MIGRATION OF
OLFACTORY ENSHEATHING CELLS
GRAFTED INTO ADULT RAT SPINAL CORD

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By

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ABSTRACT

Olfactory ensheathing cells (OECs) are non-myelinating glial cells that provide ensheathment for axons of the olfactory nerve *in vivo*. OECs have been shown to facilitate the regeneration of CNS axons, to assemble a myelin sheath around demyelinated axons, and it has been suggested OECs migrate very well within the microenvironment of the injured CNS. However, there has been no direct test of their migratory ability *in vivo*. The aims of this study were to determine whether: 1) OECs can be induced to migrate towards an ethidium bromide (EtBr)-induced focal (~1 mm long) demyelination of the spinal cord white matter; 2) OECs migrate away from a focal demyelination either into normal CNS tissue or towards a second demyelinated lesion; 3) microglial reactivity is required for the generation of the migratory signal(s) inducing OECs to migrate towards a focal demyelination; 4) OECs grafted into the subarachnoid space surrounding the spinal cord will migrate into the neuropil in the absence of demyelination.

To achieve these aims, we developed an *in vivo* model for studying the migratory ability of OECs within the adult rat spinal cord. A small focal EtBr-induced demyelination of the dorsal funiculus (unilaterally) of the spinal cord was made at variable distances from the site of a DiI-labelled OEC graft. The major findings were: i) the strength of the migratory signal(s) inducing OECs to migrate increased as the demyelinated lesion was located closer to the grafting site; ii) the OEC migration towards a distal demyelinated lesion was greatly enhanced when the cells were grafted directly into a second demyelinated lesion; iii) the cell migration occurred along a migratory path containing many reactive astrocytes and microglia; iv) the migration of OECs was significantly reduced when the microglial reactivity was dampened using minocycline; and v) OECs survived grafting into cerebrospinal fluid (i.e. subarachnoid space) and migrated into the neuropil of the brain and spinal cord. The major conclusions are that OECs can respond to migratory signal(s) arising as a result of a focal EtBr-induced demyelination and that microglia are one potential source of these migratory signal(s).

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DEDICATION

To my parents,
Hlykeria Mykolaivna Skihar and Mykola Kostyantynovych Skihar.

To all the patients who battle multiple sclerosis.

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AMPA	α-amino-3hydroxy-5-methyl-4-isoxazolepropionic acid
APP	amyloid precursor protein
ATP	adenosine triphosphate
bFGF	basic fibroblast growth factor
β-Gal	β-Galactosidase
BCS	bovine calf serum
BMCs	Bone marrow cells
BSA	bovine serum albumen
CG4	oligodendrocyte progenitor cell line
CNPase	2',3'-Cyclic nucleotide-3'phosphohydrolase
CNS	central nervous system
CSF	cerebrospinal fluid
Cx32	connexin 32
DAB	diaminobenzidine tetrahydrochloride
DiI	1,1'- dioctadecyl - 3,3,3',3' - tetramethylindocarbocyanine perchlorate
DIV	days *in vitro*
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulfoxide
DPBS	Dulbecco's phosphate buffered saline
DREZ	dorsal root entry zone
DRG	dorsal root ganglion
E1	embryonic day 1
E18	18 day embryo
EAE	experimental autoimmune encephalomyelitis
EDTA	ethylenediaminetetraacetic
EG	Enteric glia
EGF	epidermal growth factor
EtBr	ethidium bromide
FBS	fetal bovine serum
FGF	fibroblast growth factor
Gal-C	galactocerebroside
GAP-43	43 Kd growth-associated protein
GFAP	glial fibrillary acidic protein
GFP	green fluorescent protein
H_2O_2	hydrogen peroxide
HBSS	Hank's balanced salt solution
HEPES	N-2-Hydroxyethylpiperazine-Ní-2-ethanesulfonic acid
HLA	human leukocyte antigen
HS	horse serum
Ig	immunoglobulin
IGF	insulin-like growth factor
IL	interleukin
INF	interferon
i.p.	intraperitoneally

MAG	myelin-associated glycoprotein
MAPs	microtubule associated proteins
MBP	myelin basic protein
mG5	modified G5 medium
MHC	major histocompatibility complex
MMPs	matrix metalloproteases
MOG	myelin/oligodendrocyte glycoprotein
MRI	magnetic resonance imaging
MS	multiple sclerosis
MSC80	immortalized Schwann cell line
N-CAM	neural cell adhesion molecule
NGF	nerve growth factor
NSCs	neuronal stem cells
Oct-6 /SCIP/ tst-1	POU domain factor Oct-6
OECs	olfactory ensheathing cells
OMP	olfactory marker protein
ONL	olfactory nerve fiber layer
OX-18	antibody, which recognizes the MHC class 1 antigen
OX-42	antibody, which recognizes the complement type 3 receptor
p75NGFR	low-affinity neurotrophin receptor
PBS	phosphate buffered solution
PBSS	Puck's balanced salt solution
PDGF	platelet derived growth factor
PF	paraformaldehyde
PKH26	lipophilic fluorescent dye
PLP	proteolipid protein
PMP22	peripheral myelin protein 22
PNS	peripheral nervous system
PPG	picric acid/paraformaldehyde/glutaraldehyde
PSA-N-CAM	embryonic polysialic acid containing N-CAM
RV	retrovirus
SMP	skim milk powder
SV 40	simian virus 40
TGF-β	transforming growth factor-β
Th	helper T cells
TMEV	Theiler’s murine encephalomyelitis virus
TNF-α	tumor necrosis factor-α
TX-100	Triton X-100
V-CAM	vascular cell adhesion molecule
VLA	very late antigen

1 INTRODUCTION

1.1 The Primary Olfactory Pathway

The primary olfactory pathway consists of the olfactory epithelium, the olfactory nerve, and the olfactory bulb. Primary olfactory neurons are located in the olfactory epithelium, which is the epithelial component of the olfactory mucosa and lines part of the nasal cavity, and project their axons via the first cranial nerve, the olfactory nerve, to the olfactory bulb. In the bulb, the olfactory axons innervate a discrete lamina near the pial surface of the ipsilateral olfactory bulb where they form synaptic connections with neurons of the bulb in specialized areas of neuropil called glomeruli. The olfactory epithelium is the part of the adult mammalian nervous system in which it was first discovered that neurogenesis continued past the early postnatal period of development and into adulthood (Barber, 1982; Calof *et al.,* 1998; Graziadei and Monti Graziadei, 1979). New olfactory receptor neurons continue to be generated throughout the life of mammals, a process that provides new neurons to replace those lost from injury or disease (Graziadei and Monti Graziadei, 1979; Schwob, 2002). In rodents, olfactory receptor neurons have a life span of approximately one month (Costanzo and Graziadei, 1983; Graziadei and Monti Graziadei, 1978; Moulton *et al.,* 1970; Simmons *et al.,* 1981).

1.1.1 The olfactory mucosa

The olfactory mucosa lines the superior part of the nasal cavity and consists of two layers, a superficial olfactory epithelium and a deeper lamina propria (Morrisson and Costanzo, 1992). The former is an avascular pseudostratified columnar epithelium containing three principal cell types: olfactory receptor neurons, sustentacular (supporting) cells, and horizontal basal cells. The olfactory epithelium is mainly sensory in function and is specialized for the detection of odorants. The lamina propria contains numerous fascicles of olfactory axons, which are all unmyelinated, multicellular Bowman's glands and blood vessels (Getchell *et al.,* 1984); in addition to these elements being embedded within the loose connective tissue of the lamina propria, there is also an occasional small fascicle of myelinated and unmyelinated axons originating from the trigeminal nerve. Sustentacular cells of the olfactory epithelium and

Bowman's glands, the ducts of which course through the epithelium enroute to the apical surface of the epithelium, are believed to be responsible for producing the layer of mucus that covers the free surface of the olfactory mucosa (Getchell *et al.,* 1984).

Three main neuronal populations have been identified in the olfactory epithelium of adult mammals (Graziadei and Monti Graziadei, 1978, 1979), with each population representing a different stage in the maturation of newly formed olfactory receptor neurons. Globose basal cells comprise the first population and are believed to be the youngest neurons in the olfactory epithelium, with the most immature of these just beginning to emit an axon from the basal surface of the cell (Graziadei and Monti Graziadei, 1978, 1979). These cells are situated near the base of the epithelium, immediately superficial to a single layer of horizontal basal cells. The second neuronal population is made up of immature olfactory receptor neurons that have extended their axons into the lamina propria of the olfactory mucosa, with some of the axons reaching as far as the olfactory bulb. Although the neurons of this second population may respond to odor molecules, their activation does not contribute to the perception of smell because the axons of these neurons have not yet formed synaptic contacts in the olfactory bulb. Neurons of the first two neuronal populations share several phenotypic features with immature neurons from other regions of the developing nervous system, expressing for example vimentin (Schwob *et al.,* 1986), microtubule associated proteins (MAPs) characteristic of immature neurons (i.e. MAP5 and MAP1B; Schoenfeld *et al.,* 1989; Tucker *et al.,* 1989; Viereck *et al.,* 1989), the embryonic form of the neural cell adhesion molecule (N-CAM; Miragall *et al.,* 1988, 1989), and the 43 Kd growth-associated protein that is better known as GAP-43 (Verhaagen *et al*., 1989, 1990). In addition, the second progenitor cell in the continuum of globose basal cell population expresses MASH-1 (Calof *et al.,* 1996, 2002; Schwob, 2002), which is required for commitment to the neuronal lineage (Murray *et al.,* 2003). In MASH-/- mice, most of the progenitors develop along the sustentacular cell pathway instead (Murray *et al.,* 2003). Later, the third distinct subclass of globose basal cells, an immediate neuronal precursor expresses neurogenin-1 (Calof *et al*., 1996, 2002; Schwob, 2002).

Mature olfactory receptor neurons comprise the third neuronal population and are bipolar cells that possess a fusiform cell body. A single unmyelinated axon arises from the basal pole of each neuron and projects without axon collaterals to the olfactory bulb where it makes synaptic contacts with neurons in the olfactory bulb. Most of the olfactory receptor

neurons are believed to never fully mature because they retain the expression of MAP5, MAP1B (Schoenfeld *et al*., 1989; Tucker *et al.,* 1989; Viereck *et al.,* 1989), and vimentin (Schwob *et al.,* 1986); they do, however, stop expressing GAP-43 (Verhaagen *et al.,* 1989, 1990) and the embryonic form of N-CAM (Miragall *et al.,* 1988, 1989). Mature olfactory receptor neurons also exclusively express olfactory marker protein (OMP; Margolis, 1985). OMP-deficient mice showed decreased ability to respond to odorants compared with wild type mice, thus OMP was demonstrated to modulate the odor detection and signal transduction cascade in these cells (Buiakova *et al.,* 1996).

A single dendrite arises from the apical pole of the cell body of each olfactory receptor neuron and projects towards the free surface of the olfactory epithelium where it terminates in an apical knob from which extend several cilia. These olfactory receptor neurons express chemoreceptors on the surface of their dendrites, which enable the neurons to detect the presence of volatile stimuli in the surrounding environment (Getchell *et al.,* 1984). When odorants reach the superior aspect of the nasal cavity they must traverse a layer of mucus before they can bind to odorant receptors in the chemosensitive plasma membrane of the olfactory receptor neurons (Getchell *et al.,* 1984). This binding of odorant molecules by the receptors initiates a series of membrane events leading to sensory transduction and initiation of an action potential. Olfactory receptor neurons encode information about the intensity, duration and quality of the odorant and transmit this information to the brain.

At the base of the olfactory epithelium there are horizontal basal cells that lie directly against the basal lamina separating the epithelium from the lamina propria. The horizontal basal cells express the epidermal growth factor (EGF) receptor, keratin and a surface glycoprotein called alpha-N-acetyl galactosamine that binds Iβ-4 isolectin from Banderaea simplicifolia-1 (Holbrook *et al.,* 1995). These cells are attached by desmosomes to sustentacular cells and globose basal cells, and by hemidesmosomes to the basal lamina. The horizontal basal cells enfold virtually all bundles of olfactory axons within tunnels formed when horizontal basal cells arch over the basement membrane (DeLorenzo, 1957; Frisch, 1967; Graziadei and Monti Graziadei, 1978, 1979; Holbrook *et al.,* 1995). The horizontal basal cells are suggested to be the stem cell for olfactory neurogenesis (Calof and Chikaraishi, 1989; Satoh and Takeuchi, 1995).

The sustentacular cells extend from the basement membrane to the epithelial surface and are described by several authors (Getchell *et al.*, 1984; Rafols and Getchell, 1983) as modified ependymal cells. Rafols and Getchell (1983) characterized these cells in the olfactory mucosa of the salamander as two morphological types with type I resembling ependymal tanycytes and type II resembling protoplasmic astrocytes. The apical portions of the sustentacular cells are bound together with adjacent sustentacular cells or with the olfactory receptor neuron dendrites by tight junctions at the mucosal surface, where each dendrite is surrounded by sustentacular cells and thus is isolated from adjacent dendrites (DeLorenzo, 1957; Frish, 1967; Graziadei and Monti Graziadei, 1979). At the basement membrane, the basal portion of sustentacular cells lies very close to capillaries and to Bowman's glands that lie immediately on the lamina propria side of the basement membrane. Sustentacular cells are believed to (1) isolate olfactory receptor neurons from one another for electrical purposes (Graziadei and Metcalf, 1971); (2) secrete mucopolysaccharides from the apical pole (Getchell *et al*., 1984); (3) transport molecules from the lamina propria through the olfactory epithelium to its free surface and vice versa (Rafols and Getchell, 1983), and (4) guide the migration of postmitotic neurons towards the apical surface of the epithelium (Simmons *et al.,* 1981; Rafols and Getchell, 1983).

1.1.2 The olfactory nerves

The axons, arising from the basal pole of olfactory receptor neurons, course through the olfactory epithelium en route to the lamina propria where they join with other axons to form the peripheral olfactory fascicles (fila olfactoria) (Graziadei and Monti Graziadei, 1978). In the lamina propria, the unmyelinated olfactory axons are ensheathed by glial cells referred to as olfactory ensheathing cells (OECs; see Section 1.2). The olfactory fascicles eventually coalesce in the submucosa to form the olfactory nerve bundles that penetrate the cribriform plate of the ethmoid bone, enter the cranial cavity, travel within the nerve fiber layer (i.e. the most superficial layer; ONL) of the olfactory bulb, and synapse in the olfactory glomerulus. Newly formed olfactory receptor neurons send out their axons that are able to form functional connections in the adult olfactory bulb (Costanzo, 1985; Oley *et al.*, 1975; Simmons and Getchell, 1981). Connective tissue sheaths surround each olfactory fascicle within the lamina propria. Several of these olfactory fascicles and their connective tissue sheaths fuse together to form much larger fascicles, which in turn enter and usually almost completely fill each

foramina of the cribriform plate (Doucette, 1991). The olfactory fascicles then travel within the subarachnoid space bridging the gap between the foramina of the cribriform plate and the ONL of the olfactory bulb. The connective tissue sheaths of the intracranial portion of these fascicles fuse with both the dural and leptomeningeal linings of the cranial cavity (Doucette, 1991).

Olfactory axons are small, being only 0.1 to 0.5 μm in cross-sectional diameter, and never branch until they approach their target in the olfactory bulb (Graziadei and Monti Graziadei, 1978). Numerous axons are grouped together in one common mesaxon formed by the OEC (DeLorenzo, 1957). OECs are the only glial cells present in the peripheral olfactory fascicles (DeLorenzo, 1957; Doucette, 1990; Field *et al.,* 2003). The cell membrane of an OEC forms one mesaxon for a large number of axons rather than for each individual axon and separates fascicles from each other by OEC cytoplasm (DeLorenzo, 1957). The OECs of each peripheral olfactory fascicle are enclosed within a common basal lamina, which surrounds the entire fascicle (DeLorenzo, 1957; Frisch, 1967). Adjacent fascicles are separated from one another by fibroblasts and collagen (Doucette, 1993). In contrast, OECs within the ONL of the olfactory bulb are apposed to a basal lamina only where these cells contribute to the formation of the glia limitans (Barber and Lindsay, 1982; Doucette, 1984, 1986). Astrocytes, but not fibroblasts and collagen fibers, occupy the spaces between adjacent olfactory fascicles in the central nervous system (CNS; Doucette, 1984, 1986).

1.1.3 The olfactory bulb

The axons of olfactory receptor neurons enter the olfactory bulb through its ventral surface, which lies on the intracranial side of the cribriform plate immediately ventral to the surface of the frontal lobe. The olfactory axons grow through this part of the CNS to synapse with their targets (Barber and Linsday, 1982; Graziadei and Monti Graziadei, 1978). The olfactory bulb is a bilateral oblong structure attached to the rest of the brain by means of the olfactory tract. The olfactory bulb has a laminated structure and arises embryologically as an evagination of the rostral wall of the cerebral vesicle.

Proceeding from the pial surface inward, the laminar pattern of the olfactory bulb comprises the following layers: a) ONL, b) glomerular layer, c) external plexiform layer, d) mitral cell layer, e) internal plexiform layer, and f) granule cell layer. The ONL forms a superficial lamina, which extends around the entire outer circumference of the bulb and contains glial cells and fascicles of densely packed unmyelinated olfactory axons (Barber and

Linsday, 1982; Doucette, 1984, 1986, 1990). Astrocytes and OECs are the glial cells of the ONL. The synaptic terminals of the axons cluster in spherical structures know as glomeruli, and each glomerulus receives input primarily from different olfactory receptor neurons. In the glomerulus, olfactory neuron axons synapse on dendrites of the mitral, tufted and periglomerular cells. Periglomerular cells are interneurons, the axons of which synapse on the dendrites of mitral and tufted cells as well as on the cell bodies and dendrites of other periglomerular cells (Pinching and Powell, 1971). Thus, the periglomerular cells influence the transmission of the olfactory information between neighboring glomeruli. The largest (20 – 30 μm in diameter) neurons in the olfactory bulb are the mitral cells, which are arranged in a layer of one to two cell bodies thick and which are separated from the glomerular layer by their own dendrites forming the external plexiform layer. Tufted cells, also known as displaced mitral cells, can be found sparsely scattered throughout the external plexiform layer and even in parts of the periglomerular region. Dendrites and axons of the tufted cells make connections similar to those of mitral cells, respectively. However, collaterals of tufted cell axons ramify around several adjacent glomeruli, where they terminate on the cell bodies and dendrites of periglomerular cells (Pinching and Powell, 1971). The axons of mitral and tufted cells leave the olfactory bulb through the granule cell layer to enter the olfactory tract (Haberly and Price, 1977) and innervate the anterior olfactory nucleus, olfactory tubercle, amygdaloid nucleus, piriform cortex (White, 1965, Heimer, 1968; Price and Powell, 1971) and entorhinal cortex (White, 1965, Heimer, 1968). The granule cell layer forms the innermost lamina of the olfactory bulb. Granule cells extend their dendrites into the external plexiform layer where they make reciprocal dendro-dendritic contacts with mitral and tufted cells.

1.1.4 Embryonic and fetal development

The development of the olfactory system has been extensively studied in a variety of animals including mice (Hinds, 1972), frog (Byrd and Burd, 1991), ducks (Rebiere and Dainat, 1981), lungfish (Derivot, 1984), and snakes (Holtzman and Halpern, 1990). During the second week of gestation two olfactory placodes, the thick areas of cranial ectoderm situated rostrolateral to the developing cerebral vesicles, invaginate to form the olfactory pits, which will give rise to the olfactory epithelium (Graziadei, 1973; Hinds, 1972). At the base of the epithelium, processes of the epithelial cells penetrate the basal lamina and, surrounded by glial progenitor cells, extend into the mesenchyme adjacent to the olfactory epithelium (Cuschieri

and Bannister, 1975). At the end of the second week of gestation of mouse embryos, OMP can be detected using immunohistological methods in cell bodies near the surface of the olfactory epithelium (Farbman and Margolis, 1980; Graziadei *et al*., 1980a). Since only mature olfactory receptor neurons express OMP in the adult (Monti-Graziadei *et al.,* 1977), the presence of OMP in cells of developing olfactory epithelium indicates that these cells have formed synaptic contacts and have reached maturity. In the third week of gestation OMP is found in an increased number of cells at the epithelial surface, in the olfactory nerve fascicles of the lamina propria, in the ONL and by the end of the third week in the glomeruli of the bulb (Graziadei *et al*., 1980a).

During embryogenesis, olfactory receptor neurons influence the development of the olfactory bulb, the initial formation of which from the rostral wall of the cerebral vesicle occurs near the end of the second week of gestation (Hinds, 1972). At this time the number of olfactory axons and mitral/tufted cells in the olfactory bulb increases in developing *Xenopu*s larvae (Byrd and Burd, 1991). During the early developmental stages of the mouse embryos, the olfactory axons make their initial contact with the rostral wall of the cerebral vesicle, referred to as the olfactory bulb primordium (Doucette, 1989). Once the olfactory axons and peripheral glial progenitor cells of the olfactory fascicle cover the external exposed surface of the bulb primordium, they form the presumptive ONL. At this time point, which corresponds to Theiler stages 19 and 21 (Theiler, 1972), the cellular constituents of the ONL are separated from those of the mouse bulb primordium by a relatively intact glia limitans. This glia limitans eventually disappears thus leaving no physical barrier between the cellular constituents of the definitive ONL and the marginal zone of the developing olfactory bulb (Doucette, 1989).

Present at the early developmental stages, olfactory axons are found to penetrate the bulb reaching its deep intermediate zone. Here they make contact with neuronal precursor cells of the olfactory bulb and possibly influence their development (Stout and Graziadei, 1980). Later the olfactory axons retract to their final location in the olfactory nerve and glomerular layers. Olfactory afferent innervation is critical for the differentiation and maintenance of the dopamine phenotype of interneurons in the olfactory bulb (Baker and Farbman, 1993). Decreasing the numbers of the olfactory axons by partial deafferentation of the olfactory bulb results in a reduction in the number of mitral neurons in the olfactory bulb of either *Xenopu*s embryos (Byrd and Burd, 1993) or the moth, *Manduca sexta* (Hildebrand *et al*., 1997).

Complete removal of the olfactory placode in amphibians resulted in formation of a small or no olfactory bulb (Stout and Graziadei, 1980). Vasculature plays an important role in neuronal development and responds to alterations in sensory stimulations early in life.

Doucette (1989) described the development of the ONL in the olfactory bulb of mouse embryos, when Marin-Padilla and Amieva (1989) characterized the early neurogenesis of the mouse olfactory nerve. The olfactory axons are identified in the mesenchyme growing dorsocaudally toward the cerebral vesicle. Peripheral glial progenitor cells, which ensheath adjacent axons, accompany them. Olfactory axons and OECs from the same regions of the nasal mucosa establish an early association, which is maintained up to their glomerular terminal. Marin-Padilla and Amieva (1989) suggested that simple anatomical arrangement between olfactory axons and OECs allows establishing a topographic map of the sensory olfactory epithelium in the developing olfactory bulb. Thus, the axons of newly formed olfactory neurons from different nasal regions could be guided by their OEC conduits toward their target glomeruli; hence, the olfactory map may be maintained undisturbed through the animal's life span. Axons, but not the glial progenitors, penetrate into the marginal zone through small breaks in the glia limitans of the bulb primordium. Doucette (1989) suggested that the ONL of the olfactory bulb forms superficial to, rather than within, the marginal zone of the rostral wall of the cerebral vesicle. The peripheral glial progenitor cells contributed to the formation of the ONL and presumably differentiated into OECs. Arising from the peripheral nervous system (PNS), OECs are one of the two glial cell types in the ONL, with astrocytes being the second cell type and having a CNS origin. However, by Theiler stage 24 (embryonic day 18) of developing rat embryos and stage 16 of mouse embryos the OECs are the only glial cells present in the ONL (Doucette, 1989). Therefore, OECs are the only glial cells present in the OEC cultures initiated using the ONL of E18 rat olfactory bulbs.

1.1.5 Neuronal regeneration

Generation of new sensory neurons in the olfactory epithelium is an ongoing process, which continuous throughout life (Barber, 1982; Calof *et al.*, 2002; Graziadei and Monti Graziadei, 1978, 1979; Schwob, 2002). The neuronal turnover occurs both spontaneously and in response to cellular and tissue trauma (Schwob, 2002). New neurons arise as a result of the proliferation and differentiation of stem/progenitor cells located near the base of the epithelium (Graziadei and Monti Graziadei, 1978, 1983; Graziadei *et al.*, 1980b). Neurogenesis in the

olfactory epithelium is in a state of dynamic equilibrium between positive and negative regulators. Indirectly controlling the generation of new neurons, the mature neurons of the olfactory epithelium appear to be a source of negative feedback signals, which regulate the proliferation of the precursor cells. A presumptive stem cell divides asymmetrically approximately every 50 days producing another stem cell, which stays close to the basal lamina, and a neuronal precursor cell. The latter cell further divides rapidly several times producing many immature neurons, each of which migrate away from the basal lamina (Mackay-Sim and Kittel, 1991). Because in the olfactory epithelium neurogenesis is a continuous process, there are neurons at varying stages of differentiation, reflecting different stages of maturation. Therefore, there are olfactory receptor neurons with different morphological (Graziadei and Monti Graziadei, 1978), biochemical (Harding *et al.,* 1977), and physiological (Simmons and Getchell, 1981) characteristics residing within the same part of the olfactory epithelium. Caggiano *et al.* (1994) and Schwob *et al.* (1994) used a retroviral vector to label proliferating cells in the olfactory epithelium and obtained data consistent with the neurons arising from horizontal basal cells. An additional *in vivo* study suggests that a stem cell resides in the olfactory epithelium very close to the basal lamina, which is where the horizontal basal cells are localized (Mackay-Sim and Kittel, 1991). Olfactory receptor neurons can also be generated *in vitro* with the evidence suggesting they may arise from horizontal basal cells (Mahanthappa and Schwarting, 1993; Satoh and Takeuchi, 1995). However, the actual identity of the stem and progenitor cells in the olfactory epithelium is yet to be resolved.

1.2 Olfactory Ensheathing Cells

OECs are the non-myelinating glial cells that ensheath olfactory axons within both the PNS and CNS portions of the primary olfactory pathway (DeLorenzo, 1957; Doucette, 1990; Field *et al.,* 2003; Frisch, 1967). OECs are located in a region of the mammalian nervous system where axonal growth occurs throughout the lifetime of the organism. They are distributed within the lamina propria of the olfactory mucosa, the olfactory nerve, and the first two layers of the olfactory bulb (Cancalon, 1985; Doucette, 1990; Gong *et al.,* 1994). Rafols and Getchell (1983) examined the morphological relationship between the receptor neurons and nonneuronal cells, i.e. glial fibrillary acidic protein (GFAP) expressing OECs, which surround the unmyelinated olfactory axons and suggested that these OECs contribute to the guidance of

the olfactory axons to their synaptic targets in the olfactory bulb. Although Rafols and Getchell (1983) described these cells as astrocytes they were later named as OECs (Doucette, 1984).

In both the olfactory nerves and the olfactory bulbs the OECs display a very characteristic morphology (Bailey and Shipley, 1993; Doucette, 1984, 1986, 1990, 1991, 1993). Golgi in 1875 and Blanes in 1898 (as described in Ramon-Cueto and Avila, 1998) were the first to describe the glial population of the olfactory bulb of adult mammals. They observed two types of macroglia in the olfactory bulb, one stellate in morphology and the other fusiform. Stellate cells were distributed throughout the entire olfactory bulb, whereas the fusiform cells were located only in the first two layers. More recent studies have revealed the stellate cells are astrocytes and the fusiform cells are the OECs (Barber and Lindsay, 1982; Doucette, 1984, 1986, 1991, 1993). More recently, Bailey and Shipley (1993) described unipolar and irregular subtypes of astrocytes that were exclusively located in the ONL of the olfactory bulb. These astrocytes separated olfactory nerve fascicles, and had long, unbranched cytoplasmic processes (irregular subtype). The unipolar or unbranched astrocytes were different from most white matter fibrous astrocytes that elongate in a bipolar arrangement along axons in other white matter tracts.

The OECs reside within olfactory fascicles in both the PNS and CNS portions of the primary olfactory pathway, aligning their perikarya along the longitudinal axis of the fascicles (Barber and Lindsay, 1982). On the basis of ultrastructural criteria, OECs have a characteristic indented nucleus with uniformly distributed heterochromatin. Free ribosomes and large inclusion bodies are abundant in the cytoplasm as well. Compared to astrocytes, the cytoplasm of OECs is more electron dense, which may be due to the presence of more water-soluble proteins in the cytoplasm of the latter cells (Doucette, 1984). The intermediate filaments of OECs are fewer in number and are scattered throughout the cytoplasm instead of being grouped into closely packed bundles the way they are in astrocytes (Barber and Lindsay, 1982; Doucette, 1984, 1986, 1990, 1991). At the glia limitans, the plasma membranes of end-feet of OECs lack the orthogonal arrays of intramembranous particles (Barber and Lindsay, 1982; Doucette, 1991, 1993) that are so characteristic of astrocytic end-feet forming the glia limitans in other parts of the CNS (Peters *et al*., 1976).

OECs are a potential source of growth-promoting and neurite-promoting factors during development and in the postnatal period (reviewed in Ramon-Cueto and Avila, 1998). These

cells express platelet-derived growth factor (Kott *et al.,* 1994), neuropeptide Y (Ubink *et al.,* 1994), and the calcium-binding protein S100 (Cummings and Brunjes, 1995), which is suggested to facilitate the growth and survival of neurons (Bhattacharyya *et al*., 1992; Van Eldik *et al*., 1991). A neurite-promoting factor referred to as glia-derived nexin is also synthesized by both the astrocytes of the nerve fiber layer of the bulb (Scotti *et al.,* 1994) and by OECs (Reinhard, 1988; Scotti *et al.,* 1994). The low-affinity neurotrophin receptor (p75NGFR) is also expressed by OECs, with the expression of this receptor being under strong developmental regulation (Barnett *et al*., 1993; Franceschini and Barnett, 1996; Gong *et al*., 1994; Miwa *et al*., 1993; Turner and Perez-Polo, 1993, 1994; Vickland et al., 1991). At all embryonic and fetal stages of development in rodents, p75NGFR expression is associated with the OECs ensheathing olfactory axons. In neonatal rodents, OECs show an intense expression of p75NGFR at the light and electron microscope levels that decreases to almost undetectable levels in the adult. Ramon-Cueto and Nieto-Sampedro (1992) identify four subpopulations of OECs in cultures obtained from adult rat olfactory nerve and glomerular layers of the olfactory bulb. One subpopulation expresses both p75NGFR and fibronectin (5% of the total population), while a second subpopulation is p75NGFR positive and fibronectin negative (26%). The remaining two subpopulations do not express p75NGFR, although one is fibronectin positive (50%) and the other is fibronectin negative (19%).

At all developmental stages, OECs express in their plasma membranes the L1 cell adhesion molecule (Miragall *et al*., 1988), the embryonic polysialic acid containing N-CAM (PSA-N-CAM), as well as adult forms of N-CAM (Franceschini and Barnett, 1996; Miragall *et al*., 1988); they also synthesize and secrete laminin into the extracellular matrix (Doucette, 1996; Kafitz and Greer, 1997; Liesi, 1985). All of these molecules are known to be involved in cell adhesion and axonal growth (Bixby *et al*., 1988). The CAMs presumably mediate olfactory axon elongation on OECs (Barnett *et al*., 1993; Chuah and Au, 1994; Franceschini and Barnett, 1996; Key and Akeson, 1990; Miragall *et al*., 1988; Ramón-Cueto and Nieto-Sampedro, 1992) since N-CAM and L1 are only found in that part of the OEC plasma membrane in direct contact with the axons (Miragall *et al*., 1988). Only those OECs forming part of the glia limitans express PSA-N-CAM, with this molecule being present in the part of their plasma membrane that is not apposed to the basal lamina (Miragall *et al*., 1988). On the

other hand, type IV collagen and fibronectin are expressed in the region of the OEC plasma membrane that is in contact with the basal lamina (Doucette, 1995).

Vimentin is the major constituent of the OEC intermediate filaments during development and in the adult (Barnett *et al*., 1993; Franceschini and Barnett, 1996; Ramón-Cueto and Nieto-Sampedro, 1992; Schwob *et al*., 1986). Besides vimentin, OECs also express nestin in the embryo and neonate (Doucette, 1993; Valverde *et al*., 1992), and GFAP in the neonate and adult (Barber, 1982; Barnett *et al*., 1993; Franceschini and Barnett, 1996; Pixley, 1992).

