its ability to promote repair of spinal axons is even equivocal to the OB-OEC in appropriately-controlled experiments, the accessibility of these glia may render them appropriate candidates to bridge the gap – both literal and figurative - in human SCI.

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APPENDIX I:
THE 21-POINT BASSO, BEATTIE, BRESNAHAN (BBB) LOCOMOTOR RATING SCALE: DEFINITIONS OF CATEGORIES

0   No observable hindlimb movement

1   Slight movement of one or two joints, usually the hip and/or knee

2   Extensive movement of one joint OR extensive movement of one joint and slight movement of one other joint

3   Extensive movement of two joints

4   Slight movement of all three joints of the hindlimb

5   Slight movement of two joints and extensive movement of the third

6   Extensive movement of two joints and slight movement of the third

7   Extensive movement of all three joints of the hindlimb

8   Sweeping with no weight support OR plantar placement of the paw with no weight support

9   Plantar placement of the paw with weight support in stance only (i.e. when stationary) OR occasional, frequent, or consistent weight-supported dorsal stepping and no plantar stepping

10 Occasional weight supported plantar steps, no forelimb-hindlimb coordination

11 Frequent-to-consistent weight supported plantar steps and no forelimb-hindlimb coordination

12 Frequent-to-consistent weight supported plantar steps and occasional forelimb-hindlimb coordination

13 Frequent-to-consistent weight supported plantar steps and frequent forelimb-hindlimb coordination

14 Consistent weight supported plantar steps, consistent forelimb-hindlimb coordination and predominant paw position during locomotion is rotated (internally or externally) when it makes initial contact with the surface as well as just before it is lifted off at the end of stance OR frequent plantar stepping, consistent forelimb-hindlimb coordination, and occasional dorsal stepping

15 Consistent plantar stepping and consistent forelimb-hindlimb coordination during gait and no toe clearance or occasional toe clearance during forward limb advancement; predominant paw position is parallel to the body at initial contact

16 Consistent plantar stepping and consistent forelimb-hindlimb coordination during gait and toe clearance occurs frequently during forward limb advancement; predominant paw position is parallel at initial contact and rotated at lift off

17 Consistent plantar stepping and consistent forelimb-hindlimb coordination during gait and toe clearance occurs frequently during forward limb advancement; predominant paw position is parallel at initial contact and lift off

18 Consistent plantar stepping and consistent forelimb-hindlimb coordination during gait and toe clearance occurs consistently during forward limb advancement; predominant paw position is parallel at initial contact and rotated at lift off

19 Consistent plantar stepping and consistent forelimb-hindlimb coordination during gait and toe clearance occurs consistently during forward limb advancement; predominant paw position is parallel at initial contact and lift off and tail is down part or all of the time

20   Consistent plantar stepping and consistent coordinated gait; consistent toe clearance; predominant paw position is parallel at initial contact and lift off; tail is consistently up and trunk instability

21   Consistent plantar stepping and coordinated gait; consistent toe clearance, predominant paw position is parallel throughout stance, consistent trunk stability, tail consistently up

Definitions provided:

Slight: partial movement through less than half of the range of joint motion

Extensive: movement through more than half of the range of joint motion

Sweeping: rhythmic movement of hindlimb in which all three joints are extended, then fully flex and extend again; animal is usually sidelying, the plantar surface of the paw may or may not contact the ground; no weight support across the hindlimb is evident

No weight support: no contraction of the extensor muscles of the hindlimb during plantar placement of the paw or no elevation of the hind quarter

Weight support: contraction of the extensor muscles of the hindlimb during plantar placement of the paw, or elevation of the hind quarter

Plantar stepping: the paw is in plantar contract with weight support, then the hindlimb is advanced forward and plantar contact with weight support is reestablished

Dorsal stepping: weight is supported through the dorsal surface of the paw at some point in the step cycle

Forelimb-hindlimb coordination: for every forelimb step a hindlimb step is taken and the hindlimbs alternate

Occasional: less than or equal to 50%

Frequent: 51-94%

Consistent: 95-100%

Trunk instability: lateral weight shifts that cause waddling from side to side or a partial collapse of the trunk