1.2.1 Ensheathment of olfactory axons

OECs envelop multiple axons in a common mesaxon with their inner process-bearing (adaxonal) surface, which has no basal lamina (DeLorenzo, 1957; Doucette, 1990; Field *et al*., 2003; Frisch, 1967). Winding, sheet-like inner OEC cytoplasmic processes separate the unmyelinated axons into smaller groups within the inner compartment of each fascicle (Valverde and Lopez-Mascaraque, 1991). In cross section, each peripheral olfactory fascicle is seen to contain several glial cells, with the cytoplasmic processes of these cells extending towards the center of the fascicle to enclose variable numbers of olfactory axons within common mesaxons (Doucette, 1990). The outer (abaxonal) surface of OECs is smooth and faces the connective tissue, which in the PNS portion of the pathway contains fibroblasts and collagen fibers and from which they are separated by a basal lamina. This basal lamina is continuous with the epithelial basal lamina at the numerous points where small bundles of olfactory axons leave the olfactory epithelium and are first ensheathed by OECs (DeLorenzo, 1957; Doucette, 1984, 1990). Raisman (1985) suggested that the peculiar multiaxonal sheathing arrangement allows the newly growing axons to slide in along existing axonal surfaces without requiring them to make direct contact with the glial cell membrane.

Throughout their length in both the PNS (fila olfactoria) and CNS (ONL and glomerular layers) portions of the primary olfactory pathway, the olfactory axons are ensheathed by OECs, such that the axons do not contact any other glial cell type (Barber and Lindsay, 1982; Carr and Farbman, 1993; Doucette, 1984, 1986, 1990, 1991, 1993; Pixley, 1992; Raisman, 1985). Unlike Schwann cells, OECs do not ensheath individual axons; instead, the mesaxon of each OEC encloses densely packed bundles of unmyelinated axons (Barber and Lindsay, 1982; Doucette, 1993; Farbman and Squinto, 1985; Peters, 1976). Similarly, at variance with the

manner in which Schwann cells ensheath unmyelinated non-olfactory axons, OECs within the ONL of the olfactory bulb are only apposed to a basal lamina in those regions where they contribute to the formation of the external glial limiting membrane. However, similar to other peripheral nerves (Bunge *et al*., 1986), the olfactory fascicles in the lamina propria are enclosed within a basal lamina that separates the OECs from the cells and matrix of the surrounding connective tissue.

The ability of the olfactory nerves to re-establish synaptic connection with central adult neurons of the olfactory bulb in adult mammals has been attributed to OECs and to the glial arrangements at the olfactory glomerulus (Barber, 1981; Barber and Raisman, 1978; Graziadei *et al*., 1980b; Graziadei and Monti Graziadei, 1978, 1979; Wilson and Raisman, 1981). Unmyelinated axons of the spinal nerves and cranial nerves 3 to 12 cross from the PNS into the CNS and are "handed" directly from a Schwann cell to an astrocyte (Berthold and Carlstedt, 1977). In contrast, olfactory axons are never "handed" from OECs to another glial cell as the axons enter the CNS. Olfactory axons do, however, lose their glial ensheathment as they enter a glomerulus where the cytoplasmic processes of astrocytes may come into close apposition to the entering axon fascicles although the axons are never actually ensheathed by these glial cells (Doucette, 1984; Raisman, 1985). OECs extend their cytoplasmic processes in the shape of "prongs" that penetrate deeply along the periphery of each glomerulus. However, OEC processes do not enter the neuropil of the glomerulus (Raisman, 1985).

1.2.2 Embryonic and fetal development

The differentiation of OECs during embryonic and fetal development is intimately related to the development of the first cranial nerve. Progenitor cells originating from the olfactory placode give rise to OECs (Doucette, 1989, 1990; Farbman and Squinto, 1985; Marin-Padilla and Amieva, 1989). During the development of the embryo and later of the fetus, the OEC progenitors migrate away from the olfactory placodes toward the rostral wall of the telencephalic vesicle where they eventually contribute to the formation of the ONL of the presumptive olfactory bulb (Doucette, 1989; Marin-Padilla and Amieva, 1989). As these immature OECs enter the telencephalic vesicle during early embryonic development, they begin to express the N-CAM and L1 cell adhesion molecules (Miragall *et al.,* 1989). Doucette (1993) suggested that these CAMs facilitate the adhesion and growth of olfactory axons from the olfactory epithelium into the developing olfactory bulb with the growing axons being able

to use the cell surface of OECs as a substrate. On the other hand, OECs ensheath multiple axons, most of which do not come in a close contact with OEC plasma membrane (DeLorenzo, 1957; Doucette, 1990; Field *et al*., 2003; Frisch, 1967). Such arrangement of olfactory axons within an OEC mesaxon allows newly formed axons to extend to the olfactory bulb using other axons for guidance (Doucette, 1984, 1990; Raisman, 1985). These OECs also begin to express the p75NGFR (Gong *et al*., 1994) and the L14 lectin (Mahanthappa *et al.,* 1994). Although the function of p75NGFR remains to be determined, it is thought to provide a chemotactic guidance to growing olfactory axons (Doucette, 1993; Li *et al.,* 1997). Mahanthappa *et al*. (1994) showed that the L14 lectin also promotes the adhesion of primary olfactory neurons to laminin *in vitro* in an integrin-independent manner.

During the development of the rodent olfactory system, peripheral glial progenitor cells accompany and ensheath olfactory axons growing dorsocaudally toward the cerebral vesicle (Doucette, 1989). In Theiler stage 19-21 embryos, the peripheral glial progenitor cells and olfactory axons are located under the pia matter along the external surface of the bulb primordium forming a presumptive ONL immediately superficial to the bulb's external glial limiting membrane. By the first part of stage 21, the peripheral progenitor cells are identified within the presumptive ONL with an electron-dense cytoplasmic matrix filled with expanded rough endoplasmic reticulum, mitochondria, numerous free ribosoms and polyribosomes, and a well-developed Golgi apparatus. Some cells acquire bipolar shape with their long axis oriented parallel to the direction of axonal growth. Olfactory axons but not peripheral glial progenitors penetrate the marginal zone of the bulb primordium. The only glial cells found within the ONL by Theiler stage 24 of developing rat and mouse embryos are the peripheral glial progenitor cells.

1.2.3 PNS-CNS transitional zone

OECs are the only glial cells known to ensheath axons on both sides of the PNS-CNS transitional zone (Doucette, 1984, 1986, 1990, 1991; Fraher, 1999). In contrast, when myelinated primary sensory axons enter the CNS, their ensheathment abruptly changes from Schwann cell ensheathment to that of oligodendrocytes or astrocytes (Berthold and Carlstedt, 1977; Maxwell *et al.,* 1969; Steer, 1971). The relationship of unmyelinated non-olfactory primary sensory axons to surrounding glial cells is similar to that of myelinated fibers at the PNS-CNS transitional zone with the axons being ensheathed in pockets of the Schwann cells in

the PNS and those of astrocytes in the CNS. The axons of spinal cord and cranial nerve rootlets pass through pores or openings in the glia limitans at the transitional zone between PNS and CNS. Each Schwann cell within the PNS compartment is surrounded by basal lamina and collagen fibers. The basal lamina of each Schwann cell at the PNS-CNS junction is continuous with that of the glia limitans of the subpial astrocytes (reviewed in Fraher, 1999).

Each olfactory nerve rootlet travels within the subarachnoid space after leaving foramina of the cribriform plate before entering the ONL of the olfactory bulb. The basal lamina covering the external surface of each of these rootlets is continuous with that covering the external surface of the glia limitans of the ONL. Within the PNS, the OECs within each olfactory fascicle are enclosed within a common basal lamina, which surrounds the entire fascicle (DeLorenzo, 1957; Frisch, 1967). Fibroblasts and collagen fibers separate adjacent peripheral olfactory fascicles from one another (Doucette, 1993). In contrast, the OECs within the CNS portion of the primary olfactory pathway are opposed to a basal lamina only where these glial cells contribute to the formation of the glia limitans (Barger and Lindsay, 1982; Doucette, 1984, 1986). Furthermore, in the ONL astrocytes are found between adjacent olfactory fascicles instead of fibroblasts and collagen fibers (Doucette, 1984, 1986, 1993). In addition, astrocytes are excluded from the inside of these fascicles and from the PNS-CNS transitional zone of the first cranial nerve, which is where OECs have the exclusive role of forming the glia limitans (Doucette, 1991), even though elsewhere this functions is the exclusive domain of astrocytes. At the surface of the olfactory bulb, the cytoplasmic processes and plasma membrane of OECs mingle and come into close contact, respectively, with those of astrocytes, and form part of the glial limiting membrane with no intervening basal lamina between the two types of glial cell (Doucette, 1984, 1990).

1.2.4 Tissue culture protocols

OECs have attracted the attention of researchers as potential candidates for the treatment of different neurological diseases such as multiple sclerosis and spinal cord injury. When grafted into demyelinated lesions, OECs were found to form myelin around naked axons (Franklin *et al*., 1996; Imaizumi *et al*., 1998). Transplantation of these cells into a mechanically damaged spinal cord resulted in axonal regeneration, remyelination of axons in the damaged spinal cord tracts and functional recovery of locomotion in the animals (reviewed

by Boyd *et al.,* 2003; Franklin and Barnett, 2000; Plant *et al.,* 2003; Raisman, 2001; Ramon-Cueto *et al*., 2000).

OECs can be isolated from 18 day (E18) rat embryos (Boyd *et al.,* 2004; Devon and Doucette, 1992, 1995; Doucette, 1993; Doucette and Devon, 1994, 1995), newborn rats (Barnett *et al*., 1993; Chuah and Au, 1993, 1994; Franceschini and Barnett, 1996; Pixley and Pun, 1990) and from adult rats (Gudino-Cabrera and Nieto-Samperdro, 1999; Nash *et al.,* 2002; Ramon-Cueto and Nieto-Sampedro, 1992) and humans (Barnett *et al.,* 2000; Kato *et al.,* 2000). The main advantage of using embryonic tissue is that at day 18 of embryonic development all of the glial cells in the ONL of the olfactory bulb of rats are OECs (Doucette, 1989). On the other hand, cell cultures obtained from the olfactory bulbs of newborn and adult animals require additional purification procedures, such as immunopanning (Ramon-Cueto and Nieto-Sampedro, 1994) or using a cell sorter (Pascual *et al*., 2002), to eliminate contaminating cells.

Primary cultures of OECs have been obtained from different parts of the olfactory system including the olfactory mucosa, olfactory nerve, and olfactory bulb. Pixley and Pun (1990) harvested olfactory mucosa from newborn Sprague Dawley rat pups, whereas Lu *et al.* (2002) used the lamina propria of olfactory mucosa they obtained from adult rats. Devon and Doucette (1992, 1995), Boyd *et al.* (2004), Chuah and Au (1993), Barnett *et al.* (1993), Ramon-Cueto and Nieto Sampedro (1992) used the ONL of the olfactory bulb as the source of tissue to initiate OEC cultures. Another way to acquire OECs for transplantation is a creation of a cell line. Boruch *et al.* (2001) generated a cell line of OECs producing nerve growth factor (NGF) whereas Goodman et al. (1993) generated a p75NGFR-positive OEC clonal cell line from neonatal Sprague-Dawley rats using the SV40 large T antigen. In order to break intercellular connection, Ramon-Cueto and Nieto Sampedro (1992) treated their cell cultures with trypsin containing medium, whereas others utilized a combination of trypsin and collagenase (Chuah and Au, 1991; Pixley and Pun, 1990). There are advantages and disadvantages to using primary cultures or cell lines. Engineering the cell line prevents contamination present in primary cultures and provides a long-term stable supply of a purified cell population. On the other hand, OEC cell lines are unlikely to be useful in future treatments of patients with demyelinating diseases and CNS injury unless we know that grafted cells will not form tumors or cause any other disorders as a consequence of uncontrolled cellular growth.

Most studies have used pure population of OECs, whereas Li *et al.* (1997, 1998) utilized unpurified OECs and Lakatos *et al.* (2003) implemented a mixture of OECs with other cells, i.e. meningeal cells, in their experiments. Unsatisfactory results in OEC harvesting procedures or OEC transplantation studies have prompted researchers to implement additional techniques (i.e. immunoadsorbtion, enzymatic cell separation) or chemicals (i.e. growth factors). Chuah and Teague (1999) used bovine pituitary extract in order to increase the number of OECs.

A marker specific to OECs is still unavailable for identifying these cells in both *in vivo* and *in vitro* experiments. This has prompted researchers to use various cell labelling techniques. Prelabelling OECs with a nuclear fluorochrome bisbenzimide (Hoechst 33342 dye; Imaizumi *et al.,* 2000; Lu *et al.,* 2002; Pascual *et al.,* 2002; Ramon-Cueto *et al.,* 1998, 2000), a fluorescent dye 1,1'- dioctadecyl - 3,3,3',3' - tetramethylindocarbocyanine perchlorate (DiI) (Skihar and Doucette, 2001, 2003), and Cell-Tracker probes (Li *et al.,* 1997, 1998) were used before transplanting cells into the CNS of animals. Even though researchers did not find evidence that these dyes can be transferred directly to surrounding cells within the experimental time frame; macrophages could phagocytose dying OECs at the implantation site. For example, Hoechst prelabelling of cells prior to transplantation can result in false positive signals (Ruitenberg *et al*., 2002); therefore, the survival and migration of Hoechst-prelabelled transplanted cells may be overestimated. Adenoviral-mediated (Ruitenberg *et al*., 2002, 2003) and retroviral-transfer (Boyd *et al.,* 2004; Lakatos *et al.,* 2003) of LacZ or adenoviral transfer of green florescent protein (Li *et al.,* 2003) were used to identify cells with fluorescent, light, or electron microscopy. The microscopy observations revealed that adult (Li *et al.,* 2003; Ruitenberg *et al*., 2002, 2003) and neonatal (Boyd *et al*., 2004; Lakatos *et al*., 2003) rat OECs survived the implantation. Retroviral transfection of OECs with LacZ containing viral vector also allowed in-depth characterization of these cells at both the light and electron microscopic level. These observations provided unequivocal ultrastructural identification that LacZ-expressing OECs neither associated directly with central axons nor did they form myelin sheaths or basal lamina after transplantation into the damaged spinal cord of adult rats, even when regeneration and remyelination of lesioned axons were evident (Boyd *et al.,* 2004).

1.2.5 Therapeutic potential

Several approaches to enhance the remyelination of demyelinated axons in the adult mammalian CNS have been considered, with one approach being the transplantation of myelin-forming cells (Akiyama *et al.,* 2001, 2002a, 2002b; Barnett *et al.,* 2000; Franklin *et al*., 1996; Imaizumi *et al.,* 1998, 2000; Smith *et al*., 2002). One myelinating cell that is attracting increasing attention is the OEC. These glial cells have been transplanted into the electrolytically lesioned rat corticospinal tract (Li *et al.,* 1997, 1998), into proximal and distal stumps of the transected rat spinal cord with a guidance channel containing Schwann cells embedded in Matrigel replacing a transected thoracic segment (Ramón-Cueto *et al.,* 1998), into stumps of the completely transected adult rat spinal cord (Ramón-Cueto *et al.,* 2000), and into the spinal cord dorsal column that had been demyelinated by ethidium bromide (EtBr) and x-irradiated to prevent host cells from contributing to its repair (Imaizumi *et al.,* 1998). The grafted OECs adopted a myelinating phenotype that persisted throughout the lesion site (Franklin *et al.*, 1996; Imaizumi *et al.,* 1998). They assemble a myelin sheath around single regenerating or remyelinated axons. Examinations of the morphology of the remyelinating glial cells indicated substantial structural differences in comparison with that of the normal spinal cord white matter. The OECs established an end-to-end PNS type myelinating relationship with individual axonal segments. An outer sheath was formed around groups of myelinated axons; it was suggested that the outer sheath arose from the grafted OECs (Imaizumi *et al.,* 1998; Li *et al.,* 1997, 1998; Ramón-Cueto *et al.,* 1998). However, authors failed to provide evidence that transplanted OECs remyelinated axons and formed an outer sheath for these regenerating axons.

In the experiments of Ramon-Cueto *et al.* (1998, 2000), transplanted OECs were found to facilitate the regeneration of axons in descending motor pathways (i.e. corticospinal, raphespinal, and coeruleospinal). Regenerating motor fibers surrounded by OECs not only crossed the gliotic tissue formed at the proximal cut surface of the spinal cord but also grew across the scar formed between the proximal and distal stumps; some of these regenerating axons (noradrenergic and serotonergic) grew for up to 3 cm (approximately 6 spinal cord segments) distal to the lesion.

Kato *et al.* (2000) demonstrated that transplanted OECs derived from adult human olfactory nerves were capable of extensive remyelination of demyelinated axons after being

grafted into the adult rat spinal cord. Human OECs remyelinated axons in the x-irradiated and EtBr-induced demyelinated lesion (see Section 1.6.1). As had been reported previously for grafted rat OECs, the remyelinating human cells assembled a PNS-type myelin sheath around the axons (see Section 1.3.1).

OECs have features of both Schwann cells, a glial cell of the PNS, and astrocytes, one of the macroglia of the CNS (Doucette, 1990, 1995; Franklin and Barnett, 1997; Franklin, 2002b; Ramon-Cueto and Avila, 1998; Ramon-Cueto and Valverde, 1995). Similar to Schwann cells, which secrete growth-promoting and neurite-promoting factors that support the regeneration of both PNS and CNS axons (Aguayo, 1985), OECs facilitate the growth of the olfactory axons (Doucette, 1990). On the other hand, CNS glial cells and the microenvironment they create are believed to be primarily responsible for the failure of axonal regrowth in the adult mammalian CNS (Reier *et al.,* 1989; Schwab, 1990).

OECs express growth- and neurite-promoting factors such as platelet derived growth factor (PDGF; Kott *et al.,* 1994) neuropeptide Y (Ubink *et al.,* 1994; Ubink and Hokfelt, 2000), protein S-100 (Cummings and Brunjes, 1995; Franceschini and Barnett, 1996; Gong *et al.,* 1996), p75NGFR (Gong *et al.,* 1994; Treloar *et al*., 1999; Vickland *et al.,* 1991) and glia-derived netrin (Reinhard *et al.,* 1988; Treloar *et al*., 1999). Extracellular matrix and cell adhesion molecules such as N-CAM (Franceschini and Barnett, 1996), laminin (Doucette, 1996), and tenascin (Kafitz and Greer, 1998) are suggested to facilitate axonal growth and guidance in the olfactory nerve and in the ONL of the olfactory bulb, where the OECs are found.

A great amount of research data have been collected over the past decade on clinical relevance of using OEC for promoting neural repair in the CNS of adult mammals (Doucette, 1990, 1995; Franklin and Barnett, 1997; Franklin *et al*., 2002a, 2002b). OECs have been shown to promote long-distance regeneration of axons in the adult mammalian spinal cord and facilitate remyelination of CNS axons such that conduction velocities of previously demyelinated axons are restored to normal levels. OECs can be easily obtained from extracranial sources, such as from olfactory epithelium of human (Feron *et al*., 1998; Jafek *et al*., 2002; Lanza *et al*., 1993, 1994) or from similar tissue in the nasal cavity of rodents (Franklin *et al*., 2002a; Lu *et al*., 2002). Thus, patients can provide their own olfactory tissue for autologous transplantations, which will avoid unnecessary immunosuppression.

Furthermore, OECs can more easily integrate into CNS tissue after grafting into the injured or demyelinated spinal cord (Lakatos *et al*., 2000; Li *et al*., 1998) than Schwann cells (Baron-Van Evercooren *et al*., 1992; Blakemore, 1992; Duncan and Milward, 1995).

1.3 Myelinating Glia

In addition to OECs there are two additional cell types, namely oligodendrocytes (Baumann and Pham-Dinh, 2001) and Schwann cells (Bunge *et al*., 1968), possessing the ability to myelinate axons. Oligodendrocytes are myelinating glia of the CNS whereas Schwann cells perform the same role in the PNS. All three types of myelinating glia could potentially contribute to neural repair in areas where CNS myelin is damaged or absent (see Section 1.8).

1.3.1 Myelin

Myelin, which was named so by Virchow (reviewed in Baumann and Pham-Dinh, 2001), is a very important constituent of both CNS and PNS axons providing electrical insulation for these axons. Rich in lipids and low in water content, the myelin sheath has a unique segmental structure, which permits the saltatory conduction of nerve impulses, thus supporting fast nerve conduction over long distances. Myelin is very important for the development and function of the human CNS and PNS. Myelin structure and/or function are perturbed in a number of different neurological disorders of the CNS and PNS such as in multiple sclerosis and in peripheral neuropathies, respectively (Fazakerley and Buchmeier, 1993; Wekerle, 1993).

Both oligodendrocytes and Schwann cells extend cytoplasmic processes, to ensheath a segment of axon in a "jelly-roll" form (Morell *et al.,* 1994; Peters *et al.,* 1991). The cytoplasmic leaflets of each concentric layer of myelin membrane becomes closely apposed as the myelin is compacted, thus forming the major dense line. The extracellular leaflets of adjacent concentric layers of the myelin membrane fuse together to form the intraperiodic line (or minor dense lines). A narrow extracellular cleft, the periaxonal space, separates the myelin sheath from the axonal membrane. Myelin sheaths in the PNS have cytoplasmic pockets, called Schmidt-Lantermann clefts or incisures, containing cytoplasm between myelin membrane; such cytoplasmic pockets are rarely found in CNS myelin. At the inner (i.e. adaxonal) and outer (i.e. abaxonal) end of the spiral, myelin is less compact and forms inner

and outer loops, which retain small amounts of either oligodendrocyte or Schwann cell cytoplasm. Each myelin sheath, or internode, for example in rat optic nerve is about 150-200 μm in length (Butt and Ransom, 1989) and is separated by a short length of axon where there is no myelin, the node of Ranvier, from an adjacent internode (Bunge, 1968). The myelin and nodes of Ranvier ensure fast saltatory conduction allowing the nerve impulses to jump from node to node, rather than more slowly progressing along the axon as they do in unmyelinated or demyelinated fibers.

The insulating properties of the myelin sheath that facilitate the saltatory conduction of action potentials are largely due to its structure, its thickness, its low water content, and its richness in lipids (Ritchie, 1984). Oligodendrocytes and Schwann cells produce the specific constituents of myelin, such as myelin lipids and myelin proteins. Myelin lipids are represented by galactolipids (i.e. cholesterol, cerebroside, sulfatide) and phospholipids (ethanolamine phosphatides, lecithin, sphingomyelin, phosphatidylserine, phosphatidylinositol, plasmalogens). Both CNS and PNS myelin lipids comprise up to 70 % and protein molecules approximately 30% of myelin dry weight, whereas other cellular membranes have a higher ratio of proteins to lipids (Morell *et al.,* 1994, Norton, 1981). The PNS myelin has many of the same lipids as myelin of the CNS, although myelin in the PNS contains less cerebroside and sulfatide but more sphingomyelin than that in CNS (Smith *et al*., 1983). In oligodendrocytes, myelin lipids are especially rich in glycosphingolipids, galactosylceramides, in particular galactocerebroside (e.g. GAL-C) and their sulfated derivatives, sulfatides (i.e. sulfogalactosylceramides) (Raff *et al.,* 1978, Zalc *et al*., 1981). Deficiencies in some lysosomal enzymes result in an abnormal degradation of sulfatides, galactocerebrosides, or proteins and accumulation of these undegraded fatty acids. These disturbances of myelin metabolism result in leukodystrophies (Kolodny, 1993).

Myelin proteins are specific membrane components of the myelin sheath in both the PNS and CNS. The PNS and CNS myelin proteins include myelin basic protein (MBP) (Kies *et al.,* 1972) and proteolipid protein (PLP) (Folch and Lees, 1951). 2',3'-Cyclic nucleotide-3'phosphohydrolase (CNPase; Trapp *et al*., 1988) and several glycoproteins including myelin-associated glycoprotein (MAG; Quarles, 1997) and connexin 32 (Cx32) are also present in peripheral and central myelin (Scherer *et al.,* 1995). However, the expression of peripheral myelin protein 22 (PMP22) and P0 is found only in PNS myelin, whereas

myelin/oligodendrocyte glycoprotein (MOG; Brunner *et al*., 1989) can only be detected in the CNS.

MBP is a family of protein isoforms with different molecular masses and plays a major role in myelin compaction in the CNS (Wood and Moscarello, 1989). The MBP gene has been identified on chromosome 18 and assigned to 18q22-qter in the human genome (Saxe *et al*., 1985; Sparkes *et al.,* 1987). Posttranslational modifications of MBP including in particular acetylation, phosphorylation, and methylation, are important for compaction of the myelin membrane (Wood and Moscarello, 1989). In the shiverer mutant mouse, which presents with a deletion of a large part (five out of six exons) of the MBP gene (Roach *et al.,* 1985), CNS myelin lacks a major dense line, whereas PNS myelin appears normal (Privat *et al.,* 1979).

PLPs are lipid-protein complexes, so they were given the generic name of proteolipids to distinguish them from water-soluble lipoproteins. In addition to the main 25kDa isoform of PLP, there is a second PLP isoform called DM-20 (i.e. 20kDa). Both the PLP and the DM-20 isoforms are expressed in the CNS and PNS, with the former being predominant over DM-20 in CNS myelin and DM-20, on the other hand, being the dominant form in PNS myelin (Pham-Dinh *et al.,* 1991). In contrast to PLP/DM-20 expression in oligodendrocytes, neither of these lipid proteins is incorporated into Schwann cell myelin of wild type mice (Anderson *et al.,* 1997). The cohort study on patients with peripheral neuropathy who were diagnosed with Pelizaeus-Merzbacher disease, a leukodystrophy, demonstrated that expression of PLP but not DM-20 was necessary to prevent peripheral neuropathy (Shy *et al.,* 2003). DM-20 expression appears earlier than PLP during development and it is thought that it might have a role in oligodendrocyte differentiation in addition to a structural role in the formation of a myelin sheath (Ikenaka *et al*., 1992; Peyron *et al*., 1997; Timsit *et al.,* 1992). The gene coding for PLP is located on the X chromosome in humans (Willard and Riordan, 1985) at position Xq22 (Mattei *et al.,* 1986) and in mice in the H2C area (Dautigny *et al*., 1986). Jimpy mice and myelin deficient rats have sex-linked recessive mutations located in the PLP gene. In humans such spontaneous mutation occurs in Pelizaeus-Merzbacher disease, also known as an X-chomosome-linked dysmyelinating disorder, which is characterized by abnormal formation and maintenance of myelin (Nave and Boespflug-Tanguy, 1996; Seitelberger, 1995). In PLP knockout mice, oligodendrocytes can still assemble a myelin sheath around large diameter axons, but the concentric layers of myelin membrane are loosely wrapped with wide

extracellular spaces between the layers (Boison *et al.,* 1995). Therefore, PLP is suggested to be responsible for the tight adhesion of the outer leaflets of the myelin membrane surfaces leading to the formation of the minor dense line (Boison *et al.,* 1995; Duncan *et al.,* 1987, 1989). In addition, axonal swelling followed later by axonal degeneration associated with motor impairment was observed in 16 month old PLP/DM-20 null mice (Griffiths *et al*., 1998).

CNPase represents 4% of total CNS myelin protein. Although the biological role of CNPase remains obscure, this protein is present in the periaxonal membrane, inner mesaxon and in the paranodal loops of oligodendrocyte myelin (Trapp *et al.,* 1988). CNPase is also found in PNS myelin being localized similar to that of CNS myelin. CNPase is present within the oligodendrocyte plasma membrane during the earliest phase of axonal ensheathment prior to the appearance of PLP and MBP. Gravel *et al.* (1996) suggested that CNPase might play a role in the extension of oligodendrocyte cytoplasmic processes around the axons during the early stages of myelinogenesis. Over expression of CNPase in transgenic mice perturbed myelin formation, resulted in a formation of intramyelenic vacuoles, aberrant expansion of oligodendrocyte cytoplasmic processes and precocious oligodendrocyte maturation (Gravel *et al.,* 1996).

MAG, as a quantitatively minor constituent of myelin, represents only 1% of the total protein found in CNS myelin and only 0.1% in PNS myelin. MAGs are transmembrane proteins with extracellular regions that are homologous to N-CAM and other members of the immunoglobulin gene superfamily (Salzer *et al.,* 1987). Recently, MAGs have been identified as members of sialic acid binding lectins, the sialoadhesins, which preferentially bind to neuron cell surface sialoglycolipids (Yang *et al.,* 1996). In the adult rat, MAG is largely distributed across different regions of the PNS myelin internode (Meyer-Franke and Barres, 1994; Trapp and Quarles, 1982), whereas it is confined to the periaxonal collar of the myelin sheath in the CNS (Trapp *et al.,* 1989). MAG is involved in the activation of a protein tyrosine kinase Fyn and a member of the src family, during the initial stages of myelination (Umemori *et al*., 1999) and morphological differentiation of oligodendrocytes (Osterhout *et al.*, 1999). Although MAG-deficient mice develop almost normal CNS myelin, the myelin that is formed contains cytoplasmic organelles between noncompact lamellae, no periaxonal cytoplasmic periaxonal collar in most of the internodes, and an abnormal collar in the remainder (Li *et al*., 1994, Montag *et al*. 1994). Montag *et al*. (1994) suggested that MAG might participate in the

formation of the periaxonal cytoplasmic collar of oligodendrocytes and be responsible for the adhesion of oligodendrocyte processes to axons in the CNS. Two MAG proteins have been identified, large MAG and small MAG (Frail *et al*., 1985; Salzer *et al*., 1987), which differ not only in terms of their molecular weight but also in regard to their function. Pathological abnormalities of CNS myelin, but no PNS axon or myelin degeneration, are reported in large MAG mutant mice (Carenini *et al.,* 1997), although full null MAG mutants (i.e. both large and small MAGs) develop a peripheral neuropathy in addition to CNS myelin pathology (Fruttiger *et al.,* 1995). These data indicate that large MAG is the critical MAG isoform in CNS myelin, whereas small MAG is important in the PNS for the maintenance and integrity of the myelin sheath (Fujita *et al.*, 1998). Consistent with this suggestion, large MAG is expressed earlier than small MAG during CNS myelination and is the predominant isoform in adult CNS myelin, whereas small MAG is the predominant isoform in PNS myelin in both rodents (Frail *et al*., 1985; Salzer *et al*., 1987) and humans (Miescher *et al*., 1997).

MOG was first identified as the antigen responsible for the demyelination observed in animals injected with whole CNS homogenates (Lebar *et al*., 1976, 1986). Present only in mammalian species, the MOG gene is co-localized to the human major histocompatibility complex (MHC) on chromosome 6 in the human genome, and on chromosome 17 in mice (Pham-Dinh *et al.*, 1993). MOG is located in the plasma membrane of oligodendrocytes, especially on the cytoplasmic processes, and on the outermost lamellae of the myelin sheaths (Brunner *et al*., 1989). MOG is also a surface marker of mature (Birling *et al*., 1993; Coffey and McDermott, 1997; Pham-Dinh, 1993) and myelinating (Solly *et al*., 1996) oligodendrocytes. The extracellular domain of MOG, which is a member of the immunoglobulin superfamily, is believed to induce both a T cell-mediated inflammatory immune reaction and a demyelinating antibody-mediated response in the experimental autoimmune encephalomyelitis (EAE) animal model (Lassmann *et al.,* 1988).

PMP22 is a small, hydrophobic glycoprotein most prominently expressed by Schwann cells as a component of compact myelin of the PNS (Naef and Suter, 1998; Snipes *et al.,* 1992). Functionally, PMP22 is involved in myelination during the development of peripheral nerves and in the maintenance of peripheral myelin structure (Uyemura, 1993). Mutations affecting the PMP22 gene include duplications, deletions, and point mutations. Point mutations are responsible for the most common forms of hereditary peripheral neuropathies including

Charcot-Marie-Tooth disease type 1A (Patel *et al.*, 1992; Timmerman *et al*., 1992), hereditary neuropathy with liability to pressure palsies and a subtype of Dejerine-Sottas syndrome (Roa *et al.,* 1993).

P0 glycoprotein is the major transmembrane protein of PNS myelin of mammals as well as of fish and amphibia (Uyemura *et al*., 1995). In contrast to what is seen for mammalian oligodendrocytes, the oligodendrocytes of fish express P0 (Jeserich and Waehneldt, 1986). Lemke and Axel (1985), Lemke *et al.* (1988) and Lemke (1993) suggest that P0 mediates both the extracellular and the cytoplasmic compaction of myelin. The extracellular domain of P0 interconnects the turning loops of Schwann cell processes to form the intraperiod line (D'Urso *et al*., 1990; Filbin *et al.,* 1990; Schneider-Schaulies *et al*., 1990). PMP22 interacts with P0 to form complexes, which probably hold adjacent Schwann cell membranes together (D'Urso *et al.,* 1999). The intracellular part of P0 contains positively charged basic residues and is believed to interact with the phospholipids and MBP in the cytoplasmic face of apposing Schwann cell membranes, thus forming the major dense line (Kirschner and Ganser, 1980; Ding and Brunden, 1994; Martini *et al*., 1995). P0-deficient mice develop severely compromised myelin compaction, primarily at the site of the intraperiod line, whereas the major dense line continues to be formed by Schwann cells (Giese *et al.,* 1992; Martini *et al*., 1995). In the absence of MBP, Schwann cells expressing P0 are still able to form the major dense line in shiverer mice (Kirschner and Ganser, 1980; Inouye *et al*., 1985). The lack of both MBP and P0 in the double mutants results in an almost complete loss of major dense lines in PNS myelin (Martini *et al*., 1995). Therefore, the formation of the major dense line in peripheral nerve myelin is formed by both P0 and MBP (Martini *et al*., 1995).

In multiple sclerosis (MS), an immune response is directed against components of CNS myelin, including MBP (Zamvil and Steinman, 1990; Steinman, 1993, 1995) and MOG (Kerlero de Rosbo *et al*., 1993). B-cell and T-cell reactivity against PLP, CNPase and MAG have also been observed in MS patients (Oksenberg *et al.,* 1993; Steinman, 1995). The MOG gene has been identified within the major histocompatibility complex 1 region on the chromosome 6 (Daubas *et al*., 1994; Pham-Dinh *et al.,* 1993). Steinman (1993) suggested that MOG might display some immune function in the CNS. MOG can be an autoantigen as it is located on the surface of the outermost lamellae of the myelin sheath, where MOG is more exposed to antibodies than are other myelin proteins like MBP or PLP. Oligodendrocytes are

found to be susceptible to lysis by complement (Compston *et al.,* 1989). Piddlesden and Morgan (1993) suggested that the absence of the complement-inhibitory protein CD59 from the oligodendrocyte membrane contributes to this susceptibility to lysis by oligodendrocytes.

There are morphological and molecular differences between CNS and PNS myelin. Although MBP, PLP, MAG and Cx32 are expressed in both oligodendrocyte and Schwann cell myelin, oligodendrocytes also produce MOG and CNPase, whereas Schwann cells make PMP22 and P0 myelin proteins. In contrast to oligodendrocytes, which typically ensheath and myelinate multiple CNS axons, myelinating Schwann cells always have a 1:1 relationship with PNS axons. A myelinated axon is always associated with an adjacent Schwann cell, the nucleus of which gives the appearance of a "signet ring". The cytoplasmic processes of adjacent myelinating Schwann cells interdigitate at the node of Ranvier, in marked contrast to the exposure of CNS axons to extracellular space at each node (Bunge, 1968). The soma of a Schwann cell unlike that of an oligodendrocyte is circumferentially covered by a basal lamina, which is continuous with that of the adjacent internode, regardless of whether the Schwann cell is myelinating or not. This type of myelination is called peripheral or PNS-type. Schwann cell myelinated fibers are separated from each other by extracellular compartment, the endoneurium, whereas the oligodendrocyte myelinated fibers are in close contact.

1.3.2 Oligodendrocytes

Rio Hortega first introduced the term oligodendroglia in 1921 describing neuroglial cells with few processes in metallic impregnation stained brain tissue (reviewed in Baumann and Pham-Dinh, 2001). Although a major role of oligodendrocytes is the myelination of CNS axons, there are also satellite oligodendrocytes, which are found perineuronally and which are believed to regulate the microenvironment around neurons (Ludwin, 1997). A myelinating oligodendrocyte extends several cytoplasmic processes, each of which contacts and repeatedly envelops a stretch of axon with subsequent condensation of multispiral myelin (Bunge *et al*, 1962; Bunge, 1968). Adjacent myelin segments (i.e. internodes) on the same axon belong to different oligodendrocytes, with each segment being separated by a node of Ranvier. In contrast to the PNS, the axolemma is exposed to the extracelluar milieu at the nodes of Ranvier in the CNS (Bunge, 1968) and is in close proximity to astrocytic processes (Black and Waxman, 1988) and to NG2 positive glial precursor cells (Butt *et al.,* 1999). The number of axons that are myelinated by each oligodendrocyte varies according to the area of the CNS and

possibly also the species, from 40 in the optic nerve of the rat (Peters *et al*., 1991) to 1 in the spinal cord of the cat (Bunge *et al*., 1961).

Butt *et al*. (1995) classified myelinating oligodendrocytes into four types based on their morphology and on the size and thickness of the myelin sheath they formed. Type I oligodendrocytes were small cells supporting short, thin myelin sheaths for 15-30 small diameter axons. Intermediate types of myelinating oligodendrocytes were classified as types II and III. The type IV oligodendrocytes were the largest cells, forming long, thick myelin sheaths around 1-3 large diameter axons (Bunge, 1968).

Originating from migratory and mitotic precursors, oligodendrocyte progenitors differentiate progressively into postmitotic myelin-producing cells as they sequentially express developmental and then myelinating markers (Hardy and Reynolds, 1993; Pfeiffer *et al*., 1993). At the early developmental stages, oligodendrocyte precursors have been characterized by their bipolar morphology and by the expression of nestin (Gallo and Armstrong, 1995), the DM-20 isoform of PLP (Ikenaka *et al*., 1992; Peyron *et al*., 1997; Timsit *et al*., 1992, 1995), the PDGF-β receptor (reviewed in Richardson *et al*., 2000; Spassky *et al*., 2000), PSA-NCAM (Grinspan and Franceschini, 1995; Hardy and Reynolds, 1991), and CNPase. Later, PSA-NCAM and nestin are no longer expressed. As the oligodendrocyte progenitors are actively proliferating and migrating within the CNS during embryonic and fetal development, they maintain production of PDGF-β receptor, DM-20 and CNPase and begin synthesizing the GD3 ganglioside (Hardy and Reynolds, 1991), the gangliosides recognized by the monoclonal antibody A2B5 (Fredman *et al*., 1984), and the rat NG2 proteoglycan (Nishiyama *et al.,* 1996, 1999). These migrating oligodendrocyte progenitors eventually settle along fiber tracts of the presumptive white matter as pro-oligodendrocytes, which are cells of the oligodendrocyte lineage that express sulfatides and unidentified glycolipids that bind the O4 monoclonal antibody (Bansal *et al.,* 1989).

As the pro-oligodendrocytes differentiate into oligodendrocytes they become less motile (Orentas and Miller, 1996), lose their mitogenic response to PDGF-β receptor (Gao *et al*., 1998; Hart *et al*., 1989; Pringle and Richardson, 1993), and they no longer express antigenic epitopes recognized by the A2B5, GD3 and NG2 antibodies (reviewed in Baumann and Pham-Dinh, 2001). These immature oligodendrocytes are identified by virtue of their expression of Gal-C (Pfeiffer *et al*., 1993; Raff *et al*., 1978; Zalc *et al*., 1981), and by the expression of the

RIP antigenic epitope (Butt *et al*., 1995; Friedman *et al*., 1989). As the cells differentiate into oligodendrocytes they begin to express MBP (Butt *et al*., 1995). Even in the absence of neurons, oligodendrocyte progenitors can differentiate into cells that extend myelin-like membranes (Sarlieve *et al*., 1983) and express the myelin proteins PLP, MBP and MAG. Thus, the capacity of oligodendrocyte progenitors to differentiate into cells expressing a myelinating phenotype is specific to the lineage (Temple and Raff, 1986). Nevertheless, co-culture of oligodendrocyte progenitors with neurons increases gene expression of these myelin proteins in the oligodendrocytes (Macklin *et al*., 1986; Matsuda *et al*., 1997), similar to that observed *in vivo* (Kidd *et al.,* 1990). When axonal contact occurs, oligodendrocytes dramatically modify their morphology, retract cytoplasmic processes that have not made contact with an axon (Hardy and Friedrich, 1996), downregulate DM-20 expression, and begin to express the full-length isoform of the PLP gene (Trapp *et al.,* 1997). Those oligodendrocytes in white matter that do not make axonal contact undergo programmed cell death and die, thus eliminating unneeded cells (Barres *et al*., 1992). This switch from DM-20 expression to full-length PLP expression is believed to signal the beginning of the process of myelination (Trapp *et al.,* 1997).

1.3.3 Schwann cells

Professor Theodor Schwann in 1839 published his treatise on the cell theory, where he showed that in spite of a fibrous nature peripheral nerves are made up of cells, which were later named after him (reviewed in Gould, 1990). Schwann cells are the myelinating glia of the PNS.

Schwann cells develop from the neural crest (Le Douarin *et al*., 1991) and evolve through three steps (Mirsky *et al.,* 2001). First, after the transition from the neural crest Schwann cell precursors are typically found in rat and mouse nerves of 14-15 and 12-13 day old embryos, respectively. Second, precursors become immature Schwann cells that are present in 17 day old rat embryo and 15 day old mouse embryo to around birth. Subsequently, the immature Schwann cells differentiate along the peripheral nerves into two mature types with myelinating Schwann cells appearing first and mature non-myelinating Schwann cells shortly after (Mirsky and Jessen, 1996; Jessen and Mirsky, 1997, 1998). It is not known how glial cells originate from the neural crest, which also gives rise to other lineages including neurons and melanocytes. Henion and Weston (1997) suggest that while migrating to their

final destination cells of the neural crest enter distinct lineages under the influence of instructive signals. For example, β-neuregulins appear to bias neural crest cell differentiation towards the glial lineage by blocking entry of the cells into the neuronal lineage (Shah *et al.,* 1994), whereas transforming growth factor β (TGF-β) promotes the generation of smooth muscle cells and bone morphogenic protein 2 stimulates development of autonomic neurons (Shah *et al.*, 1996).

In postnatal rodents, immature myelinating Schwann cells ensheath the large axons and the non-myelinating cells accommodate small diameter axons in their membrane invaginations. Driven by axonal signals, myelinating Schwann cells carry out a large amount of membrane synthesis and ensheathment needed to form the myelin sheath (Jessen and Mirsky, 1992). Extensive changes in gene expression occur in immature Schwann cells when they are induced to myelinate an axon including up-regulation of myelin proteins such as P0, MBP, PMP22 and down-regulation of NCAM, p75NGFR and GFAP (Jessen *et al.*, 1990). Three transcriptional factors that are known to play an important role in Schwann cell myelination include Sox-10 a member of the SRY-like high motility group domain family of transcription factors (Kuhlbrodt *et al*., 1998; Southard-Smith *et al*., 1998), the POU domain factor Oct-6/SCIP/tst-1 (Arroyo *et al.,* 1998) and the zinc finger protein Krox-20 (Topilko *et al*., 1997). In mice with transiently arrested Oct-6/SCIP/tst-1 expression or permanently blocked Krox-20 transcription factors, promyelinating Schwann cells appear normal; they express a basal lamina and normal levels of MAG, MBP and P0 mRNAs. However, Schwann cells in these transgenic animals do not form a concentric compacted myelin sheath (Bermingham *et al*., 1996; Topilko *et al*., 1994). Recently, the mutation of Egr-1 (Krox-20) has been identified in patients with Charcot-Marie-Tooth disease, an inherited demyelinating neuropathy, (Warner *et al*., 1998, 1999; Timmerman *et al*., 1999).

During embryonic development, the survival of Schwann cell precursors depends on axonal signals (i.e. β-neuregulins), whereas mature Schwann cells can survive in the absence of axons (Grinspan *et al*., 1996; Li *et al.,* 1998; Mirskiy *et al.,* 2001; Trachtenberg and Thompson, 1996). This is due primarily to the presence of an autocrine growth factor loop in Schwann cells starting in rats from embryonic day 18 onwards (Meier *et al*., 1999). The most important growth factor components of autocrine survival include insulin-like growth factor (IGF)–2, PDGF-BB and neurotrophin-3 (Meier *et al*., 1999). This ability of Schwann cells to survive in

the absence of axons is critical in the context of peripheral nerve regeneration after injury, when the axons must enter the distal stump and regrow into their target tissue. Living Schwann cells are necessary for successful peripheral nerve repair, providing essential adhesion molecules and trophic factors (Fawcett and Keynes, 1990; Nadim *et al.,* 1990).

Two distinct forms of Schwann cells exist in peripheral nerves in adult mammals: myelinating and nonmyelinating Schwann cells. Schwann cells form contacts with axons, other Schwann cells and with the extracellular matrix. During differentiation, myelinating Schwann cells lose contact with all but a single axon segment, which they subsequently ensheath with cytoplasmic processes and myelinate (Zorick and Lemke, 1996). Axonal contacts are essential for initiation of myelination, which induce a Schwann cell to express myelin genes, the expression of which is influenced by extracellular matrix (Bunge *et al*., 1989, Scherer *et al*., 1994; Scherer, 1997). Schwann cells are known to produce basement membrane components including laminin, type IV collagen and fibronectin, to which they adhere via integrin receptors, dystroglycans and *N*-syndecan (Bunge *et al*., 1989; Mirsky and Jessen, 1996; Scherer, 1997). Interactions with laminin are suggested to be important for Schwann cell migration (Milner *et al.,* 1997) and myelination (Chen *et al*., 2000; Fernandez-Valle *et al*., 1994). In addition, morphological arrangements of actin cytoskeleton are also critical for Schwann cell differentiation and myelination (Fernandez-Valle *et al.,* 1997).

1.3.4 Olfactory ensheathing cells

In vivo, OECs ensheath but do not myelinate small C fibers (i.e. the olfactory axons; Doucette, 1984, 1990; Raisman, 1985). Barnett *et al*. (1993) and Devon and Doucette (1994, 1995) have demonstrated, OECs also express a nonmyelinating phenotype when grown in neuron-free cell cultures. OECs express a mixture of astrocyte-specific and Schwann-cell specific phenotypic features (Doucette, 1990, 1995; Franklin, 2002b; Franklin and Barnett, 1997; Ramon-Cueto and Avila, 1998; Ramon-Cueto and Valverde, 1995) and have been reported to perform the roles of both astrocytes and Schwann cells (Doucette, 1990, 1993; Doucette and Devon, 1995). Given the fact that OECs only ensheath unmyelinated axons *in vivo* (Barnett *et al.*, 1993; Devon and Doucette, 1992, 1995), they would be expected to express a nonmyelinating phenotype when grown *in vitro*. The media shown to be effective *in vitro* in promoting the growth and differentiation of neopallial and olfactory bulb oligodendrocyte progenitor cells failed to induce OECs to express a myelinating phenotype (Barnett *et al.*, 1993;

Doucette and Devon, 1995). Moreover, OECs in neuron-free cultures treated with dBcAMP can be induced to express GAL-C but not induced to express MBP (Doucette and Devon, 1994, 1995). In contrast, Schwann cells re-express myelin proteins (MBP and P0) when cAMP had been added to the culture medium (Lemke and Chao, 1988; Morgan *et al*., 1991; Pleasure *et al*., 1985). The results of these studies indicated that the molecular mechanisms regulating the expression of a myelinating phenotype by OECs are different from those that function in Schwann cells.

The first evidence that OECs could assemble a myelin sheath was reported by Devon and Doucette (1992), who co-cultured OECs obtained from the olfactory bulbs of E18 rat embryos with dorsal root ganglion (DRG) neurons. Co-culturing of DRG neurons with OECs resulted in the expression of a myelinating phenotype and the assembly of a PNS-type myelin sheath around the DRG neurites. Devon and Doucette (1992) used fluoredeoxyuridine to eliminate non-neuronal cells from the DRG cultures and confirmed the purity of these cultures by microscopy before adding OECs prelabelled with a lipophilic fluorescent dye (PKH26). In these co-cultures, each OEC either myelinated only one neurite or ensheathed several unmyelinated neurites at the same time. Each OEC, whether it was myelinating single neurites or ensheathing multiple unmyelinated axons, was also enclosed within its own basal lamina, a situation that does not exist *in vivo* in the primary olfactory pathway (Doucette, 1984, 1990; see Section 1.2.1). Only occasionally, two or more myelinated neurites were ensheathed by the same glial cell and were enclosed within a common basal lamina. Immunocytochemical stainings revealed that S100-positive OECs ensheathing single DRG neurites were also MBP-positive. These MBP-positive OECs assumed a bipolar morphology and formed a contiguous series of cells along the length of each DRG neurite (Devon and Doucette, 1992, 1995). Axonal contact is one essential prerequisite for Schwann cells to express a myelinating phenotype (Brunden and Brown, 1990; Brunden *et al.*, 1990), a requirement that also applies to OECs (Barnett *et al.*, 1993; Devon and Doucette, 1992, 1995; Doucette and Devon, 1994, 1995). In contrast to Schwann cells, however, OECs do not require addition of L-ascorbic acid to the growth medium for them to assemble either a basal lamina or a myelin sheath *in vitro* (Doucette and Devon, 1995).

Franklin *et al*. (1996) used an O4-positive (+ve) clonal OEC cell line they had generated using tissue from the newborn rodent olfactory bulb and grafted these cells into an

EtBr/x-irradiated (see Section 1.7.1) demyelinated area of the adult rat spinal cord. Their study provided the first *in vivo* evidence of the ability of OECs to remyelinate demyelinated CNS fibers; similar to what had been previously described *in vitro* (Devon and Doucette, 1992, 1995). This clonal cell line myelinated the axons in a Schwann cell-like fashion. Subsequent studies by Imaizumi *et al.* (1998) using primary rat cell cultures of OECs not only replicated these *in vivo* experiments but, most importantly, also showed that the OEC remyelination of axons in the dorsal columns enhanced axonal conduction across the previously demyelinated area. The grafting of OECs obtained from human OB (Bartnett *et al*., 2000), human olfactory nerve (Kato *et al*., 2000) or canine OB (Smith *et al*., 2002) into x-irradiated/EtBr-induced demyelinated rat spinal cord resulted in Schwann-like remyelination of CNS axons within the demyelinated lesion. Radtke *et al*. (2004) utilized OECs harvested from an adult H-transferase transgenic pig for transplantation into the nonhuman primate spinal cord and observed remyelination of EtBr-induced demyelinated CNS axons.

Thus, OEC remyelination was demonstrated to be functionally significant. OECs were also found capable of remyelinating electrolytically lesioned corticospinal axons that regenerated into and through the site of the lesion after grafting OECs into the lesion site (Li *et al.*, 1998). However, the remyelinating ability of OECs was questioned when the *in vitro* study of Plant *et al*. (2002) failed to identify any myelination of DRG neurites by co-cultured OECs, which were obtained using the technique of Ramon-Cueto and Nieto-Sampedro (1992). Schwann cells form more myelin sheaths in co-culture with DRG neurons than OECs in these experiments. Such lack of myelination *in vitro* by OECs, perhaps, could have been determined by the extent of the purification procedure used by Plant *et al*. (2002). p75NGFR-expressing OECs used in the study by Plant et al. might not be a subset of OECs responsible for the myelinating potential. Another possible explanation is that Plant et al. use adult OECs that may not have the same myelinating potential as embryonic OECs (Devon and Doucette, 1992, 1995). Takami *et al*. (2002) compared regenerative abilities of OECs and Schwann cells *in vivo* and found that Schwann cells but not OECs improved the functional recovery of adult rats after contusive spinal cord injury. Furthermore, OECs myelinated fewer axons than Schwann cells. However, the authors reported that only a few OECs could be identified within spinal cord tissue. Either susceptible to cytotoxicity or other unidentified causes, OECs failed to stay

around and therefore neither supported axon regeneration nor contributed to the remyelination of the regenerated axons in experiments performed by Takami *et al*. (2002).

The molecular mechanisms controlling the expression of a myelinating phenotype by OECs remain to be elucidated. Smith *et al*. (2001b) initiated a study to identify which transcription factors were expressed by OECs engaged in remyelinating spinal cord axons. Similar to Schwann cells (Blanchard *et al*., 1996; Zorick *et al*., 1999), the expression of Oct-6 /SCIP/ tst-1, Krox-20 and desert hedgehog transcription factors was detected in an EtBr/x-irradiated rat spinal cord lesion after OEC transplantation (Smith *et al*., 2001b). However, the authors did not present direct evidence that these transcription factors were expressed by grafted OECs, as opposed, for example, to invading host Schwann cells. Other studies (Barnett *et al*., 1993; Devon and Doucette, 1995; Doucette and Devon, 1994, 1995) indicate that OECs have similar, but not identical, requirements to Schwann cells for expression of a myelinating phenotype. So methodological differences, such as the age of animals from which the OECs were obtained, the method of purification, etc. might have had an impact on the results rather than the actual myelinating ability of the OECs. Moreover, there is little known yet about factors regulating the myelinating phenotype in OECs and whether all OECs even possess the ability to myelinate.

Oligodendrocytes are known to synthesize significant levels of myelin-associated molecules (i.e. Gal-C and MBP) *in vitro* even in the absence of axonal contact, provided the growth medium contains insulin, hydrocortisone, and triiodothyronine (Lopes-Cardozo *et al.,* 1989; McMorris *et al.,* 1986; Mozell and McMorris, 1991; Poduslo *et al*., 1990; Ved *et al*., 1989). When similar growth medium is used to feed neuron-free cultures of OECs, these glial cells are not induced to express either Gal-C or MBP (Barnett *et al*., 1993; Doucette and Devon 1994, 1995). These data suggest that the promoter region of the MBP gene is under different regulatory control in oligodendrocytes and OECs (Doucette and Devon, 1994, 1995). The MBP promoter is also believed to be controlled differently in oligodendrocytes and Schwann cells (Foran and Peterson, 1992; Gow *et al*., 1992). Gal-C expression by OECs in neuron-free cultures can, however, be induced by increasing the intracellular level of cAMP (Doucette and Devon, 1994, 1995), which at the same time fails to induce these cells to express MBP (Doucette and Devon, 1995).

1.4 Multiple Sclerosis

MS is a relatively common and always disabling disease of the CNS primarily affecting young adults (20 to 40 years of age) that exhibits extraordinary clinical, radiological, and pathological heterogeneity. The MS Society of Canada estimates that 50,000 Canadians have MS, with this disease affecting 1-2 people per 1000 population (MS Society of Canada website; October, 2004; http://www.mssociety.ca).

1.4.1 Neuropathology and clinical course

MS is characterized by ongoing lesion formation in the CNS and increasing cumulative damage and is now also recognized as a disease with on-going pathological processes in most patients most of the time. Autopsy studies indicate that there are individuals who never develop clinical symptoms, in spite of having neuropathologic changes typical of MS. Although no accurate data have been collected yet to estimate subclinical disease, it has been suggested that asymptomatic MS might account for up to 25% of all cases (Joy and Johnston, 2001). The majority of MS patients (~85%) develop neurological signs and symptoms over a period of several days, reach a plateau, and then show some improvement followed by a period of clinical quiescence (i.e. remission) that is variable in length but is rarely permanent (Wingerchuk *et al*., 2001). New attacks (i.e. relapses) typically consist of one or a combination of symptoms including optic neuritis, axial or limb parasthesias with neck flexion (Lhermitte's sign), weakness, gait ataxia, diplopia, and symptomatic worsening with increases in body temperature (Uhthoff symptom). The relapses of disabling symptoms repeat themselves over and over, interspersed with periods of remission; these patients are referred to as having "relapsing-remitting MS". As the disease progresses, the frequency and duration of the relapses often increase while that of the remissions usually decreases until they no longer experience any periods of remission; at this point in the progression of the disease these patients are considered to have switched from a "relapsing-remitting" to a "secondary progressive" form of MS (Prineas *et al*., 2001).

For the remaining 15% of MS patients, the disease presents first and foremost with a gradually progressive loss of neurological function that presents with no intervening periods of remission. This form of MS is referred to as "primary progressive". The appearance of, and

worsening of, the clinical exacerbations in all MS patients are usually accompanied by inflammatory infiltrates and demyelination in the brain and spinal cord white matter giving rise to the pathological hallmark of the disease, the MS plaque. Patients with primary progressive MS tend to be older. Although women are twice as likely as men to develop relapsing-remitting MS, men are as likely to develop primary progressive MS as are women.

An MS plaque is a circumscribed area of demyelination within the brain or spinal cord within which there is a relative preservation of axons. Although plaques may occur anywhere within the white matter, the periventricular area of the cerebral hemispheres, optic nerve, brain stem, cerebellum and spinal cord are among the more frequent sites (Wingerchuk *et al.,* 2001). Varying proportions of immune cells and immunoreactive substances (i.e. immunoglobulins) populate plaques, which can be classified as being active, inactive or chronic. Active plaques contain leukocytes that have invaded the CNS from the vascular system, resident activated macrophages/microglia, and reactive astrocytes located at the periphery of the plaque (Gutowski *et al*., 1999). Schwann cells may be present inside plaques, but if present are only found inside the astrocyte-poor central area of the lesion (Itoyama *et al.,* 1983). The number of plaques gradually increases over time as the disease progresses resulting in a gradual increase in the number of demyelinated axons. Demyelination and axonal pathology are important contributors to the neurological signs and symptoms of MS (Bjartmar *et al.,* 2001; Ferguson *et al*., 1997; Nowacki *et al*, 2000; Peterson *et al.,* 2001). Although it has been assumed axons and gray matter are spared in MS, recent observations indicate that MS is a destructive process leading to axonal damage (Trapp *et al.*, 1998) and brain atrophy (Jeffery *et al.,* 2000; Sailer *et al.*, 2003), even in spite of current anti-inflammatory or immunosuppressive therapies. Progressive neurological deterioration in MS patients has also been correlated with atrophy in the spinal cord (Bot *et al.*, 2004; Losseff *et al.*, 1996), cerebellum (Davie *et al.*, 1995), and cerebral cortex (Losseff *et al.*, 1996). The onset of the symptoms of MS is often associated with breakdown of the blood-brain barrier, which can be visualized by gadolinium-enhanced lesions on magnetic resonance imaging (McFarland *et al.,* 1992; Rudick *et al.,* 1996; Werring *et al.*, 2000).

Depending on cellular composition and on the relative balance between demyelination and remyelination, MS plaques can be divided into several different types (Lassmann *et al.,* 2001; Lucchinetti *et al*., 1996, 1999; 2000). Type I and II lesions are characterized by the

presence of a prominent inflammatory reaction, which is likely mediated in part by tumor necrosis factor-α (TNF-α) and by the release of reactive oxygen species by T lymphocytes and macrophages (Lucchinetti *et al*., 1996, 1999; 2000). The deposition of antibodies and complement is seen in Type II lesions but these are absent from lesions of Type I. Antibodies against various myelin proteins have been identified in cerebrospinal fluid (CSF) and in sera obtained from MS patients, although their role in the pathogenesis is not clear. For example, anti-MOG antibodies have been identified both in serum and in CSF of some MS patients (Reindl *et al.,* 1999). Hypoxic damage in oligodendrocytes is predominant in type III lesions (Aboul-Enein *et al*., 2003; Lassmann*,* 2003), which are characterized by ill-defined borders, rims of spared myelin surrounding inflamed vessels, and preferential loss of MAG. Other myelin proteins (PLP, MBP, CNP) are still present within active lesions. The Type III lesion is considered to be active because activated macrophages filled with myelin degeneration products are present. Marked degeneration of oligodendrocytes, a minor inflammatory reaction and extensive tissue destruction are present at the Type IV lesion. Similar to that of Type I and II lesions, the perivenous and radial plaque growth and simultaneous loss of all myelin protein are also present in Type IV lesions. Various degrees of remyelination are seen in both Type I and II lesions, but not in either Type III or Type IV lesions.

Depending on a number of factors such as location of lesions, disease activity, medication and genetic susceptibility, axonal loss reaches a certain threshold and becomes clinically obvious at various time-points. In longstanding lesions, an increased number of inflammatory attacks axonal injury can be substantial and results in considerable irreversible neurological impairment and disability of MS patients.

This irreversible disability that MS patients exhibit has been attributed to axonal dysfunction and loss (Davie *et al.,* 1995; De Stefano *et al.,* 1998). Acute axonal injury has been demonstrated in early lesion formation and is most pronounced in actively demyelinating plaques in part due to the inflammatory edema (Bitsch *et al*., 2000; Ferguson *et al.,* 1997; Kornek *et al.,* 2000; Trapp *et al*., 1998). Axonal injury begins at disease onset and remains clinically silent for many years. Irreversible neurological disability develops when axonal loss reaches its threshold and compensatory resources of the CNS tissue are exhausted. Accumulation of axonal amyloid precursor protein (APP), a marker of axonal injury or

dysfunction, was found in active plaques and at the border of the chronic MS lesions (Ferguson *et al*., 1997).

1.4.2 Theories on etiology of MS

The primary cause of MS remains unknown. Many investigators generally accept the concept that the immune system contributes to the destruction in multiple sclerosis and CD4 positive T cells initiate an inflammatory cascade that leads to demyelination. However, it has not yet been determined what causes MS. Therefore, researchers investigate different hypotheses such as environmental (Agranoff and Goldberg, 1974; Bach, 2002; Kurtzke, 1980), viral (Challoner *et al.*, 1995; Friedman *et al.,* 1999; Gilden, 1999; Kazmierski *et al*., 2004; Lucchinetti *et al.,* 2000), genetic (Ebers *et al*, 1995; Sadovnick *et al,* 1993; Weinshenker *et al*, 1998), vascular (Aboul-Enei *et al*., 2003; Lassman *et al*., 2001) and inflammatory (Bo *et al*, 1994; Bonetti and Raine, 1997; Dowling *et al.,* 1996; Hofman *et al.,* 1989; Merrill *et al*., 1993; Trapp *et al.,* 1999). A majority of researchers believe that a viral agent directly or indirectly triggers inflammatory and autoimmune cascades which initiate MS pathogenesis.

Epidemiological findings support an environmental hypothesis since the prevalence of MS generally increases with distance from the equator, and thus the disease occurs preferentially in areas that receive a relatively low amount of ultraviolet radiation from sunlight (Agranoff and Goldberg, 1974; Kurtzke, 1980). As a consequence, low vitamin D production is detected in the population of these areas and especially in MS patients (Bach, 2002). Other environmental factors suspected in predisposing people to the development of MS include infections (Challoner *et al.*, 1995; Friedman *et al.,* 1999; Gilden, 1999; Kazmierski, 2004; Lucchinetti *et al.,* 2000) and ecological factors (Lauer, 1997, reviewed in Wingerchuk *et al*., 2001), such as climate, soil conditions, and diet. It has been hypothesized that some infections including human herpes virus 6 (Challoner *et al.*, 1995; Friedman *et al.*, 1999, Lucchinetti *et al*., 2000; Knox, 2000; Goodman, 2003), Chlamydia pneumonia (Gilden, 1999; Sriram *et al*., 1999), and influenza virus (Kazmierski, 2004) are causative agents of MS. The genetic hypothesis is based on the evidence of a higher prevalence of MS in monozygotic twins as opposed to heterozygotic siblings (Sadovnick *et al.,* 1993). Many ethnic groups, including North American Indians, showed reduced incidence of MSdespite them living in regions with a high prevalence for this disease. Many patients with MS were tested for human leukocyte antigen (HLA) DR2, as a more susceptible locus on chromosome 6 (Ebers *et al.*, 1995).

Although HLA polymorphism is not considered to be a significant contributor, genetic factors may still be important determinants of the disease course (Weinshenker *et al.*, 1998). Polymorphisms in the interleukin (IL)-1β and IL-1β receptor antagonist genes (Schrijver *et al.*, 1999), the apolipoprotein E gene (Evangelou *et al.*, 1999), and immunoglobulin (Ig) G Fc receptor genes (Myhr *et al.,* 1999) have been also associated with MS course. MS patients with a combination of IL-1β and IL-1β receptor antagonist polymorphisms are found to have a higher rate of progression on the Expanded Disability Status Scale when compared with other possible combinations (Schrijver *et al.*, 1999). At the apolipoprotein E gene, the ε4 allelle is found to be more common in MS patients with more rapid progression of disability in MS (Evangelou *et al.*, 1999). Authors suggest that apolipoprotein E is not implicated in the early pathogenesis of MS; however, patients possessing the ε4 allelle have a reduced capacity for neuronal remodeling after relapses. Although the frequencies of two IgG Fc receptors IIA and IIIB do not differ between the MS patients and the controls, MS patients homozygous for the IgG Fc receptor IIIB neutrophil antigen 1 allele have more benign course of disease than that of patients heterozygous or homozygous for the IgG Fc receptor IIIB neutrophil antigen 2 allele (Myhr *et al.*, 1999).

There are an increasing number of studies reporting the role of an immunomediated vasculitis leading to MS plaque development (Aboul-Enei *et al*., 2003; Lassman *et al*., 2001). Activated T-cells and macrophages damage endothelial cells causing microvessel thrombosis with secondary ischemic damage of white matter. Activated microglia secrete nitric oxide (Bo *et al.*, 1994; Merrill *et al*., 1993) and cytokines TNF-α (Hofman *et al.,* 1989) and lymph toxin (Selmaj *et al*., 1991), which were found to selectively damage oligodendrocytes *in vitro* and in some MS lesions (Bonetti and Raine, 1997; Dowling *et al.,* 1996; Trapp *et al.,* 1999). Matrix metalloproteases (MMPs), a family of proteolytic enzymes including collagenasese, gelatinases, stromelysins, membrane-type metalloproteinases, matrilysin and metalloelastase, are known to be involved in remodeling of extracellular matrix of many tissues (Birkedal-Hansen *et al.,* 1993). They have been implicated in degradation of vascular basement membrane thereby facilitating leukocyte migration into inflammatory sites (Yong *et al*., 1998). The authors focused their attention on MMP-9 (gelatinase B) because it is known to degrade type IV collagen and to disrupt cell-matrix contacts. The subsequent loss of integrin signaling

leads to oligodendrocyte apoptosis (Chandler *et al.,* 1997). MMP-9 has been shown to degrade myelin basic protein of the myelin sheath (Chandler *et al*., 1995; Gijbels *et al.,* 1993).

1.4.3 Host glial cell response

The MS lesions have been characterized by the composition of demyelination, oligodendrocyte pathology, the inflammatory infiltrate, astrogliosis and axonal damage (Kornek and Lassmann, 2003; Lassmann *et al*., 1998). However, it remains unclear, which of the processes develop primarily in response to still unknown cause and which develop secondarily as a consequence of, or as a reaction to, an initiating event. The pathology of MS is associated with immunological and inflammatory processes that result in complex changes in gene expression by astrocytes and microglia in the vicinity of the lesion, in the recruitment of a number of cell types (e.g. leucocytes) into the lesion (Adamson *et al*., 1999; Al-Omaishi *et al.,* 1999; Asensio *et al*., 1999), and in the alteration of the extracellular matrix of the plaque (Gutowski *et al.*, 1999; Opdenakker, 1997). Chemokines, which are expressed in response to CNS inflammation, are known to induce a chemotactic migration of microglial cells (Badie *et al.,* 1999; Cross and Woodroofe, 1999; Yamagami *et al*., 1999). INF-γ and TNF-α are the most studied inflammatory cytokines and both molecules are expressed in MS lesions (Wingerchuk *et al*., 2001).

Heterogeneity of pathological changes in MS plaques has been previously documented (Lucchinetti *et al.,* 2000). However, one of the constant features of all plaques is the presence of microglial and astrocytic reactivity. A very recent study of patients with relapsing and remitting MS, who died during or shortly after the onset of a relapse, showed extensive oligodendrocyte apoptosis, as well as microglial and astroglial activation, in myelinated tissue in which there were few or no lymphocytes or myelin phagocytes (Barnett, 2004).

Microglia along with T cells infiltrate injured CNS tissue and play an important role in the development and progression of active MS lesions. Sriram and Rodrigez (1997) implicated macrophage/microglia as principal players in the demyelination process due to activation of these cells and release of the factors that are toxic to the oligodendrocytes and myelin. This model was supported by the findings of Chiang *et al.* (1996), who described macrophage/microglia-mediated demyelination induced by low-level chronic secretion of IL-3. The macrophages/microglia within active plaques are filled with myelin degradation products, whereas inactive plaques contain far fewer cells and the macrophages/macroglia that are

present do not normally contain myelin debris. Demyelination is believed to be occurring at the site of active plaques, whereas inactive plaques are most likely areas where some degree of tissue repair either has occurred or is actively taking place. The demyelinated lesions in patients with chronic MS have well-demarcated areas with very few cells visualized with Luxol Fast Blue staining, varying degrees of axonal loss, and persistent but minor inflammation; these lesions are referred to as chronic plaques. In recent years a harmful role of macrophage/microglia has been questioned. Diemel *et al.,* (1998) and Loughlin *et al*., (1997) suggested that macrophages can be beneficial for MS lesion and that they also may promote recovery from demyelination by the phagocytosis of myelin debris and the release of trophic factors. Macrophages were found to stimulate myelinogenesis (Hamilton and Rome, 1994) as well as remyelination (Cuzner *et al*., 1994; Loughlin *et al.,* 1997) in cell cultures. There are still questions also to whether macrophages found in MS lesions are agents of destruction or contributors to recovery. Bartnik *et al.* (2000) showed that macrophages promoted myelination in embryonic spinal cord aggregate cultures at normal and low levels of oxidative stress and on the other hand were part of the cause of neuronal damage when the levels of oxidative stress were higher.

A network of reactive astrocytes can be found in the majority of MS lesions. It is believed that astrocytes are activated by pro-inflammatory cytokines (Selmaj *et al*., 1990) and such an astrocytic reaction is not specific for MS. Although the role of astrocytic activation is not yet clear, Rosen *et al.* (1989) suggested that astrocyte sclerosis might serve as a barrier for regeneration. Other studies showed that astrocytes may be a source of growth factors, including vascular endothelial growth factor and fibroblast growth factor (FGF) 2, and could therefore support regenerative processes (Horner and Palmer, 2003; Ridet *et al.,* 1997). Reactive astrocytes present inside chronic MS plaques produce molecules such as tenascin-R and –C, which are believed to impede remyelination and axonal repair by contributng to the formation of a glial scar (Gutowski *et al*., 1999).

1.4.4 Current treatment

The treatment of MS patients is very complex and best accomplished with a multidisciplinary approach involving a neurologist, ophtalmologist, psychiatrist, urologist, gastroenterologist, nurse, physical therapist, rehabilitation specialist, and social worker. There is no cure for MS at the present time, because its cause remains

unknown. Therefore, the treatment of patients with MS is very challenging and complex. The patients with MS often develop complications such as fatigue, depression, sleep disorders, spasticity, pain, decubitus ulcers, speech and swallowing disorders, mood and cognition disorders, and others. Such complications usually require attention of a psychiatrist, physiotherapist and rehabilitation specialist.

Clinically significant acute relapses are currently being treated with the use of corticosteroids and plasma exchange to hasten recovery. Although no consensus about the optimal form, dose, route and duration of corticosteroid therapy has been accepted, corticosteroids such as methylprednisolone and prednisone have been used to reduce the rate of clinical relapses, delay progression of disability and reduce CNS lesion activity as evidenced by magnetic resonance imaging (MRI; Beck, 1992, 1995). Plasma exchange is found to enhance recovery of relapse-related neurological deficits in MS patients who show no response to high-dose corticosteroids (Weinshenker *et al*., 1999).

Phase 3 trials indicate that treatment with interferon derivatives, such as INF β-1a (Rebif®) may delay the development of clinical and MRI evidence of a second diagnosis-defining bout after a first episode of acute neurological syndromes suggestive of MS (Jacobs *et al.,* 2000). The rate of clinical relapses, the development of new lesions and increase in volume of existing lesions on MRI scans are also reduced by approximately 30 percent with administration of INF β-1b (Betaseron®, Berlex Laboratories) (Paty and Li, 1993; The IFNB Multiple Sclerosis Study Group, 1995) and INF β-1a (Avonex®, Biogen; Rudick *et al.,* 1997). INF β-1a and glatiramer acetate, a mixture of synthetic polypeptides containing glutamic acid, alanin, lysine, and tyrosine, (Copaxone®, Teva Pharmaceutical Industries) were found to reduce the frequency of relapse (Johnson *et al.*, 1995; Johnson *et al.*, 1998). In addition, INF β-1a may also delay the progression of disability in MS patients with minor disability and a relapsing form of MS (Simon *et al.*, 1998; Jacobs *et al.,* 1996; Rudick *et al.*, 1997). The specific mechanisms of action of interferons in MS are not completely understood. These agents are believed to exert their immunomodulating activities by reducing both the proliferation of T cells and the production of TNF-α, by decreasing antigen presentation, by altering cytokine production and favoring ones governed by type 2 helper T (Th) cells, by increasing the secretion of IL-10, and by reducing the passage of

immune cells across the blood-brain barrier by means of their effects on adhesion molecules, chemokines, and proteases (Rudick *et al*., 1997). Glatiramer acetate is found to promote the proliferation of Th2 expressed cytokines, compete with MBP for presentation on MHC class II molecules, thereby inhibiting antigen-specific T-cell activation, altering the function of macrophages, and inducing antigen-specific suppressor T-cells (Bornstein *et al*., 1987). An elevation of serum IL-10 levels, suppression of the pro-inflammatory cytokine TNFα mRNA, and elevation of the anti-inflammatory cytokines TGF-β and IL-4 mRNAs in leukocytes were observed in a 12 month study conducted by Miller *et al.* (1998). They suggested that the beneficial clinical effects of glatiramer acetate in MS patients may be attributed to changes in activation of T cells and a shift from a Th1 to a Th2/Th3 cytokine profile, probably leading to glatiramer acetate-driven mechanisms of bystander suppression.

1.5 Glial Cell Reaction to Injury

Following an acute insult to the CNS, a complex of morphological and metabolic changes takes place within the nervous system with the goal to repair the damaged tissue (Deckert-Schluter *et al*., 1995; Drescher *et al*., 1997; Griffin *et al*., 1992; Kreutzberg, 1996; Raivich *et al*., 1996). The injured neurons undergo crucial steps in a fight for their survival and stimulate nearby neuroglia including microglia and astrocytes for support (Kreutzberg, 1996; Raivich *et al*., 1995). The activation of microglia (Kreutzberg, 1996) and astrocytes (Eddleston and Mucke, 1993) has been described as a graded, stereotypic response, which is readily observed in stroke and ischemia, in neurodegenerative diseases, after direct or indirect axonal injury (Norenberg, 1994) or during inflammation due to infections or autoimmune disease (Perry *et al*., 1995). The production of proinflammatory cytokines, functional changes in brain vascular endothelia and a recruitment of cells of the immune system into the damage tissue has been also identified after acute nervous system injury.

1.5.1 Astrocytes

The astrocytes are considered to be the predominant neuroglial cell of the CNS and exist in two typical forms stellar-fibrillary astrocytes and protoplasmatic astrocytes. The former cells are normally located in the white matter and bear long slender processes, which stain with GFAP, a typical astrocyte cytoskeletal protein (Bignami *et al.,* 1972). The

protoplasmic astrocytes, on the other hand, are residents of grey matter, exhibit numerous short and highly branched processes and only a few of these astrocytes express GFAP. Astrocytes provide metabolically optimized nutrients to the highly energy-consuming neurons and maintain ionic balance in the extracellular space (reviewed in Tsacopoulos and Magistretti, 1996). In addition, astrocytes induce tight junctions on endothelial cells (Janzer and Raff, 1987; Pekny *et al*., 1998; Tao-Cheng *et al*., 1987), induce the ramified phenotype in microglia and blood-derived monocytes (Kloss *et al*., 1997; Schmidtmayer *et al*., 1994) and provide numerous trophic factors including PDGF (Hermanson *et al*., 1995), NGF, IGF-1, TGF-α for adjacent neurons and oligodendrocytes, particularly following injury (Eddleston and Mucke, 1993; Ridet *et al*., 1997).

In response to an injury that occurred some distance away, astrocytes became activated and increase of their synthesis of GFAP within 24 hours for example after facial nerve axotomy (Tetzlaff *et al.*, 1988). Reactive astrocytes in the adult brain appear to recover the capacity to express vimentin (Pixley and De Vellis, 1984; Schiffer *et al*., 1988; Takamiya *et al*., 1988), which normally is expressed only during embryogenesis and during the first two postnatal weeks in rodents (Pixley and De Vellis, 1984; Schnitzer *et al*., 1981; Voigt, 1989). Reactive astrocytes create a physical barrier between damaged and healthy cells. Beginning 2 weeks after axonal injury, the reactive astrocytes gradually replace microglia from the neuronal surface and begin to enwrap the perikarya of injured neurons with thin, flat cytoplasmic processes (Graeber and Kreutzberg, 1988; Guntinas-Lichius *et al*., 1997). Processes of adjacent astrocytes adhere to each other and form a multilayered stack of astrocytic lamellae, which could act as a small glia scar surrounding the axotomized neuron. Physical damage, ischemia or inflammation will lead to disruption of the blood brain barrier, collateral activation of adjacent microglia and a much stronger response of astrocytes, which will proliferate and form a massive scar (Balasingam *et al*., 1994; Giulian and Lachman, 1985). A dense network of hypertrophic astrocytes with thick, interdigitating processes and associated extracellular matrix compose the glial scars (Brodkey *et al*., 1995). It is commonly believed that the cell surface of astrocytes and the extracellular matrices in which they are embedded are more likely to hinder rather than facilitate the growth of axons *in vivo* in the adult mammalian CNS (Fraher, 1999; Reier *et al.,* 1986; Reier and Houle, 1988; Ridet *et al*., 1997). The components of the extracellular matrix such as chondroitin sulfate proteoglycans (Giftochristos and David,

1988; Lips *et al*., 1995; McKeon *et al*., 1991), tenascin (Laywell *et al*., 1992; Lips *et al*., 1995; McKeon *et al*., 1991) and collagen IV (Stichel *et al*., 1999) have been found to inhibit neurite outgrowth.

However, astrocytes are not always nonpermissive for axonal growth. In fact, there appear to be differences in the ability of astrocytes to support axonal growth. This can depend on the degree of differentiation of the cells (Baehr and Bunge, 1989; Hatten *et al*., 1991; Lucius *et al*., 1996), on whether the astrocytes have formed a two- or three-dimensional substratum for the growing axons (Fawcett *et al.,* 1989), on the region of the CNS that is injured (Alonso and Privat, 1993a, 1993b; Chauvet *et al.,* 1998; Prieto *et al*., 2000), and on the presence or absence of macrophages (David *et al.,* 1990), Schwann cells (Berry *et al.,* 1992; Dezawa *et al*., 1999) or OECs (Li *et al.,* 1997, 1998; Ramon-Cueto *et al*., 1998, 2000).

1.5.2 Microglia

Microglia are thought to be derived from infiltrating hematopoietic or mesodermal cells during the early development of the CNS (Eglitis and Mezey, 1997; Hickey and Kimura, 1988; Hickey *et al*., 1991, 1992; Kurz and Christ, 1998; Ling and Wong, 1993). The normal, resting microglia have long, ramified processes oriented parallel to the nerve fibers in the white matter, and display a stellate morphology in the gray matter (Compston *et al*., 1997). Microglia appear to participate in the immune surveillance of the nervous system. During embryonic and early postnatal (up to postnatal day 21) rat development, microglia express the complement type 3 receptor and the MHC class 1 antigen, which can be recognized by monoclonal antibodies OX-42 and OX-18, respectively (Domaradzka-Pytel *et al*., 1999). After P21, microglia within the normal CNS no longer express the MHC class 1 antigen but remain OX-42 positive. However, microglia will re-express complement type 3 receptor in response to neural damage (Graeber *et al*., 1988).

Microglia respond to neural damage by undergoing characteristic changes, which involve cellular and morphological events, such as increased cell size and number of activated cells, as well as the expression of cell surface molecules (i.e. MHC class 1 antigen; Graeber *et al*., 1988), cytokines (i.e. IL-1β, TNF-α; Bartholdi and Schwab, 1997) and growth factors (i.e. NGF, neurotrophin-3, ciliary neurotrophic factor; Eddleston and Mucke, 1993; Elkabes *et al*., 1996; Mallat *et al*., 1989). Following axotomy of the facial nerve, resting microglia become activated and then proliferate and tightly ensheath injured motoneurons with enlarged

cytoplasmic processes (Blinzinger and Kreutzberg, 1968). If an injured neuron survives and re-grows its axon, reactive microglia may revert back to the resting form. If neurons die, microglia change their form from process bearing to rounded (ameboid) macrophages, which remove the dead cells (Streit and Kreutzberg, 1988). Activated microglia develop enlarged cell processes, which give the cells a bushy appearance. Cellular hypertrophy is concomitant with upregulation of complement type 3 receptor expression (Graeber *et al*., 1988) and both are apparent by 24 hours after injury. Microglia begin to proliferate 2-3 days after injury and their numbers reach maximum levels after 4-7 days. Following an epidural application of kainic acid over the rat cerebral hemispheres, Kaur and Ling (1992) observed extensive neuronal degeneration, which elicited an increased expression of the complement type 3 receptor (marked by OX-42) and induced MHC 1 expression (marked by OX-18) in microglia. The elevated expression of the former receptor may be related to the phagocytic activity of these reactive microglia, whereas re-expression of the latter antigen was suggested to facilitate the activated microglial cells interaction with T lymphocytes as part of an immune response.

Streit *et al.* (1999) suggest that activation of microglia shortly after injury may serve to limit secondary damage to neurons, whereas chronically activated microglia may take an active role in neuron destruction. Microglial activation after acute CNS injury is described as a transient and self-limited event and it usually subsides within about one month after either traumatic or ischemic insults (Gehrmann *et al*., 1991; Morioka *et al*., 1991; Koshinaga and Whittemore, 1995). An increasing number of studies have focused on the expression of microglia-derived cytokines and growth factors following CNS injury. The mRNA levels of microglia-derived cytokines and growth factors fluctuate depending on the injury type. The expression of proinflammatory cytokines, such as IL-1β and TNF-α, is minimally upregulated following axotomy of the facial nerve (Streit *et al*., 1998), whereas a sharp increase in the expression of these cytokines is seen immediately after spinal cord injury (Bartholdi and Schwab, 1997). The upregulation of proinflammatory cytokine mRNAs is observed for a very short duration, with the levels returning to baseline levels one or two days after injury (Bartholdi and Schwab, 1997; Buttini *et al*., 1994; Streit *et al*., 1998; Taupin *et al*., 1993; Woodroofe *et al*., 1991). Streit *et al*. (1999) suggested that this transient increase in IL-1β and TNF-α expression is tightly controlled and these cytokines may function in triggering secondary events, including the production of neurotrophins, which may be beneficial to the

subsequent repair and regeneration process (Fagan and Gage, 1990; Lindholm *et al*., 1987). In contrast, prolonged increases in the production of proinflammatory cytokines (i.e. TNF-α; Probert and Akassoglou, 2001) such as occurs in chronic conditions such as MS may be detrimental to neural repair and cause secondary, irreversible damage to nervous tissue.

1.5.3 Inflammation

Reactive gliosis is a universal event after CNS injury and is a prominent pathological feature in MS where reactive astrocytes constitute the major cell type in demyelinating plaques (Raine, 1984). After injury and/or during inflammation, TNF-α and IL-6 (Selmaj *et al*., 1990) induce rapid proliferation and hypertrophy of astrocytes, which in turn secrete numerous humeral factors (e.g. TNF-α, prostaglandins, and TGF-β; Hatten *et al.,* 1991; Nieto-Sampedro *et al*., 1983; Reier and Houle, 1988). Therefore, secreted products of activated inflammatory cells contribute to the reactive gliosis found in white matter diseases of the CNS.

MS lesions are commonly characterized by the presence of inflammatory infiltrates consisting of lymphocytes and macrophages/microglia. Oligodendrocytes and the myelin sheaths they produce can be destroyed by different immunological mechanisms, such as direct interaction with cytotoxic T-cells and T-cell cytokines (Selmaj *et al.*, 1990), demyelinating antibodies (Linington *et al*., 1988; Raine, 1984), and cytotoxic cytokines (i.e. TNF-α; Probert and Akassoglou, 2001). It has been suggested that inflammation in MS is induced by the migration of activated T cells through the blood-brain barrier (Conlon *et al*., 1999). INF-γ and TNF-α, which are released as part of the inflammatory response, induce the capillary endothelial cells to express vascular cell adhesion molecule (V-CAM) and class II molecules of the MHC (Cannella and Raine, 1995). Activated T cells express integrins, particularly alpha-4 integrin, and CD4, a member of the immunoglobulin supergene family, which bind to V-CAM and MHC class II molecules, respectively. T cells express very late antigen (VLA)-4, which enables them to penetrate the endothelium. VLA-4 was found on T cells that collected around veins and capillaries and formed perivascular lymphocyte cuffs in acute MS lesions. The blockade of VLA-4 with cyclic peptides resulted in a reversion of clinical paralysis in EAE animals (Dutta *et al*., 2000). Once outside the blood vessels, the activated lymphocytes expressed alpha 1 integrin, which binds to collagen type IV in the extracellular matrix. T cells here began to secrete enzymes such as MMPs, some of which, MMP-2 and MMP-9, also called gelatinase A and B, specifically degrade collagen type IV (Brosnan and Raine, 1996; Conlon *et*

al., 1999; Gijbels *et al*., 1994). In this way activated lymphocytes gain access to the CNS where they then induce an inflammatory reaction in the white matter, creating a situation that is potentially very detrimental to CNS myelin and oligodendrocytes.

In the neuropil, activated Th1 cells may stimulate local microglia and hematopoetic macrophages, through the secretion of cytokines such as TNF-α, INF-γ, lymphotoxin-α (also known as TNF-β) and IL-12. These in turn damage myelin and cause a further release of potential antigens. These cytokines are expressed in actively demyelinating MS lesions and elevated levels of TNF-α correlate with disease activity and blood-brain barrier damage (Bitsch *et al*., 2000). However, TNF-α was recently shown to also protect neurons (Akassoglou *et al*., 1997; Liu *et al*., 1998; Probert *et al.,* 1995), to promote proliferation of oligodendrocyte progenitors and to facilitate their remyelination of CNS axons (Arnett *et al.,* 2001). Lymphotoxin-α and TNF-α then stimulate macrophages, microglia and astrocytes to produce nitric oxide and osteopontin (Steinman, 2001). Merrill *et al*. (1993) suggested that nitric oxide, one of the free radicals that in combination with antibodies, complement and TNF-α (Selmaj *et al*., 1991) mediate damage to oligodendrocytes by macroglia, plays a role in lesion formation in MS. Osteopontin, an extracellular glycosylated bone phosphoprotein, further induces Th1 cytokine production, including INF-γ and IL-12, while downregulating Th2 cytokines such as IL-10. Th1 cytokines are found during MS exacerbation whereas Th2 cytokines may reduce the size of MS lesions (Bielekova *et al.,* 2000; Chabas *et al*., 2001; Kappos *et al*, 2000; Steinman, 2001).

CD4+ helper cells are present mainly in perivascular areas of the CNS of MS patients, whereas CD8+ cells are more prominent within the MS plaques and, therefore, are thought to be more directly involved in the tissue destruction than are the CD4+ cell population (Babbe *et al.*, 2000; McCallum *et al*., 1987; Steinman, 2001; Woodroofe *et al.,* 1986). The number of CD8+ cells also correlates better with the extent of acute axonal injury than does the number of CD4+ cells (Bitsch *et al*., 2000).

A number of pro- and anti-inflammatory cytokines have been identified in the serum and CSF of MS patients. The expression of some of these markers, such as TNF-α (Bertolotto *et al*., 1999; Rieckmann *et al*., 1998), as well as molecules like adhesion molecules (Kraus *et al*., 2002; Rieckmann *et al.,* 1998), MMPs (Waubant *et al.*, 1999), chemokine receptors (Strunk *et al.,* 2000) and macrophage-related protein (e.g. MRP8/14; Bogumil *et al*., 1998), was shown

to correlate with clinical course and lesion activity identified with MRI. However, none of these markers are specific for MS and the results of different studies remain controversial and have not been adopted for use yet in clinical practice (Bertolotto *et al*., 1999, Bitsch *et al*., 1998, Carrieri *et al.*, 1998; Strunk *et al*., 2000; van Oosten *et al*., 1998).

1.5.4 Degeneration

The primary injury caused by direct mechanical trauma to the brain or spinal cord encompasses the focal destruction of neural tissue. The initial insult usually also results in a progressive wave of secondary injury, which via a complex of harmful pathophysiological mechanisms exacerbates the injury to CNS tissue and causes both necrosis and apoptosis. Following either brain or spinal cord injury, the extracellular glutamate concentration rises continuously leading, especially in an hypoxic environment (Choi, 1993), to overstimulation of ionotropic α-amino-3hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate receptors and triggering excitotoxic cell death (Doble, 1999). Precisely what factors expressed by dying neurons trigger activation of microglia remains poorly characterized. Damaged axons may release adenosine triphophate (ATP), which could activate microglial cells via purinoreceptors. The increase of ATP in culture medium was found to induce microglia membrane currents and transmembrane Ca^{2+} fluxes (Haas *et al*., 1996; Illes *et al*., 1996; Kettenmann *et al*., 1993; Walz *et al*., 1993). High levels of extracellular potassium could also be responsible for mediating certain aspects of glial cell activation. Gehrmann *et al*. (1993) described a transient potassium chloride-induced microglial activation, which was accompanied by short-lasting hypertrophy and increased OX-42 expression, but not proliferation of microglia.

In both the acute and the chronic phases of MS, demyelination and axonal degeneration have both been correlated with the severity of disabilities such as paralysis and the inability to walk (Losseff *et al.*, 1996; Trapp *et al*., 1998). During the inflammatory reaction in both MS and in EAE, lymphocytes, microglia and macrophages produce excessive amounts of glutamate, which in turn activates AMPA/kainate receptors present on oligodendrocytes and neurons (reviewed in Steinman *et al*., 2002). Glutamate via its receptors induces calcium influx and causes necrotic damage to oligodendrocytes and axons. In the EAE animal model, blocking the AMPA/kainate with quinoixalin derivatives (NBQX and MBQX) provided some protection to the oligodendrocytes and axons from immune-mediated damage, although the immune response to myelin antigens was not altered (Pitt *et al*., 2000; Smith *et al*., 2000). It

has been suggested that cytotoxic CD8+ T cells might mediate axonal damage in inflammatory lesions of MS tissue (Babbe *et al*., 2000), in EAE mice (Huseby *et al*., 2001), and *in vitro* (Medana *et al*., 2001). Within MS plaques, axons are particularly vulnerable to degeneration when exposed to nitric oxide (Smith *et al*., 2001a). In addition, inflammatory edema increases extracellular pressure contributing to axonal damage especially in CNS anatomical locations such as the spinal cord where room for tissue expansion is limited (Shi and Blight, 1996). Wujek *et al*., (2002) noted there was a 9% increase in the volume of the spinal cord during the first attack in mice with relapse-remitting EAE.

1.6 Animal Models for Studying Demyelination and Remyelination

Many animal models have been developed to study the biology and pathology of demyelination and remyelination in the CNS (Steinman, 1999), since it is not feasible to perform experimental manipulations in humans. These animal experiments provide investigators with the opportunity to study the morphological, cellular and molecular mechanisms involved in CNS demyelination and remyelination and are designed to mimic some, but not all, of the pathological and neurological sequelae of MS. The available animal models fall into the following categories: (1) neurotoxicant-induced demyelination, including EtBr (Yajima and Suzuki, 1979), lysolecithin (Hall, 1972) and cuprizone; (Blakemore, 1973), (2) autoimmune-induced demyelination, including EAE (Raine and Traugott, 1984); and (3) viral-induced demyelination, including corona-virus (Herndon *et al*., 1977) and Theiler's murine encephalomyelitis virus (TMEV) (Rodriguez *et al*., 1988).

1.6.1 Ethidium bromide

EtBr is a nucleic acid-binding neurotoxicant that has often been used to induce demyelination because of its effectiveness in killing CNS glial cells, but not axons, when injected into white matter. This nucleic acid chelating agent reacts with the nuclei of those cells residing in the vicinity of the injection (Blakemore, 1984; Yajima and Suzuki, 1979). When injected in the dorsal column of the spinal cord, EtBr demyelinates axons of the dorsal column sensory pathways by killing all the glial cells, including the oligodendrocytes, astrocytes and microglia, in the vicinity of the injection. However, axons are spared from the toxic effects because their cell bodies lie elsewhere, specifically within the dorsal root ganglia situated some distance from the site of EtBr injection. Within three days, the EtBr-induced

demyelinating lesion is composed of naked, demyelinated axons, and a total absence of glial cells. During lesion repair, invading macrophages scatter among the demyelinated axons and around blood vessels, and cluster around disintegrating myelin sheaths to clean up the debris. In addition, oligodendrocyte progenitor cells, astrocytes and Schwann cells all migrate into the lesion site from the surrounding tissue to assist in neural repair. The oligodendrocytes contribute to the remyelination of the demyelinated axons in the central part of such EtBr lesions, while Schwann cells remyelinate axons in the subpial areas and astrocyte-free regions (Felts and Smith, 1996; Yajima and Suzuki, 1979). Blakemore and Patterson (1978) were the first to expose the animal's spinal cord to x-irradiation prior to injection of EtBr, using 40 Grays of x-irradiation to suppress the spontaneous repair of the lesion by host glial progenitor cells. Such a lesion provides an excellent environment in which the myelinogenic properties of grafted myelin-forming cells can be tested (Akiyama *et al*., 2001, 2002a, 2002b; Blakemore and Crang, 1985; Franceschini and Barnett, 1996; Franklin *et al.,* 1996; Groves *et al*., 1993).

1.6.2 Lysolecithin

Another neurotoxicant-induced animal model of demyelination is one using lysolecithin (lysophosphatidyl choline), which was first used by Hall and Gregson (1971) for tissue in the PNS. Numerous investigators have subsequently injected lysolecithin into the CNS to permeabilize the plasma membranes of cells (Blakemore *et al.*, 1977; Blakemore, 1978; Waxman *et al*., 1979). Due to its permeabilizing property lysolecithin affects oligodendrocytes, thus resulting in demyelination in the immediate vicinity of the injection site (Blakemore, 1978).

Woodruff and Franklin (1999) compared lesions induced by either EtBr or lysolecithin and found that a significant proportion of axons remained demyelinated for a longer time after an EtBr injection than after a lysolecithin-induced demyelination. Although, EtBr-induced demyelinated lesions remyelinated more slowly than after lysolecithin injection, exposure to the latter toxin did cause axonal damage.

1.6.3 Cuprizone

The neurotoxicant cuprizone, bis–cyclohexanone-oxaldihydrazone, is a copper chelator, causes copper deficiency resulting in liver and brain tissue damage (Blakemore, 1972, 1973; Ludwin, 1978; Suzuki and Kikkawa, 1969). Rats, guinea pigs, and hamsters acquired a severe spongiform encephalopathy after being on an 8 week cuprizone (0.2%) diet (Carey and

Freeman, 1983). In Wister male rats, only oligodendrocyte perturbation and intramyelinic edema were observed (Love, 1988). However, after a short 4 or 6 week exposure to a diet containing cuprizone C57BL mice developed acute demyelination. This occurred as a result of the apoptotic death and depletion of mature oligodendrocytes within the corpus callosum (Armstrong *et al*., 2002; Arnett *et al.*, 2001; Hiremath *et al*., 1998; Mason *et al*., 2000, 2001; Morell *et al*., 1998) and the superior cerebellar peduncle (Komoly *et al*., 1987; Stidworthy *et al*., 2003). Cuprizone treatment has been also used as a model for investigating the damage to oligodendrocytes in Swiss, CD1, and ICI mice (Bakker and Ludwin, 1987; Blakemore, 1974, Ludwin, 1978). Oligodendrocytes are considered to be the main CNS target of cuprizone because copper is required in high levels for proper mitochondrial function and oligodendrocytes are much more metabolically active (Hiremath *et al*., 1998; Morell *et al*., 1998) than other cell types in the central nervous system (Blakemore, 1973; Cammer and Zhang, 1993). When cuprizone was used in SJL mice before inducing EAE by PLP peptide administration, it was found to inhibit both T-cell activation and function and to attenuate the severity of the EAE (Emerson *et al*., 2001).

The cuprizone-induced demyelination is presumably mediated by microglia/macrophages (Hiremath *et al.,* 1998) since the blood-brain barrier remains intact and there is no lymphocyte-induced inflammatory reaction (Bakker and Ludwin, 1987; Kondo *et al*., 1987; McMahon *et al*., 2002). After cuprizone removal from the diet, oligodendrocyte progenitors start to proliferate and migrate from the subventricular zone. They accumulate within the demyelinating lesions and differentiate into mature oligodendrocytes, which in turn remyelinate the demyelinated axons (Mason *et al*., 2000). However, prolonged maintenance of C57BL mice on the cuprizone diet resulted in the failure of remyelination (Mason *et al*., 2004). Newly regenerated mature oligodendrocytes along with the resident progenitors become progressively depleted within the chronically demyelinated corpus callosum.

1.6.4 Experimental autoimmune encephalomyelitis

The basis for the development of models of acute EAE emerged from "paralytic accidents", which were noted by Stuart and Krikorian (1928), Rivers *et al*. (1935), Kabat *et al*. (1947) after rabies immunizations in which spinal cord inflammation led to clinical paralysis (reviewed by Steinman, 1999). An animal model of acute disseminated EAE by Rivers (1935) was developed in monkeys once immunization against brain tissue was identified as the cause

of the demyelination and paralysis (reviewed by Steinman, 1999). A mixture of brain material with complete Freund's adjuvant, an emulsion made from killed mycobacteria tuberculosis, was used by Kabat *et al*. (1947) to produce highly reproducible white matter inflammation, demyelination and acute paralysis (reviewed by Steinman, 1999).

Although the actual mechanisms responsible for the extensive CNS demyelination in MS remain to be elucidated, it is believed the pathology is related to autoimmunological phenomena induced by viral infection or by exposure to an unknown allergen (see Section 1.5.2). Once sensitized to an antigen present on CNS (but not PNS) myelin, lymphocytes enter the neuropil and induce macrophages to engulf CNS myelin. Myelin breakdown products further stimulate the immune response in the form of a positive feedback loop.

The presence of autoimmune antibodies against myelin components in the sera of MS patients lends support to the use of EAE as an animal model for MS (Raine *et al*., 1974, 1977; Steinman, 1999). Experimental animals can be immunized with MBP (Raine *et al.,* 1974, 1977), or with a synthetic peptide analog of MBP that contains only the essential amino acids (Fallis *et al*., 1989), with PLP, or with MOG (Steinman, 1999). Then, adoptive transfer of T-cells sensitized to myelin antigens is required to induce demyelination in the CNS. When induced in the guinea pig (Raine *et al*., 1974, 1977; Stone and Lerner, 1965; Traugott *et al*., 1982) or mouse (Brown and McFarlin, 1981; Lublin *et al*., 1981), EAE presents the neurological features of chronic CNS demyelination. Experimental animals with EAE display neurological signs such as weight loss, mouth wetting, some urinary and fecal incontinence, and mild hindlimb paraparesis. Incomplete recovery and progressive worsening follow after each relapse of demyelination until the animals often eventually develop quadriparesis or spastic paraparesis (Raine and Traugott, 1983).

These clinical features are the result of plaques of chronic demyelination scattered throughout the white matter. T cells, immunoglobulin, and complement play a role in the demyelinating pathogenesis in EAE. T cells reactive to myelin and antibodies to MBP, MOG, and PLP can be detected in the CSF and in demyelinated areas from brains of animals with EAE (Steinman, 1996). Adhesion molecules, cytokines, chemokines, HLA molecules, and metalloproteases are also critical participants in the development of inflammatory lesions (Steinman, 1999). Treatment with antibodies to α-integrin, CD4+ve T cells reactive to myelin proteins, and to the cytokines IL-6 and TNF-α have been found to reverse paralysis in EAE

(Conlon *et al*., 1999). Targeting of these molecules is now in use in clinical trials on MS patients (Steinman, 1999). As a consequence of these studies, Copaxone, a synthetic polymer analog of MBP (Arnon, 1996) and INF-β (Ruuls *et al*., 1996) were found to be effective in reducing the relapse rate in MS. On the other hand, INF-γ, also a critical cytokine tested in clinical trials, was found to exacerbate disease, and a monoclonal antibody to TNF-α and a soluble TNF-α receptor also the worsened clinical symptoms in MS patients (Steinman, 1999).

Although the EAE animal model gives rise to clinical signs comparable to those seen in MS, such as demyelination and a sparing of astrocytes and microglia, EAE-induced lesions are numerous and scattered all over the CNS with no experimental control over their location and size. In order to control where and how big the demyelinated lesions are, it is preferable to inject a single dose of EtBr, which results in the demyelination of the axons without inducing Wallerian degeneration.

1.6.5 Theiler's virus

Theiler's murine encephalomyelitis virus is a single-stranded RNA virus that belongs to the *Piconaviride* family and was first described by Theiler in 1937 (reviewed in Oleszak *et al*., 1995). This virus has been reported to cause neurological and enteric diseases in susceptible strains of mice, such as SJL (Dal Canto *et al*., 1996; Lipton and Jelachich, 1997; Monteyne *et al*., 1997; Oleszak *et al*., 1995; Tsunoda and Fujinami, 1996). After intracranial inoculation with the Theiler's virus DA strain, these mice develop a biphasic disease. Acute disease occurs within 3 to 12 days postinfection followed by late chronic demyelinating disease developing at 30 to 40 days postinfection. Later, the animals die being unable to completely clear the virus from their bodies (reviewed by Dal Canto *et al*., 1996; Monteyne *et al*., 1997; Oleszak *et al.,* 1995; Tsunoda and Fujinami, 1996). In contrast, in resistant strains of mice, such as C57BL/6, the animals develop only the early acute phase of the disease without progression into late chronic demyelinating disease and these mice also clear the virus completely from their bodies by about 3 weeks postinfection (Lipton, 1975; Lorch *et al*., 1981).

Both susceptible and resistant strains of mice develop acute polioencephalitis with destruction of neurons to a variable degree, extensive mononuclear cell and lymphocyte infiltration in the CNS (reviewed by Begolka *et al*., 1998; Drescher *et al.*, 1997; Oleszak *et al.*, 1995; Rodriguez *et al*., 1988). Variably intense and multifocal demyelinating lesions of the cerebral hemisphere and spinal cord white/grey matter, mononuclear cell infiltrates in the

spinal cord, progressive spinal cord atrophy and axonal loss were observed in DA-induced susceptible mice 30 to 40 days after inoculation. The inflammatory areas were concentrated in the regions of the diencephalon, the hippocampus, the basal ganglia, the anterior horn of the spinal cord and leptomeninges with sparing of the white matter during the early acute phase of the disease. The monocytes and T lymphocytes infiltrated small and medium-sized parenchymal blood vessels forming perivascular cuffings and foci of ischemic-type coagulative necrosis. During the late chronic stage, myelin loss, myelin-laden phagocytic macrophages, axonal swellings, perivascular and leptomeningeal infiltrates were detected within the demyelinating lesions, which involved the spinal cord anterior, lateral, and dorsal columns with preferential susceptibility of the thoracic segments.

1.7 Cell Transplantation: A Potential Therapeutic Approach to Treating Multiple Sclerosis

Remyelination by transplanted myelin-forming cells has been explored extensively in animal experiments as a possible approach for achieving repair in demyelinating diseases. There are several different cell types for such transplantation, including oligodendrocytes and their progenitors, Schwann cells, OECs, bone marrow cells, neuronal stem cells, tanycytes and enteric glia. Franklin and Barnett (2000) reviewed the properties of the ideal cell for transplantation including an ability to remyelinate CNS axons, availability of cell source, migratory and survival capabilities, compatibilities with astrocytes, and potential vulnerability of the myelin produced by the cell candidate.

Oligodendrocytes are normally responsible for CNS myelination and are the cells that are typically targeted by the different approaches being used to induce demyelination in animal models. Therefore, the ability of oligodendrocytes in these animal models to sufficiently respond to demyelination is severely compromised. This leaves oligodendrocyte progenitor cells and stem cells as the main glial contributors of the CNS to the remyelination of the axons. There is a wealth of experimental evidence demonstrating the production of new myelin in animals after transplantation of oligodendrocyte lineage cells (Barnett *et al*., 1993; Duncan *et al.,* 1992; Franklin and Blakemore, 1997; Groves *et al.*, 1993; Warrington *et al*., 1993). Human oligodendrocyte progenitors have also been shown to myelinate congenitally dysmyelinated axons in the mouse brain (Windrem *et al*., 2004). Remyelination by transplanted cells was also

accompanied by both improved conduction (Utzschneider *et al*., 1994) and functional recovery (Jeffery *et al*., 1999). Therefore, oligodendrocyte progenitors would be the most suitable for cell transplantation in MS. However, the isolation of oligodendrocytes from neural tissue is a complex process, which not only yields low cell numbers but also lacks broad ethical approval. Although Penderis *et al*. (2003) found no depletion of oligodendrocyte progenitors after repeated episodes of focal EtBr-induced demyelination in the rat caudal cerebellar peduncle, several authors have suggested that repeated demyelination in MS results in the depletion of oligodendrocyte progenitors (Niehaus *el al*., 2000; Wolswijk, 2002). Furthermore, the ongoing immunological process directed against myelin and oligodendrocytes might attack the transplanted cells.

Schwann cells normally myelinate only PNS axons; however, it has been known for many years that in some conditions these cells can and will remyelinate axons within the confines of the CNS (Blakemore and Crang, 1985, 1989; Blakemore *et al.*, 1995). In addition to experimental data, Schwann cells contribute to the spontaneous remyelination in MS (Ghatak *et al*., 1973; Itoyama *et al*., 1983, 1985). Although Schwann cells myelinate only a single axon in contrast to oligodendrocytes, it has been shown that transplantation of Schwann cells into demyelinated spinal cord can restore normal conduction properties of CNS axons (Honmou *et al*., 1996). In order for these cells to contribute to remyelination, unlike mature oligodendrocytes, the Schwann cells must migrate from the PNS towards the demyelinated area or be grafted into the lesion. The Schwann cell migration towards the demyelinated area was observed only when astrocytes were absent from a lysolecithin-induced lesion in irradiated rat spinal cord (Harrison, 1985). Similar to what is found in animal models, Schwann cells can sometimes be identified inside the astrocyte-poor central area of MS plaques (Itoyama *et al.*, 1983). Unlike oligodendrocytes, human Schwann cells are readily accessible from the sciatic nerve, where peripheral myelin is intact in MS patients. Schwann cells can be expanded *in vitro* (Rutkowski *et al*., 1995; Van den Berg *et al*., 1995) and used after cryopreservation (Kohama *et al*., 2001). Therefore autologous Schwann cells could be harvested from an MS patient, expanded under appropriate conditions *in vitro* and injected directly into demyelinated lesions. The advantages of using Schwann cells are the accessibility of these myelinating cells, the possibility of autologous transplantation without the need of subsequent immunosupression, and the potential for avoidance of immune attacks against CNS myelin in MS. However,

Schwann cells are not a normal cellular component of the undamaged CNS. These cells migrate into areas of the damaged CNS, where they remyelinate axons only in astrocyte depleted areas of the CNS (Blakemore, 1975). Another potential problem for the use of Schwann cells is the inhibitory effect of astrocytes on Schwann cell remyelination of CNS axons (Franklin and Blakemore, 1993; Harrison, 1985; Woodruff and Franklin, 1999). In addition, MS plaques are disseminated throughout the CNS neuropil, thus Schwann cells would be required to migrate to many lesions. These cells have shown limited migratory ability when injected into an animal's spinal cord in the presence of a toxin-induced demyelinated lesion (see Section 1.8.2).

Bone marrow cells (BMCs) have recently also been considered as a potential candidate for transplantation as part of a therapeutic approach in the treatment of demyelinating diseases. BMCs are known to be a source of circulating erythrocytes, platelets, monocytes, granulocytes, and lymphocytes (Phinney *et al*., 1999). BMCs can be obtained from an individual's bone marrow and, therefore, they have been under the scope of researchers as a potential candidate for autologous cell therapies. Several studies have used BMCs isolated from rat femoral bones for transplantation into the demyelinated (x-irradiation/EtBr) spinal cord of adult rats (Akiyama *et al*., 2002a; Sasaki *et al*., 2001). Sasaki *et al*. (2001) observed predominantly a peripheral pattern of myelination, reminiscent of that of Schwann cells, after BMC transplantation, whereas Akiyama *et al*. (2002a) reported both peripheral and central types of remyelination of CNS axons, with a concomitant improvement in conduction velocity. Although x-irradiation/EtBr-induced demyelination has been reported to lead to delayed endogenous remyelination (Blakemore and Crang, 1985; Honmou *et al*., 1996), there is a possibility that host Schwann cells were recruited from outside the lesion area as a result of performing the injection procedure of BMCs. Thus, host Schwann cells could have produced the peripheral type of remyelination seen in rats that received BMC transplant. This interpretation would be consistent with BMCs secreting various growth factors that could have created an environment suitable for Schwann cell migration and remyelination. Admittedly, BMCs have been also reported to differentiate into non-hematopoietic and non-lymphopoietic cells (Caplan, 1991; Owen, 1988; Prockop, 1997) and recently also to produce neurons and astrocytes when grown in cell culture (Woodbury et al., 2000). *In vivo* experiments have shown that BMCs possess the capacity to differentiate into astrocytes when transplanted into normal (Azizi *et al*., 1998) and

ischemic (Eglitis et al., 1999) brain or lead to neuronal differentiation when injected intravenously (Brazelton *et al*., 2000; Mezey and Chandross, 2000). Therefore, the fate of transplanted BMCs requires further study to investigate further possible lineages these cells might take in their response to a new environment.

Tanycytes present in the mediobasal hypothalamus and pituicytes from the neurohypophysis were found to express a distinctive set of immunological markers in common with OECs and nonmyelinating Schwann cells, namely p75NTR, O4 antigen, estrogen receptor-α type, and IGF-1 (Gudino-Cabrera and Nieto-Sampedro, 2000). Gudino-Cabrera and Nieto-Sampedro (2000) suggested that OECs, tanycytes, and pituicytes share a common phenotype and are three members of a family of Schwann-like macroglia of the CNS. They referred to these three glial cells as aldynoglia, a term derived from a Greek word meaning "to make grow". It appears that tanycytes and pituicytes, like OECs, provide a more hospitable microenvironment for growing axons in the CNS, thus making them ideal candidates for glial cell transplantation into areas of CNS injury. Tanycytes have been shown to myelinate DRG neurites *in vitro* (Gudino-Cabrera and Nieto-Sampedro, 2000), and also promote the growth of CNS axons after grafting into the CNS of adult rats (Prieto *et al*., 2000). Although tanycytes and pituicytes have been shown to promote axonal regeneration and myelination of DRG neurites, the harvesting procedure of these cells in humans is complicated and would require a challenging neurosurgical manipulation.

Dogiel (1899) was the first to describe the glial cells of the enteric nervous system (Brehmer *et al*., 1999). Enteric glia (EG) have been shown to facilitate the regeneration of dorsal root axons through the normally non-permissive environment of the dorsal root entry zone (DREZ) into the spinal cord and as well as an incomplete functional recovery of the cutaneous trunci muscle reflex after injury (Jiang *et al*., 2003). However, EG can only be obtained by means of an abdominal surgical procedure, which can lead to unnecessary postsurgical complications.

Neuronal stem cells (NSCs) are multipotent cells, which have been identified in the developing and adult CNS (Gage *et al*., 1995; Johe *et al*., 1996; Lois and Alvarez-Buylla, 1993; Palmer *et al*., 1997; Reynolds and Weiss, 1992). Grafts of normal mouse clonal NSCs injected into the brain ventricles on newborn shiverer mice have been shown to effectively reverse the pathology and improve some symptoms of the mutant strain (Yandava *et al*., 1999). The

researchers found that most of the transplanted cells migrated through the brain and differentiated into oligodendrocytes. The injection of NSCs derived from wild type mouse either intravenously or into the cerebral ventricles of C57/BL mice with MOG-induced EAE resulted in a marked increase in the numbers of oligodendrocyte progenitors within demyelinated areas, a decreased extent of demyelination and axonal loss, and functional recovery of the mice receiving such transplants (Pluchino *et al*., 2003). Transplantation of human NSCs into demyelinated rat spinal cord resulted in axonal remyelination similar to that of Schwann cells (i.e. PNS-type myelin) and improvement of conduction velocities of the remyelinated axons (Akiyama *et al*., 2001).

NSCs can be isolated from the adult human brain only during a neurosurgical procedure, such as collection of a biopsy (Kukekov *et al*., 1999). Addition of growth factors such as EGF and basic fibroblast growth factor (bFGF) to culture medium is required to expand these cells *in vitro* (Cattaneo and McKay, 1990; Gensburger *et al*., 1987; Hammang *et al*., 1997; Kalyani *et al*., 1997; Svendsen *et al*., 1996). Mouse NSCs have been shown to differentiate into both neurons (Brustle *et al*., 1995; Stemple and Anderson, 1992; Svendsen *et al*., 1996) and oligodendrocytes (Hammang *et al*., 1997; Milward *et al*., 1997) when transplanted into the embryonic and neonatal CNS but only into astrocytes when grafted into the adult CNS (Lundberg and Bjorklund, 1996). Therefore, these cells differentiate along the different cell lineage depending on environmental signals. Fetal tissue is considered to be the best current tissue source for human neural stem cells; however, unresolved ethical issues are a major concern in future clinical applications. The other important issue is that stem cells are highly proliferative and thus also bear the risk of tumor formation (Bjorklund *et al.,* 2002). Although stem cell research has a lot of questions to be answered, the potential of such an approach should not be neglected.

OECs are the glial cells that ensheath the olfactory axons of the first cranial nerve (Doucette, 1984, 1990, 1995; Ramon-Cueto and Avila, 1998; Ramon-Cueto and Valverde, 1995). Although *in vivo* OECs express only a nonmyelinating phenotype, Devon and Doucette (1992, 1995) demonstrated their myelinating ability *in vitro,* which was independently confirmed *in vivo* by several other research groups (Franklin *et al*., 1996; Imaizumi *et al*., 1998; Li et al., 1997, 1998; Lakatos *et al*., 2003). The regenerative effort of most types of axons within the CNS fails to support more than a minimal amount of growth (Kiernan, 1979; Reier

and Houle, 1988; Fawcett, 1998), but olfactory axons are usually, in the absence of experimental intervention, successful at reinnervating the olfactory bulb (Graziadei and Monti Graziadei, 1979; Barber and Lindsay, 1982; Doucette *et al*., 1983; Doucette, 1990). It appears the growth of olfactory and non-olfactory axons is favored by a permissive OEC environment (Doucette, 1990, 1995; Ramon-Cueto and Valverde, 1995; Franklin and Barnett, 1997; Fawcett, 1998; Ramon-Cueto and Avila, 1998).

A number of studies over the past several years have demonstrated the ability of OECs to support the growth of non-olfactory CNS axons *in vivo* (Lustgarten *et al*., 1991; Ramon-Cueto and Nieto-Sampedro, 1994; Smale *et al*., 1996; Li *et al*., 1997, 1998; Perez-Bouza *et al*., 1998; Ramon-Cueto *et al*., 1998; Navarro *et al.*, 1999). OECs were shown to facilitate the regeneration of primary afferent axons after rhizotomy across the DREZ of the spinal cord (Ramon-Cueto and Nieto-Sampedro, 1994; Navaro *et al*., 1999), cholinergic fibers of the fimbria/fornix pathway across a cavity formed after aspiration of a large portion of the fiber pathway (Smale *et al*., 1996), lesioned corticospinal tract axons (Li *et al*., 1997, 1998; Nash *et al.,* 2002) and after spinal cord transection injury (Ramon-Cueto *et al*., 1998, 2000). Therefore, OECs have been receiving increased attention as possible candidates for neural repair through the grafting of myelinating glia.

Cellular interactions within demyelinating CNS lesions are complex and ultimately determine whether oligodendrocytes or Schwann cells will assume primary responsibility for remyelinating the axons. Astrocytes are one of the key players in determining which glial cell type will remyelinate axons in each demyelinated area of the CNS (Blakemore *et al*., 2003). Cells of the oligodendrocyte lineage are given such preference in the presence of astrocytes. However, it is unlikely that any one glial cell type can correct all of the problems in injured CNS tissue. A cell that can adopt several different roles is needed. OECs were demonstrated to possess this feature, being able to switch their phenotype from that resembling an astrocyte to one more like that of a myelinating Schwann cell (Devon and Doucette, 1992, 1995; Doucette, 1995). Due to their highly malleable phenotype, OECs will very likely be able to combine the roles of astrocytes and Schwann cells when transplanted into a lesion cavity (Doucette, 1995). Even if OECs may not promote axonal growth better than Schwann cells, or any other potential candidate, OECs might differ from Schwann cells in restoring the glia limitans and blood brain barrier and allowing maximal re-entry of axons into the host tissue.

Schwann cells fail to integrate into the CNS, which in part is due to astrocytic perturbation of the neuron-Schwann cell interaction and a consequent lack of ensheathment and myelination (Blakemore and Crang, 1989; Franklin *et al*., 1992; Guenard *et al*., 1994). The migratory ability of Schwann cells within the CNS parenchyma is also limited, being mostly confined to the vicinity of the graft when they are transplanted into the cerebral hemisphere even of newborn shiverer mice (Baron-Van Evercooren *et al*., 1992). Eventually, grafted Schwann cells are usually extruded from the CNS, even after remyelinating axons in the spinal cord of mice are exposed to an intracisternal injection of cholera toxin B (Jasmin *et al*., 2000). Several authors suggested that astrocytes inhibit the migration of Schwann cells within the parenchyma of the CNS (Fawcett and Asher, 1999; Franklin and Blakemore, 1993). Schwann cells and astrocytes have been shown to avoid one another when they are co-cultured in the same culture dish. Astrocytes either grown together with Schwann cells *in vitro* (Lakatos *et al*., 2000) or after transplantation of the latter cells into the adult rat spinal cord were induced to increase the expression of axon growth inhibitory chondroitin sulfate proteoglycans (Lakatos *et al*., 2003).

The repair of the CNS damage in patients with demyelinating disorders remains a major challenge for physicians and neuroscientists. Therapeutic cell transplantation has shown potential in promoting CNS repair. However, what cell or combination of different cells is the best for transplantation? The cell candidate should be of a nature that avoids the need for immunosupression and this can be achieved if the cells were obtained from the patient. Other important issues are the cell source, the approach for cell harvesting with minimal damage to the patient, the efficiency of both the harvesting technique and the tissue processing to yield a large enough number of cells. The chosen cell has to have the ability to form a myelin sheath around demyelinated axons, even in the presence of activated host cells (i.e. astrocytes and microglia) and even within an inflammatory environment. In addition, the transplanted cells will be required to migrate to multiple demyelinated areas as seen in MS and remyelinate axons at least in those located in the vitally important parts of the CNS like brainstem.

1.8 Migration of Myelinating Glia Within Central Nervous System

If glial cell transplantation is to be considered a realistic therapy for the repair of demyelinated CNS lesions in disorders such as MS, a number of important questions have to be

resolved, among which is the ability of each glial candidate to migrate following implantation into the CNS. This is a very important issue in MS since transplanted cells will be required not only to grow and myelinate within the extent of a single demyelinated area but also to migrate between at least several plaques so that a minimal number of cell injections will be needed to repair multiple lesions.

1.8.1 Oligodendrocyte Migration

The migratory abilities of oligodendrocytes and their progenitors have been studied extensively both *in vitro* (Armstrong *et al*., 1990; Milner *et al*., 1997) and *in vivo* (Fruttiger *et al*., 1999; Milner and Ffrench -Constant, 1994; Milner, 1997; Niehaus *et al*., 1999; Osterhout *et al*., 1999). The oligodendrocyte progenitors have been shown to be highly motile (Gansmuller *et al*., 1991; Small *et al*., 1987). Growth factors such as PDGF (Armstrong *et al*., 1990; Fruttiger *et al*., 1999; Osterhout *et al*., 1999) and bFGF (Milner *et al*., 1997) promote oligodendrocyte progenitor migration either *in vitro* (Armstrong *et al*., 1990; Milner *et al*., 1997) or *in vivo* (Fruttiger *et al*., 1999; Osterhout *et al*., 1999). In contrast to oligodendrocyte progenitors, more mature oligodendrocytes bear multiple processes, which probably impede the migratory capabilities of these cells. Oligodendrocyte maturation correlates with down-regulation of integrin expression (i.e. α v β 1, α v β 3, α v β 5 and α v β 8) by oligodendrocytes, receptors known to play an important role in cell migration (Milner and ffrench-Constant, 1994; Milner, 1997). Endogenous oligodendrocytes have been observed migrating from the subventricular zone toward the lysolecithin-induced lesion in the corpus callosum of adult rat (Nait-Oumesmar *et al*., 1999).

Transplantation of cells of the oligodendrocyte lineage has also shown that progenitor cells migrate further *in vivo* than do the more mature cells (Warrington *et al*., 1993; Archer *et al.*, 1997). When grafted into uninjured CNS tissue, oligodendrocyte progenitor cells migrated for up to 3-4 mm in the rostral-caudal direction from the injection site in the spinal cord of the myelin deficient newborn rat (Tontsch *et al*., 1994) and for 20 mm along the dorsal columns of Shiverer rat pups (Archer *et al*., 1997). When CG4 cells (oligodendrocyte progenitor cell line) were transplanted into the normal rat spinal cord, their migration and survival was found to be limited to areas of the injection site (Franklin *et al*., 1996). In contrast, following transplantation into the x-irradiated spinal cord of adult rats, CG4 cells migrated from the grafting site (i.e. ventral funiculi) through both white and grey matter towards the x-

irradiation/EtBr-induced demyelinated lesion in the dorsal funiculi (Franklin *et al*., 1996). Baron-Van Evercooren *et al*. (1996) observed migration of transplanted oligodendrocyte progenitors from the grafting site towards a lysolecithin-induced demyelinated lesion in adult wild type and Shiverer mouse spinal cords. Migrating cells were identified along the meninges, perivascular space, as well as the outer surface of myelin sheaths in CNS wild type and Shiverer mouse white matter. In summary, it has been shown that the more immature cells of the oligodendrocyte lineage migrate the best within the CNS are.

1.8.2 Schwann Cell Migration

Schwann cells have been reported to migrate into the CNS and myelinate CNS axons in a number of developmental and pathological studies (reviewed in Franklin and Blakemore, 1993). Blakemore (1975) observed Schwann cells migrating along perivascular spaces and reaching demyelinated axons after an injection of lysolecithin into the spinal cord of adult rats and after the consequential destruction of the glia limitans in this area of the CNS. The migratory ability of Schwann cells grafted into the cerebral hemisphere of newborn Shiverer mice is mostly confined to the vicinity of the graft (Baron-Van Evercooren *et al*., 1992). Schwann cells from an immortalized cell line (MSC80) but not Schwann cells that were obtained from the sciatic nerves of newborn rats migrated towards a focal lysolecithin-induced demyelinated lesion of the Shiverer mouse spinal cord (Baron-Van Evercooren *et al*., 1992, 1993). The migrating MSC80 cells used the ependyma, meninges, and blood vessels as the main migratory routes rather than white matter tracts. Limited migration of Schwann cells along perivascular routes and intrafascicularly within the fimbria was also noted after grafting primary Schwann cells into the hippocampus (Brook *et al*., 1993; Raisman *et al*., 1993). These Schwann cells extended long fiber-like cytoplasmic processes that extended singly amongst and parallel to the orientation of the host glial cells and the axons in the fimbria (Raisman *et al.*, 1993). Although the transplanted Schwann cells induced a transient but marked hypertrophy of host fimbrial astrocytes, the migration of the donor Schwann cells did not appear to be impeded. In other studies describing Schwann cell migration, blood vessels (and the glia limitans) were also found to be favored routes of migration (Blakemore, 1984; Blakemore *et al*., 1986; Langford and Owens, 1990). Laminin, a glycoprotein constituent of blood vessel basal lamina, has been reported to facilitate migration of Schwann cells *in vitro* (McCarthy *et al.,* 1983). Iwashita and Blakemore (2000) created areas of persistent demyelination in the

dorsal funiculus of the rat thoracolumbar spinal cord by injecting EtBr into white matter exposed to x-irradiation. Their results indicated that significant numbers of transplanted Schwann cells were not attracted to migrate through normal tissue towards the demyelinated area (Iwashita and Blakemore, 2000). Schwann cells also show poor long-term survival after transplantation into either normal or x-irradiated adult white matter (Iwashita *et al*., 2000). Fawcett and Asher (1999) suggest that astrocytes inhibit the migration of Schwann cells via the secretion of chondroitin sulphate proteoglycans.

1.8.3 Olfactory ensheathing cell migration

Several studies have reported that OECs have the ability to migrate within the microenvironment of the injured spinal cord when Hoechst prelabelled cells were grafted very close to the site of injury (Navarro *et al.,* 1999; Ramon-Cueto and Nieto-Sampedro, 1994; Ramon-Cueto *et al*., 1998, 2000). Transplanted neonatal OECs expressing β-Galactosidase (β-Gal) were found to migrate from the DREZ into the dorsal horn and adjacent dorsal funiculus (Riddell *et al*., 2004). However, other reports presented evidence for little migration of virally-infected OECs that were grafted directly into the cystic cavity of a spinal cord contusion injury (Boyd *et al*., 2004; Plant *et al*., 2003). These data may indicate that OECs migrate best within the injured CNS when grafted close to, but not within, the area of tissue injury. OECs may also prefer to stay within the lesion site when grafted directly into it and help the host tissue facilitate a more hospitable environment for axon regeneration. Takami *et al.* (2002) grafted virally infected OECs into the cystic cavity of the compression injury of the spinal cord in the adult rat but after 12 weeks found no β-Gal+ve OECs within or around the damaged area; therefore, this study raises the question as to whether the OECs actually survived being grafted into the lesion cavity. When mouse OECs derived from the lamina propria of the olfactory mucosa were grafted into the DRG of adult rats they migrated towards the DREZ of the spinal cord but did not cross into the CNS and also failed to promote the regeneration of dorsal root axons across the DREZ (Ramer *et al*., 2004).

In contrast to the limited migration of OECs observed in spinal cord injury, these cells have been reported to migrate within the large demyelinated (x-irradiation/EtBr) area induced in the dorsal funiculi of the adult rat spinal cord (Imaizumi *et al*., 1998). OECs grafted into a focal electrolytically induced lesion of the corticospinal tract at the upper cervical segment of the rat spinal cord not only infiltrated the lesion area but also extended beyond it for at least 2-3

mm in the caudal direction (Li *et al*., 1997, 1998). Interestingly, when cat OECs were transplanted into the medial longitudinal fasciculus of adult cats immediately caudal to a transection, Gudino-Cabrera *et al.* (2000) found the cells migrated retrogradely towards the abducens nucleus but failed to find any anatomical or functional evidence for the regeneration of the axonal projection from the abducens nucleus towards the oculomotor nucleus.

2. MATERIALS AND METHODS

2.1 Olfactory Ensheathing Cell Cultures

2.1.1 Dissection of embryos

Breeding colonies of adult Wistar rats were kept over one night in an animal room with a 12/12 hour light/dark cycle (Doucette, 1993). The next morning the females were checked for the presence of a vaginal plug; if present, this day was designated embryonic day 1 (E1). The pregnant rats were placed in a chamber filled with 5% CO_2 for 5 -10 min and then cervically dislocated. Using sterile techniques, the uterine horns of pregnant rats (E18) were exposed by transabdominal cesarean section, fetuses dissected from the surrounding tissue, and immersed in a room temperature bath of Hank's balanced salt solution (HBSS). To reduce developmental variation, only embryos at Theiler stage 23 (Theiler, 1972) of development were used for these experiments. Immediately after dissection, embryos were observed under a dissecting microscope in order to determine their developmental stage and separate the embryos at Theiler stage 23 from those of younger or older stages (1-2 embryos per litter). Four phenotypic features were used to identify embryos at Theiler stage 23 of development. The first feature was the presence of hair follicles over all the skin of the embryos and a second feature was the complete separation and divergence of the digits in both the forelimbs and the hind limbs. A third feature was an ear pinna that covered more than half of the external auditory meatus and the fourth feature was the presence of an umbilical hernia. The Theiler stage 23 embryos were decapitated, their heads transferred to a 1:1 mixture Dulbecco's Modified Eagle's Medium (DMEM; 4.5 g/L of glucose; Sigma-Aldrich Canada Ltd.) and F12 containing 10% fetal bovine serum (FBS; Hyclone, Logan, UT) and then split in half along the sagittal midline.

2.1.2 Disaggregation of tissue and setting up cell culture

The olfactory bulbs were dissected from embryo heads as described in Doucette (1993, 2001). Each head was transferred into a 60 mm Petri dish containing growth medium (DMEM/F12/10% FBS). Under a dissecting microscope, the heads were fixed upright with a pair of Dumont Forceps inserted into the tissue lying to either side of the nose. The skin,

subcutaneous tissue, and fibrous tissue of the cranium were cut between the presumptive cerebral hemispheres and cerebellum and then between the right and left cerebral hemispheres along the midline, beginning posteriorly and continuing as far forward as the presumptive nasal bones of the skull. The prongs of the Dumont Forceps were placed between the cerebral hemispheres and the cerebellum to fix the embryo's head while cutting it with the scalpel in the sagittal plane, thus completely separating each head into a right and left half. Using a pair of spring scissors, the olfactory nerves were cut along the ventral surface of the bulb. The exposed olfactory bulbs were dissected out of their position in the anterior cranial fossa by cutting the olfactory peduncle. The bulbs were collected in a 60 mm Petri dish, while the rest of the embryo's head was discarded. This dissection procedure for obtaining the olfactory bulbs was repeated for each fetus.

The ONL was dissected from each olfactory bulb as follows. The prongs of one pair of forceps were placed inside the bulb's cut end that previously had been attached to the olfactory peduncle. The free edge of the outer ONL was grasped with a second pair of forceps and gently peeled off the deeper layers of the bulb primordium. The ONLs were collected in a growth medium (DMEM/F12/10% FBS). This peeling procedure removed almost all of the ONL from the entire circumference of the bulb, with the exception of an occasional small tag of olfactory bulb tissue. The dissected ONLs were gently forced through a Nitex mesh (75 μm pore size) to disaggregate the cells mechanically. The resulting cell suspension was diluted to a final volume of 40 ml in DMEM/F12/10% FBS. Five milliliters of the cell suspension were plated inside each of eight 25 cm^2 Falcon flasks. For immunostaining, 2 ml of the cell suspension were placed into each of ten 35 mm Falcon dishes with glass coverslips inside and 5 ml of the remaining cell suspension were plated inside each of four 25 cm^2 Falcon flasks. All cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air.

2.1.3 Purification and expansion of cell culture

Beginning with the first feed after 3-4 days *in vitro* (DIV), twice a week (for 2 weeks) the cultures were fed with a modification of the G5 medium (mG5) of Bottenstein (1985). The mG5 medium consisted of DMEM (4.5 g/l of glucose) as the basal medium, to which insulin (5 mg/ml), transferrin (50 mg/ml), selenium (5.2 ng/ml), hydrocortisone (3.63 ng/ml), and biotin (10 ng/ml) were added. Any meningeal fibroblasts contaminating the sparse primary cell cultures did not grow very well in this serum-free condition. In comparison to the G5 medium,

the mG5 medium contained neither epidermal growth factor nor fibroblast growth factor, which were found not to be mitogenic for fetal OECs (Doucette and Devon, 1995). After 10-14 days in mG5, the medium was changed to DMEM/F12/10% FBS and the cultures were fed with this medium until they approached confluency.

2.1.4 Harvesting cells and long-term storage of cells

Cell cultures were rinsed three times with Puck's balanced salt solution (PBSS) to remove all traces of serum. The cultures were then incubated in 0.025% trypsin/0.01% ethylenediaminetetraacetic acid (EDTA)/PBSS for 5 min at room temperature. Using the phase contrast microscope to monitor the progress of the trypsinization, the flasks were lightly tapped on the countertop several times. The trypsin solution was then triturated to assist in dislodging the cells. The resulting cell suspension was centrifuged at the relative centrifugal force of 160 G (800 RPM, for 10 min) and then resuspended in DMEM/F12/10% FBS. In order to determine the number of viable cells, 400 μl of the cell suspension was stained with 0.3% Nigrosin dye. Dead or dying cells were stained purple-black by the Nigrosin dye, whereas living (i.e. viable) cells remained unstained. The number of viable (i.e. Nigrosin-excluding) cells were counted in four corners of an Improved Neubauer Levy Hemacytometer (Hausser Scientific Partnership, Horsham, PA, 19044). The number of viable cells per ml was calculated using the following formula:

$$\frac{\text{Total count}}{4} \times 10 \times 1000 \times \frac{5}{4} = \text{number of viable cells/ml.}$$

Using this count, the cell suspension was diluted in DMEM/F12/10% FBS to the desired cell concentration and 1 ml of the resulting cell suspension was placed into each of several Nalgene cryovials ($\approx 1 \times 10^6$ cells per 1 ml of medium). To each vial, 110 μl of dimethyl sulfoxide (DMSO) was added prior to sealing the cryovials and placing them into a NALGENE Cryo 1°C Freezing Container (Nalge Company, Rochester, N. Y., USA) containing isopropyl alcohol. The NALGENE Cryo Container was stored overnight in a -70°C freezer. The following morning, the cryovials were transferred to the vapor phase of liquid nitrogen in a Locator (JR Plus, Cryo Biological Storage System) for long-term storage.

2.1.5 Prelabelling cells with DiI

When the cell cultures approached confluency the cells were ready to be labelled by DiI. The cell cultures were first trypsinized, as described in Section 2.1.4, after which the resulting

cell suspension was incubated in DMEM/F12/10% FBS containing 40 μg/ml of DiI for 1 hour at 37°C in a 5% CO_2 atmosphere. At the end of the 1 hour incubation the cell suspension was centrifuged at 160 G (for 10 min) and resuspended in DMEM/F12/10% FBS. Following a second centrifugation and resuspension in DMEM/F12/10% FBS, a cell count was performed to determine the number of viable cells (see Section 2.1.4) and the DiI-prelabelled OECs were frozen in aliquots of ~$1x10^6$ cells per 1 ml, as described in Section 2.1.4.

2.2 Immunofluorescent Staining of Cell Cultures

Some OEC cultures initiated at the time of the initial dissection were plated into 35 mm dishes containing uncoated glass coverslips to assess the phenotype of the cells in the primary cultures. In addition, when some of the OEC primary cell cultures were close to being confluent (e.g. ~80%) they were trypsinized (see Section 2.1.4) and a cell suspension of OECs was plated into 35 mm dishes at a density of $5x10^4$ cells per ml to examine the immunocytochemical phenotype of the cells. All cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. The primary cultures were fed with mG5 medium for 14 days followed by DMEM/F12/10% FBS for an additional 14-21 DIV and harvested after a total of 28-35 DIV. The secondary cultures were fed with DMEM/F12/10% FBS for the entire 10-14 DIV prior to harvesting the cultures for staining.

2.2.1 Intracellular antigens

Immediately after the coverslips were removed from the dishes, the cells were fixed in either Zamboni's fix, consisting of 0.2% picric acid and 4% paraformaldehyde in 0.2 M phosphate buffer (pH 7.4), or in a mixture of 0.2% picric acid/4% paraformaldehyde/0.05% glutaraldehyde (PPG) in 0.2 M phosphate buffer solution (PBS, pH 7.4) for 15 min at room temperature. The Zamboni's fixative was used when the cell culture was to be stained with mouse anti-nestin; the PPG fixative was used when staining the cultures with the rabbit anti-S100β antibody. After fixation, the cell cultures were rinsed twice in 0.03M PBS (30 sec each at room temperature), followed by a third PBS wash for 30 min at 4°C and a fourth PBS rinse for 1 min at room temperature. The cells were then incubated for 5 min in a solution of 0.2% Triton X-100 (TX-100) in order to permeabilize the cell membrane and were then washed in PBS for 10 min. A 10 min blocking in 2% skim milk powder (SMP) was followed by a 30 min incubation at room temperature in a 1:1 mixture of the following antibodies: mouse anti-nestin (1:100) and

rabbit anti-S100β (1:200). These antibodies were diluted in 1% SMP/0.2% TX-100 in 0.03 M PBS. The control sections were incubated in diluent only.

The cell cultures were rinsed in 0.03 M PBS for 10 min. Appropriate FITC and Texas Red-conjugated secondary antisera were then applied in a 1:1 mixture on the cell cultures for a 30 min incubation at room temperature. The secondary antibodies included: swine anti-rabbit IgG FITC conjugated (1:100, Dimention Labs, Dako A/S, Denmark) and goat anti-mouse IgG Texas Red conjugated (1:100, Bio/Can Scientific, Mississauga, ON, Canada).

The cells were washed in PBS for 5 min before incubation in a Hoechst solution (diluted 1:50) for 15 min at room temperature. The cells were then given a final rinse in PBS for 5 min. The coverslips were mounted onto slides using Citifluor (Marivac Ltd, Halifax, NS, Canada) medium (a Glycerol/PBS solution). After excess Citifluor was removed from around the edges, the coverslips were sealed with clear nail polish.

2.2.2 Live method

The coverslips were removed from the culture dishes and incubated in DMEM medium containing 5% horse serum (HS) and 15 mM N-2-Hydroxyethylpiperazine-Ní-2-ethanesulfonic acid (HEPES) for 5 min at room temperature. The cells were then incubated for one hour in rabbit anti-mouse p75NGFR (1:100) diluted in DMEM/5% HS/15 mM HEPES. The control sections were incubated in diluent only.

The coverslips were washed four times (5 min each at 4^{o}C) with Dulbecco's phosphate buffered saline (DPBS). This solution contained 2.68 mM potassium chloride, 1.47 mM potassium dihydrophosphate, 0.14 M sodium chloride, 8.1 mM di-sodium hydrophosphate, 0.49 mM magnesium chloride and 1.19 mM calcium chloride dissolved in distilled water. Then the cells were fixed by immersion in Zamboni's fixative (pH 7.4) for 15 min at room temperature. The cells were washed twice in DPBS (30 sec each at room temperature), followed by a third wash in DPBS for 30 min at 4°C and a fourth DPBS rinse for 1 min at room temperature. The swine anti-rabbit FITC-conjugated secondary antiserum (1:100) was then applied to the cell cultures for a 30 min incubation at room temperature. The cell cultures were then washed with four changes of DPBS for 5 min each at 4°C before incubation in a Hoechst solution (see Section 2.2.1). The cells were then given 4 final rinses in DPBS for 5 min each, after which the coverslips were mounted onto slides using Citifluor medium (see Section 2.2.1).

2.2.3 Live method in combination with intracellular antigens

After being removed from the culture dishes, the coverslips were incubated in DMEM medium containing 5% HS and 15 mM HEPES for 5 min at room temperature. The cells were then incubated for one hour in rabbit anti-mouse p75NGFR (1:100) diluted in DMEM/5% HS/15 mM HEPES. The control sections were incubated in diluent only.

The coverslips were washed with four changes in DPBS for 5 min each at 4°C and then fixed by immersion in Zamboni's fixative (pH 7.4) for 15 min at room temperature. The cells were washed twice in DPBS (30 sec each at room temperature), followed by a third wash in DPBS for 30 min at 4°C and a fourth DPBS rinse for 1 min at room temperature. The cultures were then incubated for 15 min in a solution of 0.2% TX-100, 5% HS and 0.1% BSA in 0.03 M PBS (pH 7.4).

These cell cultures were fixed with Zamboni's fixative for immunostaining with mouse anti-nestin. Following a 15 min fixation at room temperature, the cells were washed twice in DPBS (30 sec each at room temperature), followed by a third wash in DPBS for 30 min at 4°C and a fourth DPBS rinse for 1 min at room temperature. The coverslips were incubated for 15 min in a solution of 0.2% TX-100, 5% HS and 0.1% bovine serum albumen (BSA) in 0.03 M PBS (pH 7.4). Preincubation in TX-100 was required to permeabilize the cell membranes. A 30 min incubation in the mouse anti-nestin (1:100) antibody followed immediately after the TX-100 incubation.

The cell cultures were rinsed in DPBS four times for 5 min each. Appropriate FITC and Texas Red-conjugated secondary antisera (see Section 2.2.1) were then applied to the cell cultures for a 30 min incubation at room temperature. Swine anti-rabbit IgG FITC conjugated secondary antiserum (1:100) was used in a 1:1 mixture with a goat anti-mouse IgG Texas Red conjugated secondary antiserum (1:100).

The cell cultures were washed with four changes of DPBS for 5 min each at 4°C before incubation in a Hoechst solution (see Section 2.2.1). The cells were then given 4 final rinses in DPBS for 5 min each. The coverslips were mounted onto slides using Citifluor medium (see Section 2.2.1).

2.3 Retroviral Infection

All procedures involving handling of the retroviral supernatant and cells infected with the retrovirus (RV) were performed in accordance with requirements and standards of the Biosafety Code of the University of Saskatchewan (endorsed by the Board of Governors on March 29, 2001), the Laboratory Biosafety Guidelines (Laboratory Center for Disease Control, Health Protection Branch, Health Canada, Second Edition, 1996), as well as the Policies of the Department of Health, Safety and Environment and the Biosafety Advisory Committe of the University of Saskatchewan. Use of the RV in these experiments was covered under Biosafety Permit #ANA-02. All of the *in vitro* work done with the RV was done in a Biosafety Level 2 hood in a designated Biohazard room.

2.3.1 Production and collection of retrovirus

The BAG replication-deficient RV was constructed by Price et al. (1987). They cloned the Escherichia coli Lac-Z gene, which produces an indelible and innocuous marker enzyme called β-galactosidase, into a vector (pDOL) that was derived from the Moloney murine leukemia virus (Mo-MuLV). In the pDOL vector, the wildtype Mo-MuLV LTR provides the promoter to drive stable expression of the Lac-Z gene in infected cells. Downstream from the Lac-Z gene they cloned the simian virus 40 (SV 40) early promoter and the Tn5 neo gene to provide a selectable marker for removing uninfected cells from cell cultures infected with the BAG replication-deficient RV.

The ψ (PSI) 2 BAG alpha cells, the packaging cell line for this replication-deficient RV, were purchased from the American Type Culture Collection company (Catalog # CRL 9560, Lot # 206280, ATCC, Manassas, VA, USA) and were used to produce supernatant containing a high titer of RV. The ψ 2 BAG alpha packaging system, which was developed from the NIH/3T3 cell line, provides viral envelopes to package the BAG replication-deficient RV so it can be used to infect other cells (e.g., the OECs). Once the BAG RV has infected an OEC it is unable to spread to adjacent non-infected cells due to the absence of the genetic machinery in the OEC genome that is required to make a viral envelope.

Vials of frozen ψ 2 BAG alpha cells were thawed in a 37°C water bath. The cells were transferred into a DMEM medium containing 10% bovine calf serum (BCS) and 1% antibiotic-antimycotic (Sigma-Aldrich Canada Ltd, Oakville, ON). After the cell suspension was centrifuged at 160 G for 10 min, the cell pellet was resuspended in a desired volume of

DMEM/10% BCS/1% antibiotic-antimycotic. The resulting cell suspension was plated into 25 cm^2 flasks, which were gassed with 5% CO_2 for approximately 1 min and then tightly closed with sealed caps. Cultures of ψ 2 BAG alpha cells were fed with DMEM/10% BCS/1% antibiotic-antimycotic medium twice a week. When the cell cultures became confluent, the growth medium was removed and discarded and fresh DMEM/10% BCS/1% antibiotic-antimycotic medium was added to each flask. Within 2-3 days the medium in the flasks had turned yellow reflecting the high density and metabolic activity of the cells, which in turn indicated a high titer of RV was present in the medium. This yellow-colored medium was collected from the cell cultures every 2-3 days, stored in sterile 50 ml plastic centrifuge tubes at 4°C, and filtered through a 0.45 μm low protein binding filter. Aliquots of filtered RV-containing medium were either used right away or frozen and stored at -80°C until needed.

2.3.2 Titration of retrovirus

NIH 3T3 cells were used to determine the viral titer in the ψ 2 BAG α conditioned medium. The NIH 3T3 cells were purchased from the American Type Culture Collection (ATCC Catalog # CRL-1658). Vials of frozen NIH 3T3 cells were thawed in a 37°C water bath and then the cells were transferred into a DMEM/10% BCS/1% antibiotic-antimycotic medium. After the cell suspension was centrifuged at 800 RPM for 10 min, the cell pellet was resuspended in the desired volume of DMEM/10% BCS/1% antibiotic-antimycotic medium. The resulting cell suspension was plated into 60 mm dishes at a density of 5 x 10^4 cells/ml. The day after the cells were plated, the growth medium was replaced with DMEM/10% BCS/1% antibiotic-antimycotic medium to which was added one of several different volumes of RV supernatant and 8 μg/ml polybrene; the cell cultures were placed in the incubator at 37°C for 2.5 hours. The NIH 3T3 cells were infected with logarithmic dilutions (10^{-1} through 10^{-5}) of retroviral supernatant. In order to ensure an even distribution of the virus over the surface of the cell culture, the dishes were agitated every 30 min throughout the 2.5 hour incubation period. After 2.5 hours of incubation, a sufficient volume of DMEM/10% BCS was added to each dish to dilute the concentration of polybrene in the growth medium to 2μg/ml. The infection of NIH 3T3 cells with RV containing supernatant was repeated daily for an additional two days. These NIH 3T3 cell cultures were trypsinized (see Section 2.1.4) immediately upon finishing the infection. Cell suspensions were collected separately from each dish, centrifuged and resuspended in DMEM/10% BCS. The resulting cell suspensions, derived from infections with logarithmic

dilutions of RV supernatant, were diluted 1:20 in DMEM/10%BCS containing 400 μg/ml geneticin (G 418 sulfate, Life Technologies, Gibco BRL); each cell suspension was plated into three 60 mm dishes. The cell cultures were fed with fresh DMEM/10% BCS containing 400 μg/ml geneticin twice a week for the next 10-14 days. At the end of the culture period, the growth medium was removed from the dishes and the cells were fixed in methanol for 15 min at room temperature. The fixed cells were allowed to air dry, after which they were stained with 0.45% Coomassie brilliant blue (color index = 42660) dissolved in Methanol/acetic acid for 1 min at RT and rinsed with distilled water. The average number of virus-expressing Geneticin-resistant colonies was determined using the following formula:

$$\frac{\text{number of colonies} \times \text{dilution factor}}{\text{volume of RV supernatant}} = \text{number of colony forming units/ml}$$

2.3.3 Infection of olfactory ensheathing cells

The OEC cultures were initiated as described in Section 2.1 and grown in 25 cm^2 Falcon flasks. The cell cultures were fed with mG5 medium as described in Section 2.1.3. Infection with RV began on the day the cell cultures were switched from mG5 medium to serum-containing medium. One control flask from each set of cell cultures was fed with the DMEM/F12/10% FBS medium containing no RV. One ml of the retroviral supernatant in DMEM/10% BCS medium containing 8 μg/ml polybrene was added to each 25 cm^2 Falcon flask designated for RV infection and the cell cultures were incubated for 2.5 hours at 37°C. At the end of this time 3 ml of DMEM/F12/10% FBS were added to each flask after which the flasks were returned to the incubator for 21.5 hours. Fresh RV/polybrene solution was added daily, as just described, for 2 weeks. The control flask was treated identically on a daily basis, but received no exposure to RV.

2.3.4 Geneticin selection of infected cells

After the 2 week period of daily exposure to RV containing medium, the RV-infected and uninfected cell cultures were fed twice a week with DMEM/F12/10% FBS medium containing geneticin (400 μg/ml); control (uninfected) cell cultures were fed in an identical manner. Close monitoring of control cultures determined when all uninfected cells were killed, which usually occurred within 4-5 days of being first exposed to the geneticin. The RV-infected OEC cultures were then expanded with DMEM/F12/10%FBS medium that contained no geneticin, until they

approached confluence. Prior to harvesting, 400 µg/ml of geneticin was added to the growth medium for the last 3-4 days.

2.3.5 x-Gal staining of OEC cultures

Some RV-infected OEC cultures were stained with x-Gal solution to confirm that the cells were expressing β-galactosidase. The following steps were performed in the Biohazard hood. The growth medium was aspirated from the dishes prior to rinsing them 3 times with 0.03M PBS at room temperature. The cell cultures were then fixed for 5 min at room temperature with a 0.2% glutaraldehyde solution (in 0.03M PBS) and washed 3 more times with 0.03M PBS (room temperature). Once the cells were fixed, the following steps could be carried out in the general lab. The OEC cultures were incubated with a solution containing 0.1% x-Gal (5-bromo-4-chloro-3-indolyl-β-d-galactoside; Gibco BRL, Grand Island, N.Y. 14072 USA) in 20 mM potassium ferricyanide, 20 mM potassium ferrocyanide, and 2 mM MgCl dissolved in 0.03M PBS for 4 hours at 37°C. These cultures were then rinsed 3 times with 0.03M PBS and stained for 2 min at room temperature with a solution containing 3.7 mM Nuclear Fast Red (5-chloro-2-methoxybenzenediazonium chloride hemi (zinc chloride) salt; Sigma-Aldrich, Oakville, ON) and 75 mM aluminum sulfate dissolved in distilled water. After a final wash with distilled water, the OEC cultures were dehydrated in an ascending series of ethanols (70%, 95%, 100%), cleared in xylene for 2 min and then mounted on slides using Shandon xylene substitute mountant.

2.4 Surgery

All surgical procedures were performed under sterile conditions. Animal care was provided in accordance with the requirements of the Canadian Council on Animal Care, and the experimental protocols were evaluated and approved by the Protocol Review Committee, which is a Subcommittee of the University Committee on Animal Care and Supply (University of Saskatchewan). Animals had free access to food and water throughout the period of study.

2.4.1 Anesthesia and analgesia

Thirty six adult (3-4 months old) female Wistar rats weighing 210-280 grams (U of S Laboratory Animal Service Unit) were anesthetized intraperitoneally (i.p.) with sodium pentobarbital (Somnotol; MTC Pharmaceuticals, Cambridge, Ontario, Canada) at a dose of 55 mg per kg body weight (Groups 1-4; see Table 4-1). In addition, Buprenex (Buprenorphine

hydrochloride, Reckitt & Colman Products Limited, Hull, England), an opioid analgesic, was administered intramuscularly before surgery was started at a dose of 0.05 mg per kg body weight.

An additional 21 rats were anaesthetized by inhalation of Halothane (MTC Pharmaceuticals, Ontario, Canada) (Groups 5-8; see Table 1). The induction of anesthesia was achieved by a mixture of 3% Halothane in 100% oxygen using a Boyle anaesthetic machine (Ohmeda, Mississauga, ON). After 5-10 min, the animals were immobile and unresponsive to a pinch test on their hind paws (pedal reflex). A single intramuscular injection of Buprenex (0.05 mg per kg body weight) was then administered prior to making the skin incision. The concentration of 3% Halothane was maintained during the initial parts of the surgery until it was time to begin the laminectomy. Beginning with the laminectomy, and continuing through the EtBr and cell grafting, the Halothane concentration was kept at 2-2.5%; closure of the wound was done using 1-1.5% Halothane.

During the course of the entire surgery, each animal was closely monitored (every 3-5 min) for their respiration, heart rate, selected reflexes, and the color of their paws. For all of the rats in Groups 1-8 they continued to breath at a normal rate, their heart rate seemed appropriate (as judged by palpation), pedal and tail pinch reflexes were absent, and the paws remained pink in color throughout the duration of each operation.

2.4.2 Laminectomy

An operation field was designated on the midline of the back. The rats were placed onto a reusable heating pad (Safe and Warm Incorporated, Seattle WA, USA) to maintain normal body temperature. The shaved operating field was washed with a 1:1 mixture of Betadine antiseptic solution (10% povidone-iodine topical solution UPS) and 70% ethanol. Using a #23 Almedic stainless steel scalpel blade, a 3-5 cm longitudinal incision was made through the skin and the superficial fascia along the posterior midline, immediately superficial to the spines of the lower thoracic vertebrae. In the surgical area, the muscles located on the dorsal surface of the spine were cut and moved laterally. A laminectomy was performed of the designated thoracic vertebra, after the appropriate spinous process had been removed using bone rongeurs. The dura mater was cut longitudinally within the hole created by the laminectomy.

2.4.3 Injection of ethidium bromide

A 32 Gauge Hamilton needle was connected to a 5 μl Hamilton syringe, which was then mounted onto a rodent stereotaxic apparatus using the syringe holder of a KD Scientific Syringe Pump (KDS 310). A previously prepared stock solution of 0.03% EtBr dissolved in distilled water was loaded into the 5 μl Hamilton syringe. The 32 Gauge needle was lowered so it just touched the spinal cord surface, as seen through a Nikon SMZ-1B stereoscopic dissecting microscope. After zeroing the XYZ coordinates of the stereotaxic apparatus, the needle was slowly lowered into the right dorsal funiculus to a depth of 1 mm, approximately 1 mm to the right of the dorsal spinal artery. The needle was left in place for 1 to 2 min after being inserted into the cord, prior to initiating the injection of EtBr. Using the KDS 310 syringe pump, 1μl of 0.03% EtBr was injected over 10 min using a flowrate of 0.10 μl/min. After completion of the injection, the needle was left in the spinal cord for 1-2 min prior to being slowly withdrawn. The syringe with attached needle was immediately rinsed with dH_2O, followed by a couple of rinses with acetone and then a few final rinses with dH_2O.

2.4.4 Grafting DiI-labelled olfactory ensheathing cells

Immediately before surgery, cryovials were withdrawn from the locator and thawed in a bath of lukewarm water for approximately 1 min. The cell suspension was then slowly added to medium in a Falcon centrifuge tube to dilute the DMSO, followed by centrifugation at 160 G (for 10 min) and resuspension in medium (x2). After the last wash in medium, the total number of viable cells was determined as described in Section 2.1.4. The cell viability averaged 73% (see Table 2.1).

The cell pellet was resuspended in a grafting solution containing 1 μg/ml of $CaCl_2$, 1 μg/ml of $MgCl_2$, 0.1% Glucose in 0.1M PBS; the final cell concentration was 50,000 cells per μl. This cell suspension was stored at 4°C for no longer than 1 hour prior to grafting. This cell suspension was loaded into the 5 μl Hamilton syringe, as just described for the EtBr. The injection of cells into the dorsal funiculus was done using the same flowrate setting, as previously described for EtBr (see Section 2.4.3). A 1 μl volume of cell suspension, containing 50,000 DiI-labelled OECs, was injected over a period of 10 min. After completion of the injection, the needle was left in the spinal cord for 1-2 min prior to being slowly withdrawn. The syringe with attached needle was immediately rinsed with dH_2O, followed by a couple of rinses with acetone and then a few final rinses with dH_2O.

Table 2.1 Cell counts of viable OECs before and after being frozen in liquid nitrogen

The cell culture number	Total number of viable cells frozen	Total number of viable cells when thawed	% Viability
K 427 ONL	1,152,600	703,125	61%
K 428 ONL	881,400	687,500	78%
K 429 ONL	863,000	725,000	84%
K431 ONL	1,001,600	781,250	78%
K 447 ONL	1,218,750	775,000	71%
K 462 ONL	1,127,300	721,500	64%
Mean number:		739,375	73%

2.4.5 Grafting retroviral-infected olfactory ensheathing cells

The procedure for grafting of RV-infected OECs into the dorsal funiculus of the rat spinal cord (n=1) was identical to that described for DiI-labelled OECs in Section 2.4.4.
In order to graft RV-infected OECs onto the pial surface of the rat spinal cord (n=3), the Anesthesia and Analgesia (Section 2.4.1) and Laminectomy (Section 2.4.2) parts of the surgical procedure were the same as previously described. The cell suspension was prepared for transplantation and loaded into the 5 μl Hamilton syringe similar to that described in Section 2.4.4. The 30 Gauge 90° needle was lowered so it just touched the spinal cord surface, as seen through a Nikon SMZ-1B stereoscopic dissecting microscope. The stereotaxic apparatus was zeroed, so the needle tip was inserted into the slit in the dura and touched the dorsal surface of the spinal cord under visual observation. The needle was slowly moved 1 mm to the right of the dorsal spinal artery so the right lip of slit in the dura surrounded the needle's tip without the needle being injected into the right dorsal funiculus. A 1 μl volume of cell suspension, containing 50,000 RV-infected OECs, was injected over a period of 10 min at the same flowrate setting described for EtBr (see Section 2.4.3). After completion of the grafting, the needle was left in position adjacent to the dorsal surface of the spinal cord for 1-2 min prior to being slowly withdrawn.

2.4.6 Wound closure and postoperative care

The needle was slowly withdrawn 1 to 2 min after either the cell grafting or EtBr injection had been completed. The muscles along the spine were sutured with Ethicon 4-0 surgical silk, and washed with a 3% solution of hydrogen peroxide (diluted in dH_2O) for antiseptic purposes. The skin wound was closed with 3-4 silk sutures, washed with Betadine antiseptic solution diluted 1:1 with 70% ethanol, and painted with a 2% Xylocaine local anaesthetic gel (Lidocaine Hydrochloride Jelly, USP Sterile Topical Anesthetic, Astra Pharma Inc., ON, Canada).

Each animal was monitored every 5 to 10 min during the first hour of the postoperative period for their respiration rate and for the color of their paws. The Group 1-4 rats were kept on a heating pad for 2-3 hours either until the pad had reached room temperature or until the rat had started to move around in the cage, whichever came first. Any bedding was kept away from the animal's face to avoid inhalation of small particles. The rats were observed each hour postsurgically for the next three hours and also the following morning to ensure they were

drinking water and eating food. On the first postoperative day the rats were transferred back to the animal facility room. Postoperative monitoring was done once a day until the rats were killed by an overdose of Somnotol and perfused for necropsy (see Section 2.5). During this monitoring, particular attention was directed towards the sutures and the healing of the wound. No problems were observed with the sutures on any of the rats. Postoperative wounds healed with no complications and were replaced by soft white linear scars after 2-3 weeks. By this time, the sutures were no longer observed and the new fur grew enough to cover the postoperative scar. On a daily basis, the rats were also checked for their mobility, alertness, food and water consumption, and the condition of their fur. The surgical manipulations did not cause noticeable mobility problems in any of the rats included in the Groups listed in Table 4-1.

With the exception of several animals, all rats were drinking water, eating food, and actively moving around in the cage during the postoperative period. Their activity, general appearance and behavior appeared to be normal. There were only three rats after somnotol anesthesia noticed lying in the cage for more than 30 min after surgery; these rats were barely moving but were breathing normally. These animals received 5 ml injections of saline i.p. one time each and their condition improved over the next two hours. Another three rats suddenly stopped breathing either during the last steps of the surgery or shortly after the operation, which might have been due to Halothane side effects, myocardial and respiratory depression, or the animal's individual sensitivity to Halothane. These animals were cervically dislocated and are not included in the animal numbers listed in Table 4-1. There were two additional rats whose entire body jerked when the needle was inserted into the dorsal funiculus of the spinal cord. Such a whole body reaction was likely due to the needle reaching the corticospinal tract located at the depths of the dorsal funiculus. During the postoperative period, it was noted that the right hind limb of these rats was paralyzed. Following necropsy, the lesion occupied the entire dorsal funiculus including the corticospinal tract. The tissue sections of these rats were excluded from analysis and the rats are not included in the animal numbers listed in Table 4-1.

2.4.7 Minocycline treatment

Six rats injected with EtBr at T10 and OECs at T12 (Group 6; see Table 4-1) and six additional rats that were injected with EtBr at both T10 and T12 and OECs at T12 (Group 7; see Table 4-1) received a 0.5 ml i.p. injection of either minocycline (45 mg/Kg in 0.9% saline; n=3 for each of Groups 6 and 7; Sigma-Aldrich, Oakville, ON) or vehicle (i.e. 0.9% saline; n=3 for

each of Groups 6 and 7) daily at approximately 24 hour intervals for the first 4 weeks after EtBr injection. The abdomen of all rats was examined each day prior to giving these daily injections for evidence of tenderness or other adverse reactions to either the minocycline or saline injections. To minimize the discomfort of daily injections, a new sterile needle was used for each injection. The minocycline and vehicle treated rats (Groups 6 and 7) were kept in the animal facility for an additional four weeks after the last minocycline or vehicle injection. A 4 week treatment time frame was chosen to ensure the anti-inflammatory property of minocycline was active for a sufficiently long period of time.

2.5 Necropsy of Tissue

2.5.1 Perfusion of the rats

Between 2 to 8 weeks after the last operation (i.e. Grafting DiI-labelled OECs) the rats were deeply anaesthetized with an i.p. injection of 0.35 c.c. Somnotol (~90 mg/kg body weight). The rat's chest cavity was opened transabdominally to expose the heart, after which the apex of the left ventricle was cut off with scissors. A needle, which was connected to the tubing of a Variable Flow Tubing Pump (VWP Scientific Products, VWR Canlab), was inserted into the left ventricle and pushed up into the ascending aorta. Blood exited the cardiovascular system via an additional incision in the wall of the right atrium; this incision was made immediately after the perfusion had begun. Approximately 300 ml of a 1% solution of sodium nitrite dissolved in 0.03 M PBS (pH 7.4) was passed through the vascular system to flush out the blood. The rat was then perfused with approximately 250 ml of a freshly made cold solution of 4% paraformaldehyde (PF) in 0.03 M PBS (pH 7.4) over a period of 30-40 min.

2.5.2 Dissection of spinal cord

When the perfusion was done, the skin and muscles were cut longitudinally along the posterior midline from the skull to the tail base. The exposed spinous processes and vertebral laminae were removed using bone rongeurs through the whole length of the spinal column. The spinal cord with its short rootlets were gently dissected out, immersed in fresh fixative (4% PF) and stored overnight at 4°C. After approximately 18-20 hours fixation, the spinal cords were immersed in a 30% solution of sucrose dissolved in 0.03 M PBS (pH 7.4) for 2-3 days at 4°C. The spinal cords were then ready for sectioning on a sliding microtome.

2.5.3 Cutting and storage of tissue sections

The rat thoracic spinal cord segments (T8-T12) were identified by counting the short rootlets left after dissection, beginning in the cervical area and proceeding caudally. These segments were individually separated using a scalpel blade. The specimen holder of a sliding microtome (American Optical Company, Scientific Instrument Division, Buffalo, New York, USA) was frozen in dry ice prior to application of Tissue-Tek O.C.T. 4583 compound (Sacura Finetek U.S.A., Inc., Torrance, CA 90504 USA) onto its surface. One spinal cord segment at a time was placed onto the prepared specimen holder, covered with O.C.T. medium, and frozen by means of dry ice. The sliding microtome was used to cut coronal sections of 40 μm thickness. Serial coronal sections were collected and placed in a 96 well storage plate filled with cryoprotectant (a solution of 0.11 M sodium phosphate monobasic and 0.38 M sodium phosphate dibasic in distilled water, containing 3% Glycerin and 3% Ethylene Glycol). Free floating sections were stored in this cryoprotectant at -20°C until they were mounted onto subbed slides for cell counts or for immunohistochemical staining.

2.5.4 Immunohistochemical staining

The 40 μm thick sections of spinal cord were removed from cryoprotectant and washed three times in 0.03 M PBS (30 sec each at room temperature), followed by a fourth PBS rinse for 30 min at 4°C. The sections were then incubated for 30 min in a solution of 3% SMP and 0.1% TX-100 in 0.03 M PBS (pH 7.4); preincubation in SMP/TX-100 was required to block non-specific binding sites and to permeabilize the cell membranes.

Incubation in primary antibodies was done overnight at 4°C. The following antibodies were used: rabbit anti-mouse p75NGFR (1:9000, Chemicon International Inc., Temecula, CA 92590, USA), rabbit anti-cow GFAP (1:11000, Dimension Labs, Dako A/S Denmark), mouse anti-rat MHC Class I (Clone OX-18, 1:9600, Secotec Product Datasheet, Kidlington, Oxford, England), mouse anti-CD11b (Clone MRC OX-42, 1:3200, Serotec Product Datasheet, Kidlington, Oxford, England), and mouse anti-Vimentin (Clone V9, 1:1000, Sigma Immuno Chemicals, St. Louis, MO 63178, USA). The primary antibodies were diluted in 0.03 M PBS. The control sections were incubated in diluent only.

The following day, the sections were washed with two changes of 0.03 M PBS and then blocked again in a solution of 3% SMP/0.1% TX-100 in 0.03M PBS for 30 min. Appropriate biotinylated secondary antisera were then applied to the sections for a 30 min incubation at room

temperature. The biotinylated secondary antibodies included: goat anti-rabbit IgG (1:200, Vectors Labs, Burlingame, CA 94010 USA) and horse anti-mouse IgG (1:150, Vectors Labs, Burlingame, CA 94010 USA).

The sections were then incubated for 15 min in a 1% solution of hydrogen peroxide (H_2O_2) in 30% methanol to quench the enzymatic activity of endogenous peroxidase. An additional wash in two changes of 0.03 M PBS (pH 7.4) was followed by incubation in a 3% solution of SMP in 0.03 M PBS (pH 7.4) (30 min). The Vectastain Elite ABC kit (Vector Laboratories, Inc., Burlingame, CA 94010 USA) was used to make the Avidin DH (reagent A)/ biotinylated horseradish peroxidase H (reagent B) cocktail; 8µl of each reagent per ml were diluted in 0.03 M PBS (pH 7.4) 30 min prior to being used. The sections were incubated in this solution for one hour at room temperature.

After two washes in 0.03 M PBS (pH 7.4), the sections were rinsed in 0.175 M sodium acetate (pH 6.8) for 10 min. A solution of 0.0185 M diaminobenzidine tetrahydrochloride (DAB) and 0.1586 M nickel sulfate ($NiSO_4$) in 0.175 M sodium acetate (pH 6.8) was made one hour prior to use and kept in the dark. Immediately after adding 85 µl of a 30% solution of hydrogen peroxide to each 100ml of DAB/$NiSO_4$ solution, the sections were immersed in DAB/$NiSO_4$/H_2O_2 for 5 min in the dark.

The sections were then rinsed once (for 10 min) in 0.175 M sodium acetate (pH 6.8), given a final rinse in 0.03 M PBS (pH 7.4) for 10 min, and mounted onto subbed (1% gelatine, 0.1% chrome alum) slides. The sections were left to air dry for approximately 18-20 hours, after which they were dehydrated in an ascending series of ethanols (70%, 95%, 100%). The sections were cleared in xylene for 2 min and then coverslipped using Shandon xylene substitute mountant (Shandon, Pittsburgh, PA 15275, USA).

2.5.5 Data collection and statistical analysis

Four sections were randomly chosen from each of the caudal, middle, and rostral portions of each spinal cord segment (i.e T8-T12), mounted onto a microscope slide, and coverslipped using Citifluor antiquenching mounting medium. All tissue sections to be used for cell counting were stained with Hoescht dye (1:50 dilution) prior to mounting to ensure that the only DiI+ve profiles counted were those associated with a nucleus. Each tissue section was examined to determine whether it contained any DiI+ve cells, to identify the specific location in which the cells were found, and to count the total number

of labelled cells within the dorsal funiculus. For statistical analysis the mean cell count for each group of 4 sections was computed. The initial data analysis showed no differences between the data collected from the rostral, middle and caudal portions of each spinal cord segment for each Group. Therefore, these data were combined and plotted as one bar for each spinal cord segment and for the statistical analysis of the data. The data collected from Groups 1-5 (see Table 4.1) were statistically compared using a one-way analysis of variance (Prism program). The independent variable was the presence and location of one or two EtBr-induced demyelinated lesions (i.e. the experimental groups). The data on cell counts obtained from each spinal cord segment (i.e. T8-T11) and for each survival time (2, 4 or 8 weeks) were compared separately for statistical significance. The significance level was set at $p < 0.05$. Multiple post hoc comparisons between the different Groups (see Table 4.1) were performed with Bonferonni's multiple comparison post-test (Prism program) with the significance level being set at $p < 0.05$. The data collected from Groups 6 and 7 (see Table 4.1) were statistically compared using the Mann-Whitney U test (Prism program) and a significance level set at $p < 0.05$.

2.5.6 Luxol fast blue staining of tissue sections

The 40 μm thick spinal cord sections were removed from cryoprotectant and washed in 0.03M PBS for 10-20 min. The sections were then mounted onto gelatine-chrome alum subbed coverslips and were left to air dry for approximately two hours. Tissue sections were washed in 0.03M PBS for 10 min and then in distilled water for 10 min. The sections were dehydrated in 70% and 95% ethanol for 2 min each. Tissue sections were then incubated in a solution containing 1.05% Luxol Fast Blue (sulfonated copper phthalcyanine; Sigma-Aldrich, Oakville, ON), 90% ethanol and 10% acetic acid for 16 hours at 37°C. After washing in distilled water twice, the sections were incubated in a 0.05% lithium carbonate solution in distilled water for 20 sec, followed by 70% ethanol and another rinse in distilled water. This was then followed by quick rinses in 70% ethanol and then 90% ethanol, followed by two changes of isopropyl alcohol for 3 and 2 min, respectively. The tissue sections were quickly rinsed in a 1:1 mixture of xylene and absolute alcohol. The sections were cleared with three changes in xylene and then mounted on slides using Shandon xylene substitute mountant.

2.5.7 x-Gal staining of tissue sections

The 40 μm thick sections of the brain, olfactory bulbs, cerebellum, brain stem, cervical and thoracic spinal cord of Group 8 rats were stained with x-Gal solution to determine the β-galactosidase expression in OECs after either intraspinal implantation or pial surface grafting. Tissue sections were removed from cryoprotectant and washed in 0.03M PBS for 10-20 min. The sections were then mounted onto gelatine-chrome alum subbed coverslips and were left to air dry for approximately two hours. Tissue sections were incubated in a 0.1% x-Gal solution for 48 hours at 37°C. The sections were rinsed 3 times with 0.03M PBS and then stained for 2 min at room temperature with a 0.1% Nuclear Fast Red solution in distilled water also containing 5% ammonium sulfate. After a final wash with distilled water, tissue sections were dehydrated in an ascending series of ethanols (70%, 95%, 100%). The sections were cleared in xylene for 2 min and then mounted on slides using Shandon xylene substitute mountant.

3. SPECIFIC AIMS

Multifocal demyelinated lesions in white matter are the main characteristic pathologic features of MS, which is the most common CNS demyelinating disease in humans. Although the pathology of MS is believed to be related to immunological phenomena induced by viral infection or exposure to unknown allergens (Agranoff and Goldberg, 1974; Bach, 2002; Challoner *et al*, 1995; Friedman *et al,* 1999; Gilden, 1999; Kazmierski et al., 2004; Kurtzke, 1980; Lucchinetti *et al.,* 2000), the mechanisms responsible for the extensive CNS demyelination and controlling whether remyelination occurs remain to be elucidated. In order to enhance remyelination in the adult mammalian CNS, several approaches involving the transplantation of myelinating glia have been considered (Barnett et al., 1993; Blakemore and Crang, 1985, 1989; Blakemore *et al*, 1995; Duncan *et al.,* 1992; Franklin and Blakemore, 1997; Groves *et al.*, 1993; Warrington et al., 1993). The cells of choice have been oligodendrocytes, Schwann cells and OECs (see Section 1.7). Although *in vivo* OECs provide ensheathment for the unmyelinated axons of the first cranial nerve, these glial cells can be induced to express a myelinating phenotype both *in vitro* (Devon and Doucette, 1992, 1995) and after grafting into demyelinated areas of the adult mammalian spinal cord (Franklin et al., 1996; Imaizumi et al., 1998; Li et al., 1997, 1998; Lakatos *et al*., 2003). It was the neurite-promoting and remyelinating abilities of the OECs that first attracted investigators to graft these cells into damaged CNS tissue (Lustgarten *et al*., 1991; Ramon-Cueto and Nieto-Sampedro, 1994; Smale *et al*., 1996; Li *et al*., 1997, 1998; Perez-Bouza *et al*., 1998; Ramon-Cueto *et al*., 1998; Navarro *et al.*, 1999).

The demyelinated lesions in MS are scattered throughout the white matter of the CNS, making it impractical to graft myelin-forming cells into each demyelinated area as part of a therapeutic approach to remyelinating the axons. A more sensible approach would entail grafting the cells into a smaller number of sites and having the glial cells migrate towards each of several demyelinated areas. The purpose of my Ph.D. research project was to examine the

migratory ability of one type of myelin-forming cell, namely the OECs, after they were grafted into the spinal cord of adult rats and to identify the conditions under which they would be induced to migrate towards a focal area of demyelination. For these experiments the ensheathing cells were prelabelled with a fluorescent dye (i.e. DiI) prior to grafting so the cells could be identified for cell counting after they had mingled with host cells in spinal cord segments rostral to the graft. The specific aims of my research project were:

Specific Aim # 1	To determine whether OECs will be induced to migrate towards a focal area of demyelination in the spinal cord of adult rats when the cells are grafted into normal CNS white matter.
Specific Aim # 2	To determine whether the grafted OECs migrate away from a demyelinated area of spinal cord into normal CNS tissue.
Specific Aim # 3	To determine whether the grafted OECs migrate away from a demyelinated area of spinal cord towards a second demyelinated area.
Specific Aim # 4	To determine whether microglial reactivity contributes to the migratory signal inducing OECs to migrate towards a focal demyelination of CNS white matter.
Specific Aim # 5	To determine whether OECs grafted onto the pial surface of the spinal cord will migrate into the neuropil in the absence of demyelination.

4. RESULTS

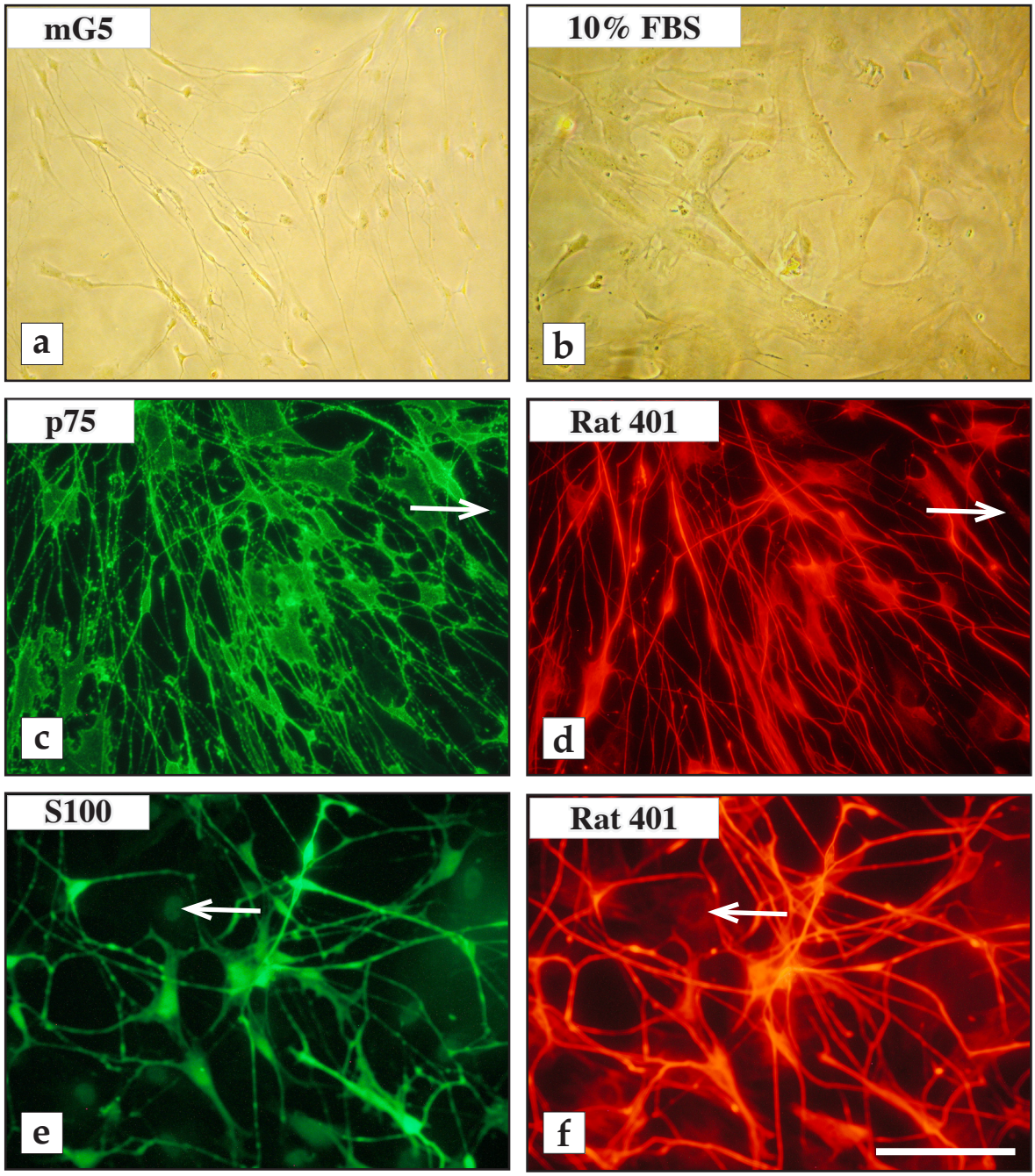
4.1 Olfactory Ensheathing Cell Cultures

At 3 DIV sparsely distributed flat and fusiform cells, either singly or in groups of a few cells, comprised the cell cultures initiated from the tissue obtained from the ONL of the E18 rat olfactory bulb. Over the next 10 DIV, as the cell cultures were fed twice a week with the mG5 serum-free medium, the morphology of the cells comprising the culture changed to predominantly a fusiform, bipolar or tripolar morphology. The bipolar and tripolar cells were small phase bright cells, whereas the somata of the fusiform cells were flatter (Fig. 4.1a). A very small number of large, flat cells, which were most likely meningeal fibroblasts, were present in the cell cultures when the growth medium was first changed from one containing serum to the mG5 medium; the mG5 medium supported an increase in the number of fusiform, bipolar and tripolar cells at the expense of the larger, flatter cells, which were usually almost completely absent from the cell cultures after 2 weeks (i.e. after ~10 days in mG5). The growth medium for the cell cultures was then switched back to DMEM/F12/10% FBS, which was used to expand the cell cultures over the next 20 to 30 DIV until they approached confluency. In the serum-containing medium the cells acquired a flatter multipolar and fusiform morphology (Fig. 4.1b).

To assess the purity of the cell cultures they were double immunostained with a monoclonal antibody to nestin in combination with either polyclonal antisera to p75 or to S100. Almost all of the cells in these cultures were both S100+ve (Fig. 4.1e) and nestin+ve (Fig. 4.1f). Likewise, the majority of the cells in these cultures co-expressed the p75 NGF receptor on their cell surface, including the somata as well as the cytoplasmic processes (Fig. 4.1c), and the intermediate filament protein nestin (Fig. 4.1d). There were only a small number of cells not expressing any of these markers (indicated by arrows in Fig. 4.1c-f).

FIGURE 4.1: Morphology and Phenotype of OECs *in vitro*.

Phase contrast micrographs showing OECs in serum-free medium (a) and in serum containing medium (b). Double immunostaining of OECs showing their co-expression of p75NGFR (c) and nestin (d), and of S-100 (e) and nestin (f). Cells that expressed none of these markers (the white arrows) were rare in any of the cell cultures used for this thesis. Bar = 100 μm


mG5
a
10% FBS
b
p75
c
Rat 401
d
S100
e
Rat 401
f

4.2 The Animal Model For Studying OEC Migration

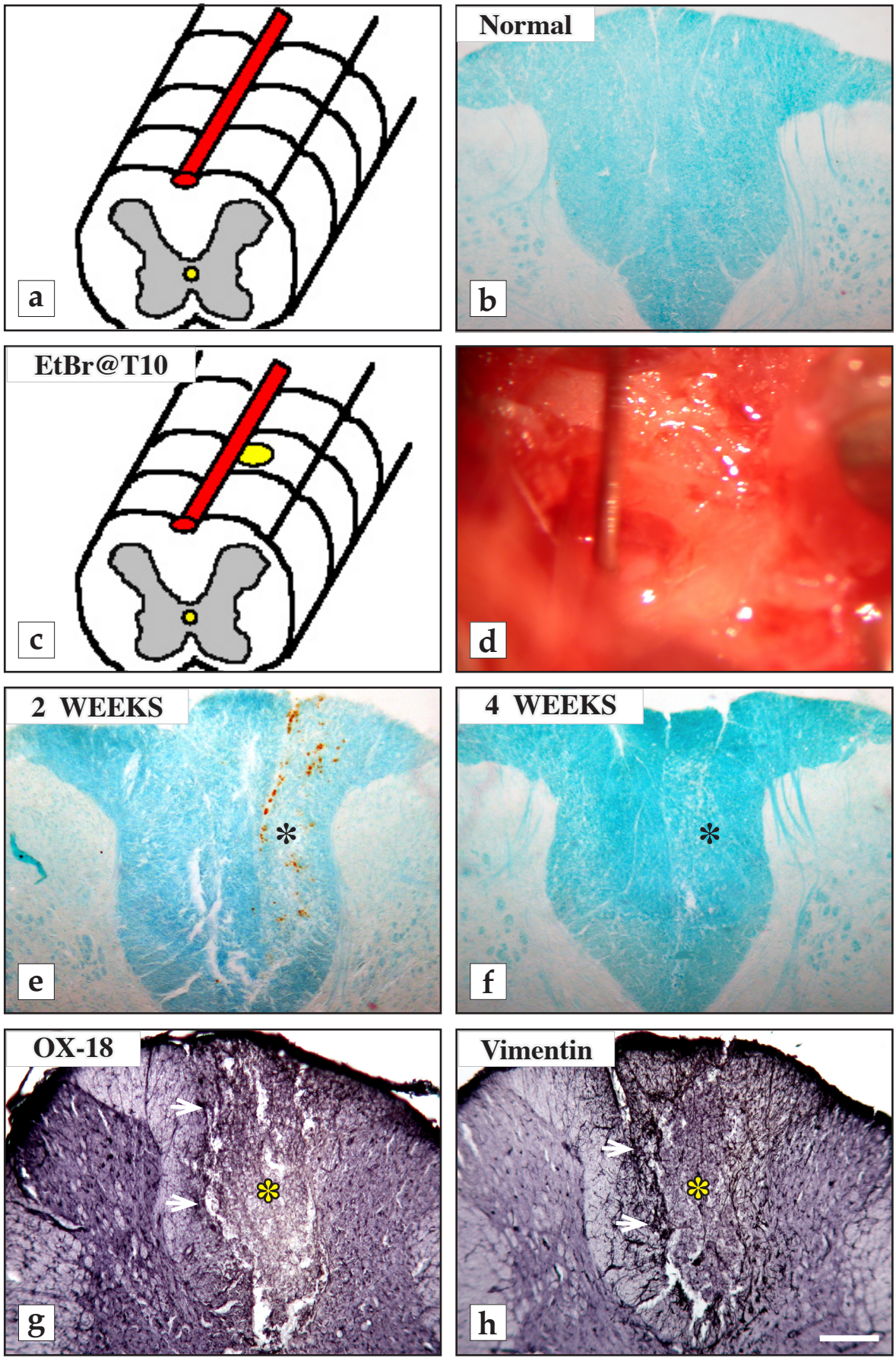
4.2.1 The Ethidium Bromide Lesion

The 0.03% EtBr solution used in this research project created a demyelinated lesion that remained confined to the right dorsal funiculus of the spinal cord with no detectable involvement of the contralateral funiculus. Practically the entire right dorsal funiculus was demyelinated at the epicenter of the EtBr injection, with the rostral-caudal extent of the damage extending for approximately 1 mm; this length corresponded to approximately 20% of the length of a spinal cord segment. Previous studies have used x-irradiation to impede host glial cells in their repair of demyelinated area of white matter (see Section 1.6.1) but in the model used here the cell migration of OECs was allowed to occur in conjunction with the host glial cells being allowed to contribute to the neural repair. With this concentration of EtBr, demyelination was still evident at 2 weeks after injury (Fig. 4.2e) but by 8 weeks this region of white matter had been almost entirely remyelinated (Fig. 4.2f).

The monoclonal antibody OX42 recognizes the CR3 complement receptor on the surface of resting and phagocytic microglia while the monoclonal antibody OX18 recognizes the rat class 1 major histocompatibility complex antigen on the surface of phagocytic microglia. Both of these antibodies were used in this research project to reveal the extent of the microglial reactivity to the cell graft. The spinal cord tissue was also stained with a monoclonal antibody to vimentin because astrocytes in the adult mammalian CNS do not normally express detectable levels of vimentin, unless they are induced to do so in response to brain or spinal cord injury. At 2 weeks after injury in a cross-section through the segment of injury, OX-18+ve reactive microglia (Fig. 4.2g) and vimentin+ve reactive astrocytes (Fig. 4.2h) were confined primarily to the demyelinated lesion within the right dorsal funiculus, with only an occasional reactive glial cell being found in the left dorsal funiculus or in the medial part of the right dorsal horn. Since this 0.03% EtBr concentration created an acceptably small, demyelinated lesion that was confined to one-half of the dorsal funiculus, this concentration was used in all subsequent experiments.

FIGURE 4.2: A small, focal EtBr-induced demyelinated lesion.

A schematic diagram (a) and a Luxol Fast Blue stained coronal section (b) of the normal adult rat spinal cord. c) A schematic diagram of an EtBr-induced demyelinated lesion at T10 (yellow circle). d) A micrograph showing the needle for injecting EtBr inserted into the right dorsal funiculus at T10. Luxol Fast Blue stained coronal sections of the T10 spinal cord segment two (e) and eight (f) weeks after injecting EtBr into this white matter. The OX-18+ve microglial (g) and vimentin+ve astrocytic (h) reactivity at the lesion site at two weeks after EtBr injection. Asterisks in c-h) denote the location of the EtBr-induced lesion. The white arrows indicate the astrocytic (g) and microglial (h) reactivity along the medial border of the EtBr-induced lesion. Bar = 400 μm applies to all figures except a) and c).


a
Normal
b
EtBr@T10
c
d
2 WEEKS
e
4 WEEKS
f
OX-18
g
Vimentin
h

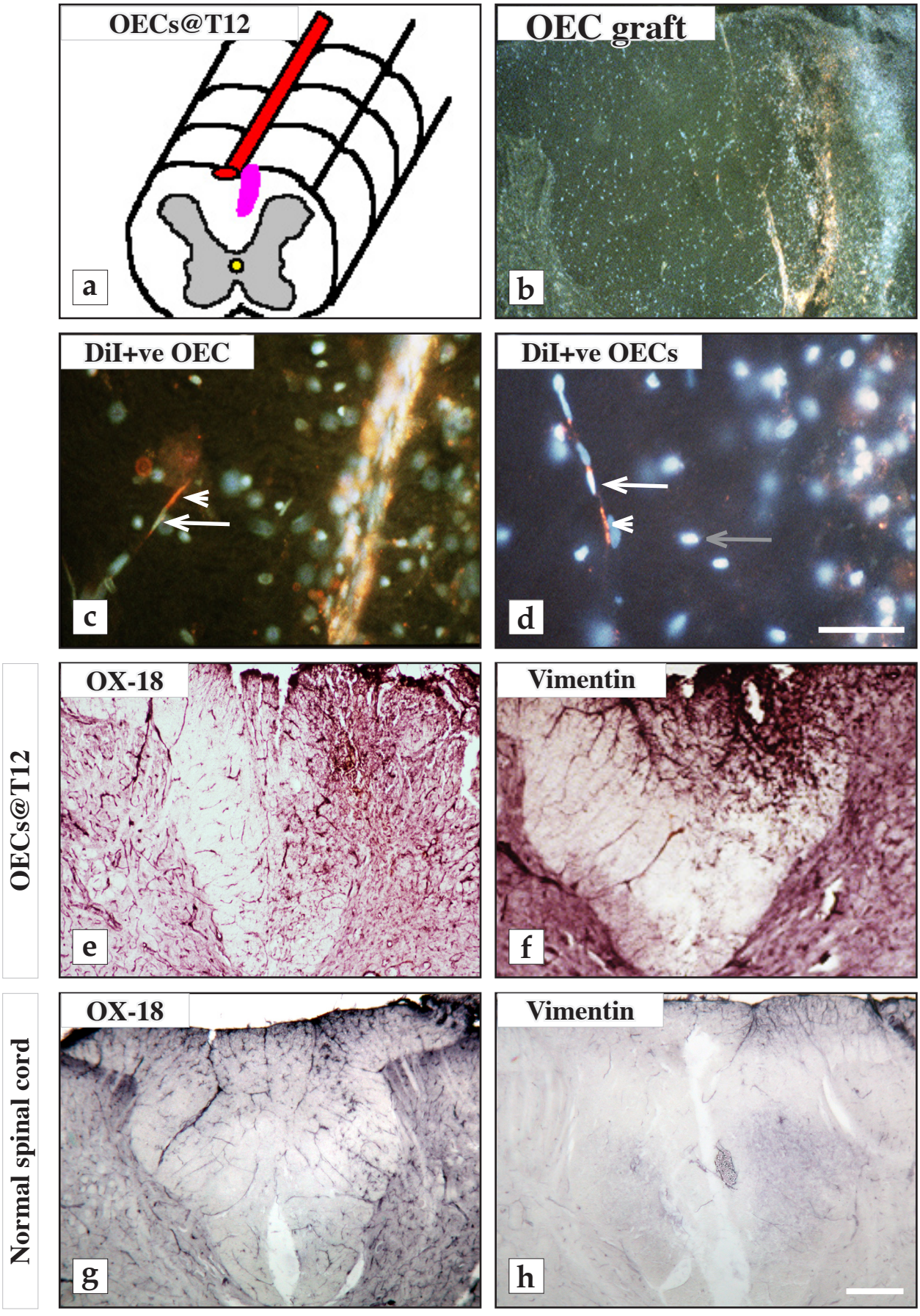
4.2.2 The Cell Graft

One µl of cell suspension containing 50,000 DiI-labelled OECs was stereotactically injected into the right dorsal funiculus of the T12 spinal cord segment (Fig. 4.3a & b). The needle was inserted immediately lateral to the dorsal spinal artery and was lowered into the right dorsal funiculus to a depth of 1 mm. Macroscopically, the grafting site could be identified on the pial surface of the cord by a pink discoloration of approximately 1-1.5 mm in diameter that covered the surface of the right dorsal funiculus of the T12 spinal cord segment. Once the T12 spinal cord segment was cut in the coronal plane into 40 µm thick sections, a similar pink discoloration was also seen to be present throughout much of the depth of the right dorsal funiculus. When these coronal sections were examined with a dissecting microscope (~ 10x magnification) twenty to twenty-five of the sections (i.e. a length of 800-1000 µm) through the midportion of T12 were seen to contain this pink discoloration of the right dorsal funiculus. The remainder of the coronal sections cut from the T12 spinal cord segment, both rostrally and caudally to the grafting site, did not contain visible discoloration of the dorsal funiculi. Microscopically, a compact mass of DiI+ve OECs could be seen with the fluorescent microscope; this DiI+ve OEC cell mass was seen within the right dorsal funiculus of each coronal section through the midportion of the graft (Fig. 4.3b). In addition to this compact mass, single as well as small groups of DiI+ve OECs were found within both dorsal funiculi and both dorsal horns of T12 (Fig. 4.3c).

Two weeks after grafting DiI+ve OECs into the right dorsal funiculus at the midpoint of the T12 spinal cord segment, OX-18+ve reactive microglia (Fig. 4.3e) and vimentin+ve reactive astrocytes (Fig. 4.3f) were detected within and around the location of the cell graft. These reactive microglia and astrocytes were localized predominantly within the right dorsal funiculus between the midline septum and the right dorsal horn and extending from the pial surface down to about the level of the corticospinal tract. No OX-18+ve microglial cells were seen in the dorsal funiculus of the T12 spinal cord segment in control animals, which received neither EtBr injection nor OEC graft

FIGURE 4.3:DiI+ve OECs grafted into the dorsal funiculus of T12.

a) A schematic diagram representing a graft of OECs (purple oval) in the dorsal funiculus of the T12 spinal cord segment. b-d) DiI+ve OECs in the right dorsal funiculus of T12 showing the graft at low power (b) and single cells at high power (c, d). For quantitation of cell migration, these sections were stained with Hoechst dye and each DiI-labelled OEC with a red fluorescent cell body and cytoplasmic processes (white arrowhead) and a blue Hoechst-stained nucleus (white arrow) was counted. Most of the cells with blue Hoechst-stained nuclei (grey arrow) were DiI-ve and were considered to be host cells. OX-18+ve reactive microglia (e) and vimentin+ve reactive astrocytes (f) at the site of an OEC graft at the level of T12 two weeks after grafting. OX-18 (g) and vimentin (h) immunostaining of the T12 spinal cord segment of an adult rat that received no cell graft or EtBr injection. Bar in 'd' = 100 μm and applies to figures c) and d). Bar in 'h' = 100 μm and applies to figures b) and e-h).


OECs@T12
a
OEC graft
b
DiI+ve OEC
c
DiI+ve OECs
d
OECs@T12
OX-18
e
Vimentin
f
Normal spinal cord
OX-18
g
Vimentin
h

(Fig. 4.3g). Vimentin staining revealed occasional activated astrocytes in the neuropil only adjacent to pia matter (Fig. 4.3h).

4.2.3 Using DiI to Identify Grafted Cells

After grafting, the DiI-prelabelled OECs were identified for counting in the tissue sections of the spinal cord by virtue of the red fluorescence of their cell bodies and cytoplasmic processes (Fig. 4.3c & 4.3d). All of the spinal cord tissue sections that were to be used for generating cell counts of DiI+ve cells were stained with Hoechst dye, which stains all of the nuclei in the sections and can be visualized using a DAPI filter which shows the blue fluorescent nuclei. For the red fluorescence to be counted as a DiI+ve cell it had to be associated with a Hoechst+ve nucleus, as illustrated by the arrows in Fig. 4.3c and 4.3d. For the purposes of the cell counts, DiI-negative/Hoechst+ve nuclei were assumed to belong to cells of the host animal. The majority of the DiI fluorescence was confined to fusiform- and spindle-shaped cells (see Fig. 4.3c & 4.3d), and these were the ones that were counted in the experiments of Sections 4.3 to 4.6. Occasionally, red fluorescent particles were found scattered throughout the dorsal funiculi of the tissue sections, being present for the most part within and around the cell graft. These fluorescent particles might have been the remains of dead or dying cells that had been grafted into the spinal cord or the phagocytosed remnants of these cells within the microglia or macrophages that responded to the insertion of the needle into the spinal cord. There was no indication of any other possible leakage of DiI into the intercellular space; for example, there was no DiI staining of the axons of DRG neurons or the corticospinal tract, both of which are located within the dorsal funiculi at the grafting site.

4.3 OEC Migration In The Absence Of Demyelination

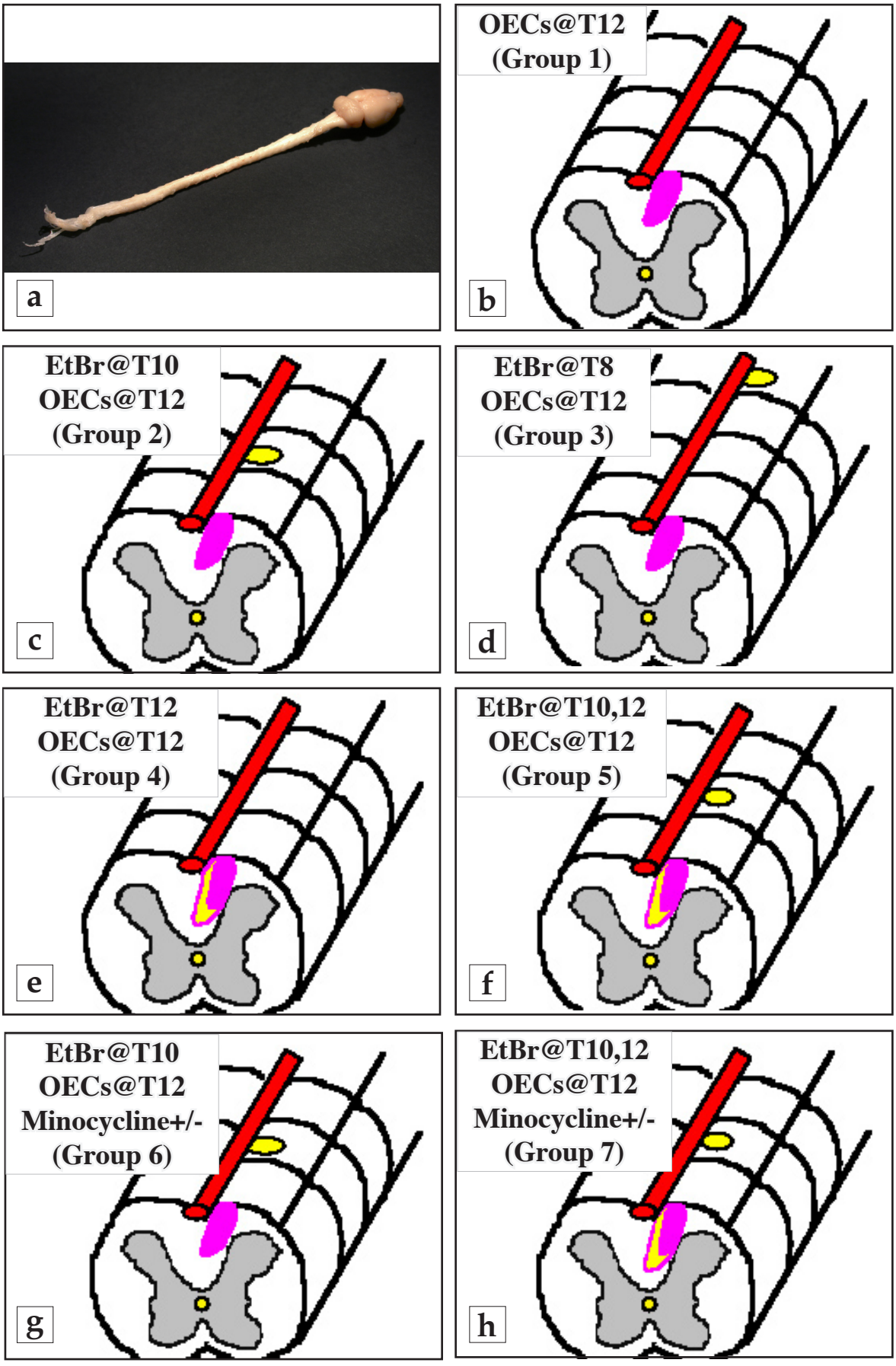
OECs were grafted into the right dorsal funiculus of the T12 segment of a control group of rats (Group 1; see Table 4.1), which received only an OEC graft (Table 4.1; Fig. 4.4a & 4.4.b). The rats were killed 2, 4, or 8 weeks after grafting, the spinal cords were sectioned in the coronal plane (40 μm thick), and the tissue sections stained with Hoechst dye to label the nuclei, as described in Section 2.5.5 and 2.5.6.

Table 4.1 The experimental groups used to address each Specific Aim

Experimental Groups:	Number of Animals Used	Thesis section where results can be found	Type of Anesthesia
Group 1 rats (OEC at T12)	9	4.3	Somnotol
Group 2 rats (EtBr at T10 – OECs at T12)	9	4.4	Somnotol
Group 3 rats (EtBr at T8 – OECs at T12)	9	4.4	Somnotol
Group 4 rats (EtBr at T12 – OECs at T12)	9	4.5	Somnotol
Group 5 rats (EtBr at T10+12 – OECs at T12)	9	4.5	Halothane
Group 6 rats (EtBr at T10 – OECs at T12) - minocycline treatment - saline treatment	 3 3	4.6	Halothane
Group 7 rats (EtBr at T10+12 – OECs at T12) - minocycline treatment - saline treatment	 3 3	4.6	Halothane
Group 8 rats RV-labelled OECs at T12 pial surface	3	4.7	Halothane

FIGURE 4.4:Schematic diagrams summarizing the experimental groups.

a) The brain and spinal cord of an adult female Wistar rat; b) A schematic diagram representing a graft of OECs (purple oval) in the dorsal funiculus of the T12 spinal cord segment (OECs at T12; see Table 4.1). c) and d) Schematic diagrams representing an EtBr injection (yellow circle) at either T10 (c; EtBr at T10/OECs atT12; see Table 4.1) or T8 (d; EtBr at T8/OECs atT12; see Table 4.1) and the grafting of OECs into the T12 spinal cord segment (purple oval). e) and f) Schematic diagrams representing an OEC graft (purple oval) directly into an EtBr-induced demyelination at T12 (yellow oval) in the absence (e; EtBr at T12/OECs atT12; see Table 4.1) or the presence (f; EtBr at T10, T12/OECs atT12; see Table 4.1) of a second EtBr lesion located at T10 (yellow circle). g) and h) Schematic diagrams representing the experimental design for EtBr injection (yellow circle and yellow oval) and OEC cell graft (purple oval) used to test the effect of minocycline treatment on OEC migration.


a
OECs@T12
(Group 1)
b
EtBr@T10
OECs@T12
(Group 2)
c
EtBr@T8
OECs@T12
(Group 3)
d
EtBr@T12
OECs@T12
(Group 4)
e
EtBr@T10,12
OECs@T12
(Group 5)
f
EtBr@T10
OECs@T12
Minocycline+/-
(Group 6)
g
EtBr@T10,12
OECs@T12
Minocycline+/-
(Group 7)
h

4.3.1 The Cell Graft

The cell grafts were easily identified by virtue of their blue, Hoechst-stained nuclei and red, DiI+ve cell bodies and cytoplasmic processes (Fig. 4.3c). More than 200 DiI+ve OECs were present in the dorsal funiculus of each coronal spinal cord section through the midpoint of the rostral-caudal extent of the graft. These DiI+ve cells were so closely packed together that it was impossible to obtain an accurate count of the number of cells. At all survival times a very small number of DiI+ve cells were also found scattered throughout the dorsal funiculus of the T12 spinal cord segment.

4.3.2 Host Glial Cell Reactivity

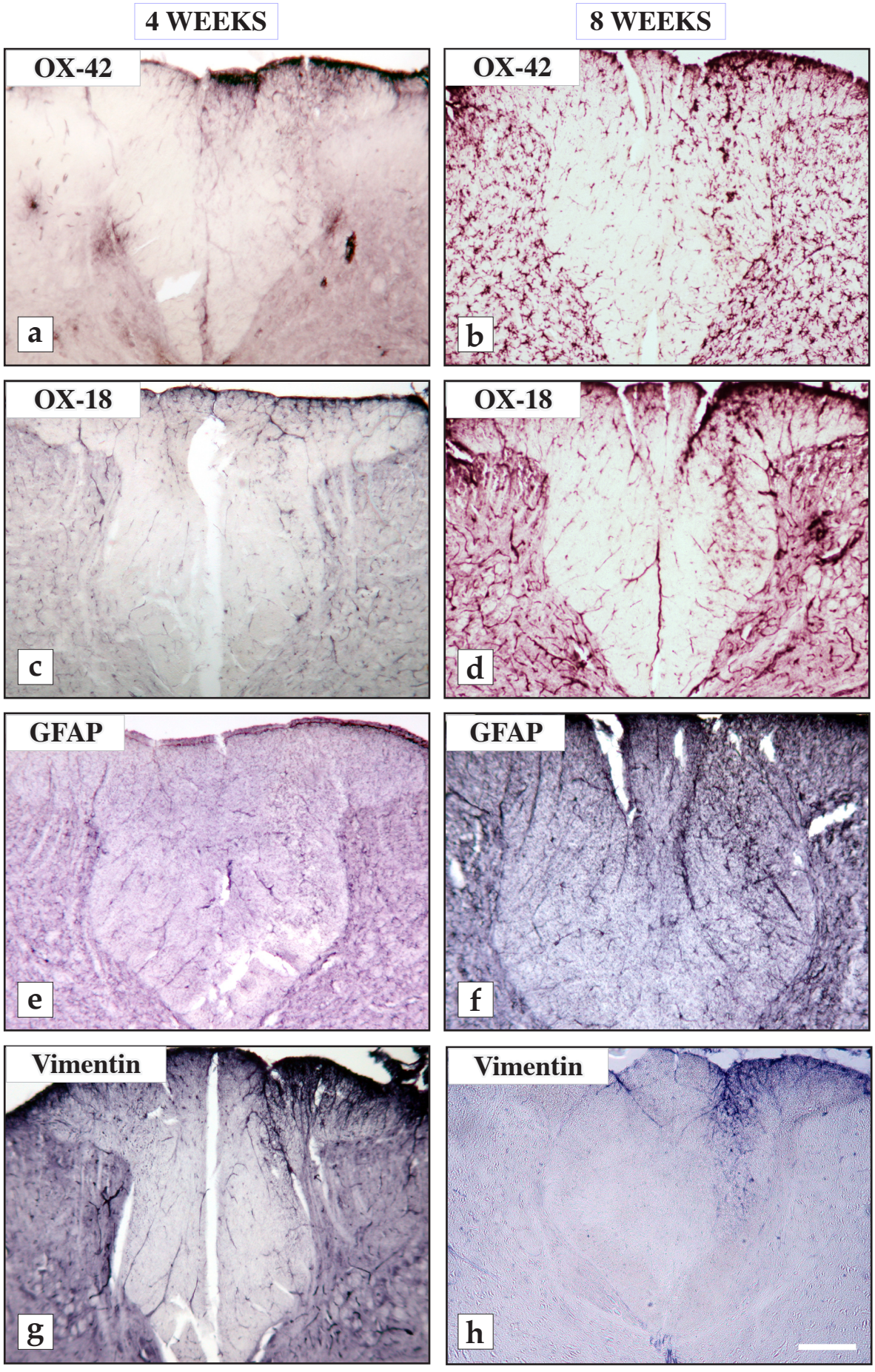
Both reactive microglia and astrocytes were present in the dorsal funiculus of the T12 segment of the spinal cord, presumably in response to the placement of the cell graft (Fig. 4.3e & 4.3f). The vimentin+ve reactive astrocytes possessed an intensely stained cell body and several long cytoplasmic processes, whereas the OX-18+ve cells had small round somata and a few short, branched cytoplasmic processes. In more rostral segments of the spinal cord in these control rats, the microglial and astrocytic reactivity was less and less apparent in segments further away from the graft and with increasing survival times, but was always confined to the ipsilateral dorsal funiculus. Thus, OX-18+ve reactive microglia and vimentin+ve reactive astrocytes were found within the right dorsal funiculus of T12 at two weeks after grafting (Fig. 4.3e & f). Four (Fig. 4.5a, c, e &g) and eight (Fig. 4.5 b, d, f & h) weeks after cell grafting at T12, OX-42- (Fig. 4.5 a & b) and OX-18-expressing (Fig. 4.5 c & d) microglia as well as GFAP+ve (Fig. 4.5 e & f) and vimentin+ve (Fig. 4.5 g & h) astrocytes were also seen within the right dorsal funiculus of T11, occupying the area between the pial surface and the corticospinal tract.

4.3.3 DiI+ve Cells Rostral to Cell Graft

Tissue sections from the rostral, middle and caudal thirds of each spinal cord segment rostral to T12 (i.e. T11, T10, T9 and T8) were randomly chosen for fluorescent microscopy. Four sections from each third of the segment (twelve per segment) were

FIGURE 4.5: Microglial and astrocytic reactivity in the dorsal funiculus of T11 in Group 1 rats.

OX-42+ve (a, b) and OX-18+ve (c, d) microglia, and GFAP+ve (e, f) and vimentin+ve (g, h) astrocytes in the dorsal funiculus of T11 at 4 (a, c, e, g) and 8 weeks (b, d, f, h) after grafting OECs at T12 in Group 1 rats. Minimal microglial and astrocytic reactivity is seen at T11 when the only experimental manipulation was grafting OECs one spinal cord segment caudally. Bar = 400 μm


4 WEEKS
8 WEEKS
OX-42
a
OX-42
b
OX-18
c
OX-18
d
GFAP
e
GFAP
f
Vimentin
g
Vimentin
h

used for cell counts. DiI+ve OECs were present within the dorsal funiculus of each spinal cord segment from T11 to T8 (the most rostral segment examined in this study). The average number of these cells within the dorsal funiculus of T11 varied from 17.1 per 40 μm tissue section at 2 weeks survival (Fig. 4.6a), to 30.9 after 4 weeks (Fig. 4.6b) and to 7.6 per tissue section at 8 weeks after grafting (Fig. 4.6c). In more rostral spinal cord segments (i.e. T8-T10) the numbers of DiI+ve OECs in the Group 1 rats always averaged less than 8 per 40 μm tissue section regardless of survival time, except for the average cell counts at T10 at the 4 week time point where an average of 12.1 cells per tissue section were counted (Fig. 4.7 - 4.9). The small numbers of migrating DiI+ve OECs that were seen in each spinal cord segment rostral to the cell graft were found on the pial surface and along the septi that lay between the fiber tracts comprising the dorsal funiculi (Fig. 4.10a & b).

4.4 Inducing OECs To Migrate Towards A Focal Demyelination

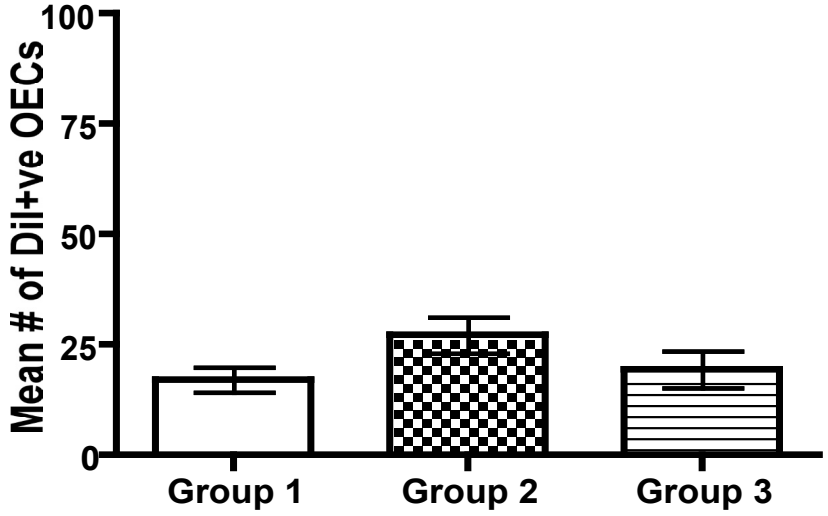
To address specific aim #1, a gliotoxin-induced demyelination of axons was accomplished by stereotactically injecting 1 μl of a 0.03% solution of EtBr into the right dorsal funiculus of either the T10 (Group 2 rats) or T8 (Group 3 rats) spinal cord segment of separate groups of adult rats (see Table 4.1). Three days after the EtBr injection, DiI+ve OECs were grafted into the right dorsal funiculus of the T12 spinal cord segment of each rat (Fig. 4.4c & d).

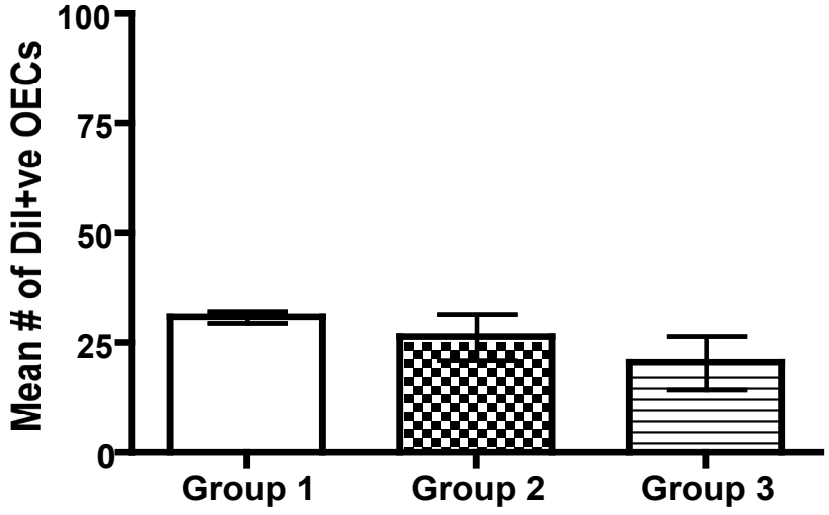
4.4.1 The Cell Graft

In Groups 2 and 3 rats the cell grafts had a similar appearance to that described in Section 4.3.1 for the control group (i.e. Group 1 rats) that had received only a cell graft (data not shown). This cell graft consisted of a closely packed DiI+ve collection of OECs. Although there were more than 200 DiI+ve OECs present in each coronal spinal cord section through the midpoint of the rostral-caudal extent of the graft, no attempt was made to obtain an accurate quantitation since it was impossible to distinguish individual cells throughout much of the compact mass. DiI+ve OECs were also found scattered within the plane of the section in the neural tissue outside of the main body of the graft.

FIGURE 4.6: Number of DiI+ve OECs in the dorsal funiculus of T11 in Groups 1-3 rats.

Histograms depicting the mean number (+/- SEM) of DiI+ve Cells (Y-axis) in the dorsal funiculus of the T11 spinal cord segment of control rats (OECs@T12; Group 1) or rats injected with EtBr at either T8 (EtBr@T8/OECs@T12; Group 3) or T10 (EtBr@T10/OECs@T12; Group 2) at 2 (a), 4 (b) or 8 weeks (c) after grafting of OECs at T12 ($n = 3$ for each Group at each survival time). A one-way analysis of variance was applied to the data with the data for each survival time being analyzed separately for statistical significance. The resulting value of F (6.56) was statistically significant only at the 8 weeks (c) time point ($p < 0.05$). Multiple post hoc comparisons between Groups 1-3 at the 8 week time point were performed with Bonferonni's multiple comparison post-test revealing a significant difference between Groups 1 and 2 ($p < 0.05$; 1 asterisk).


Mean # of DiI+ve OECs
100
75
50
25
0
Group 1
Group 2
Group 3


Mean # of DiI+ve OECs
100
75
50
25
0
Group 1
Group 2
Group 3

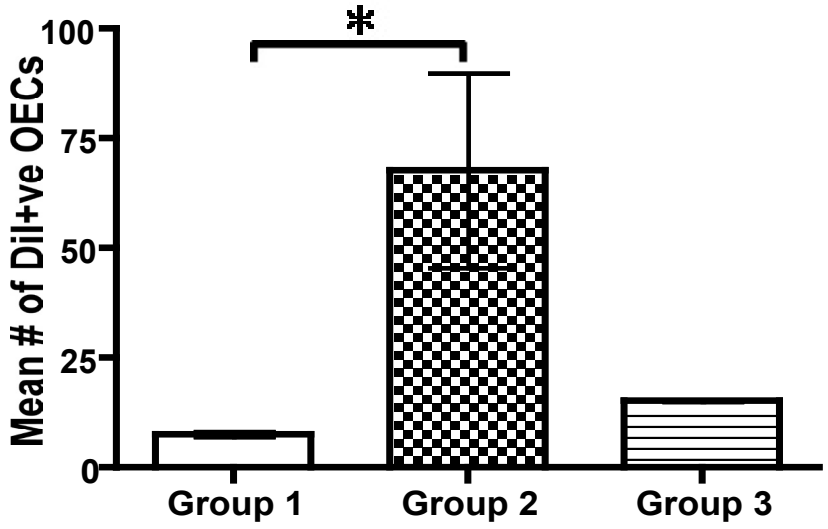
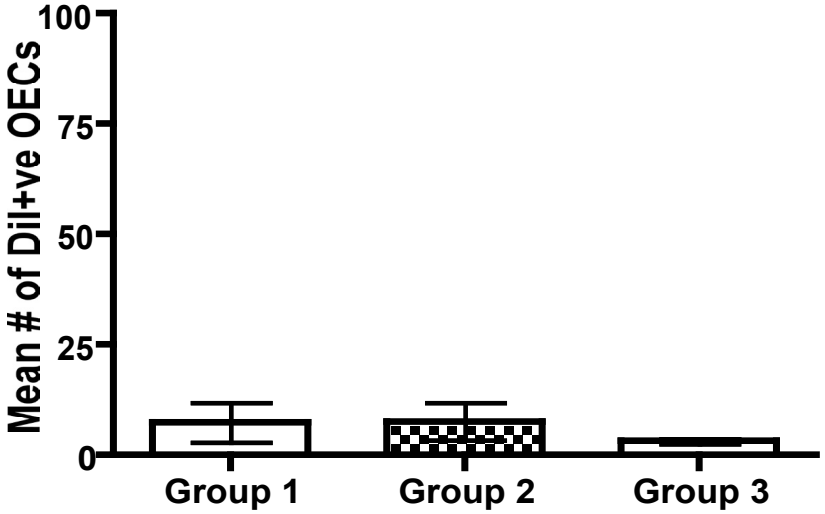
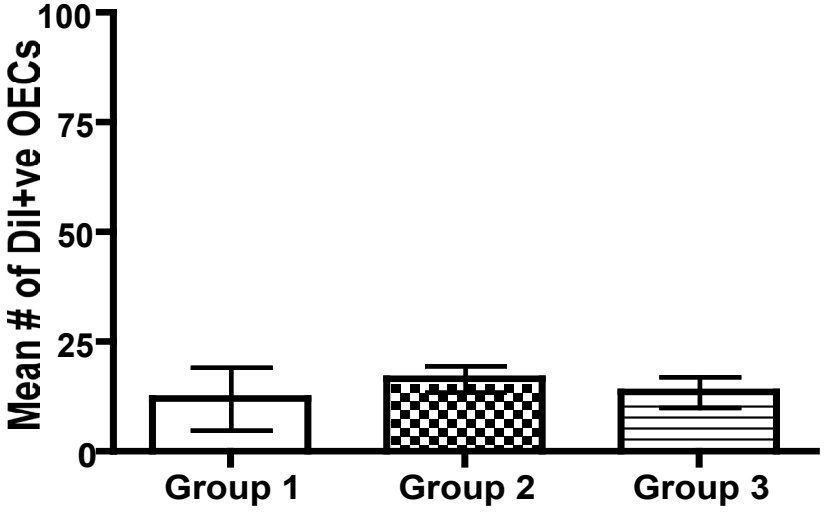

*
Mean # of DiI+ve OECs
100
75
50
25
0
Group 1
Group 2
Group 3

FIGURE 4.7: Number of DiI+ve OECs in the dorsal funiculus of T10 in Groups 1-3 rats.

Histograms depicting the mean number (+/- SEM) of DiI+ve Cells (Y-axis) in the dorsal funiculus of the T10 spinal cord segment of control rats (OECs@T12; Group 1) or rats injected with EtBr at either T8 (EtBr@T8/OECs@T12; Group 3) or T10 (EtBr@T10/OECs@T12; Group 2) at 2 (a), 4 (b) or 8 weeks (c) after grafting of OECs at T12 (n = 3 for each Group at each survival time). A one-way analysis of variance was applied to the data with the data for each survival time being analyzed separately for statistical significance. The resulting values of F were not significant at any time point ($p > 0.05$).


Mean # of DiI+ve OECs
100
75
50
25
0
Group 1
Group 2
Group 3


Mean # of DiI+ve OECs
100
75
50
25
0
Group 1
Group 2
Group 3

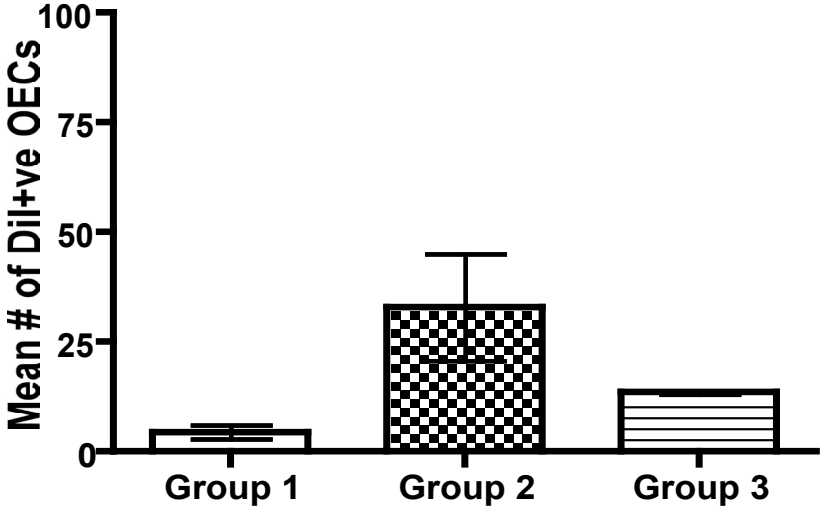
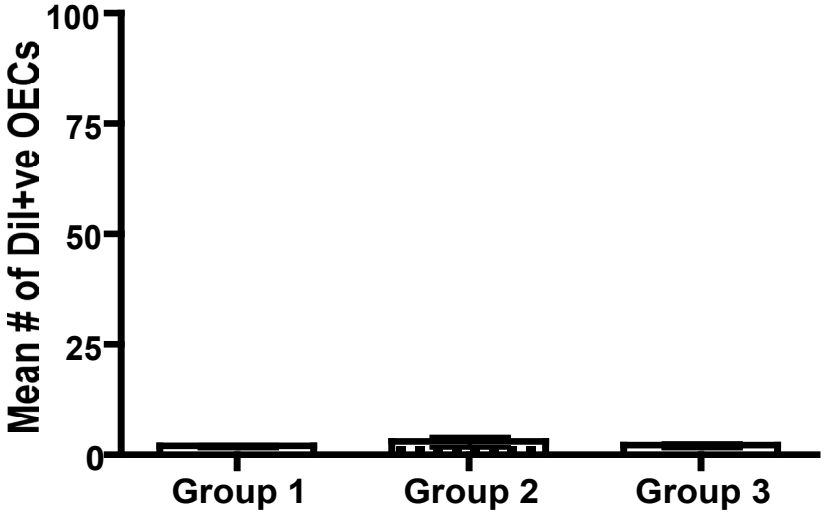
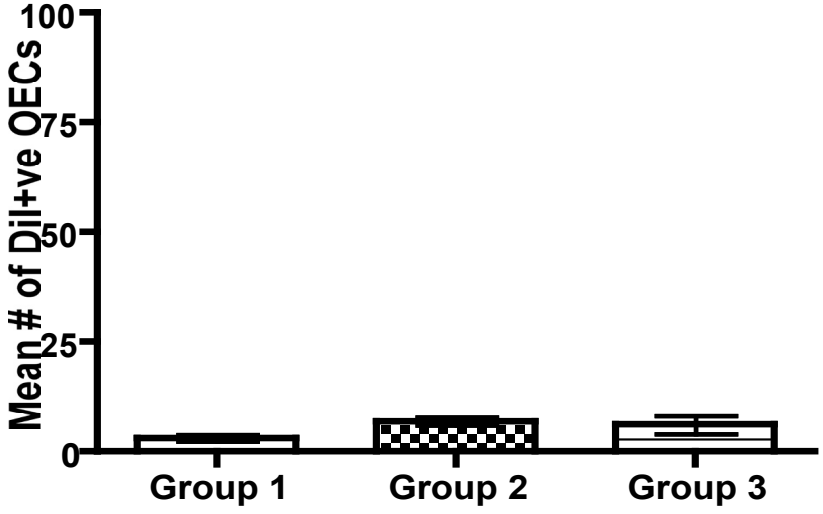

Mean # of DiI+ve OECs
100
75
50
25
0
Group 1
Group 2
Group 3

FIGURE 4.8: Number of DiI+ve OECs in the dorsal funiculus of T9 in Groups 1-3 rats.

Histograms depicting the mean number (+/- SEM) of DiI+ve Cells (Y-axis) in the dorsal funiculus of the T9 spinal cord segment of control rats (OECs@T12; Group 1) or rats injected with EtBr at either T8 (EtBr@T8/OECs@T12; Group 3) or T10 (EtBr@T10/OECs@T12; Group 2) at 2 (a), 4 (b) or 8 weeks (c) after grafting of OECs at T12 ($n = 3$ for each Group at each survival time). A one-way analysis of variance was applied to the data with the data for each survival time being analyzed separately for statistical significance. The resulting values of F (8.182) were statistically significant only at the 8 weeks time points ($p < 0.02$). Multiple post hoc comparisons between Groups 1-3 were performed with Bonferonni's multiple comparison post-test revealing a significant difference between Groups 1 and 2 at 8 weeks survival ($p < 0.05$; 1 asterisk).


Mean # of DiI+ve OECs
100
75
50
25
0
Group 1
Group 2
Group 3


Mean # of DiI+ve OECs
100
75
50
25
0
Group 1
Group 2
Group 3

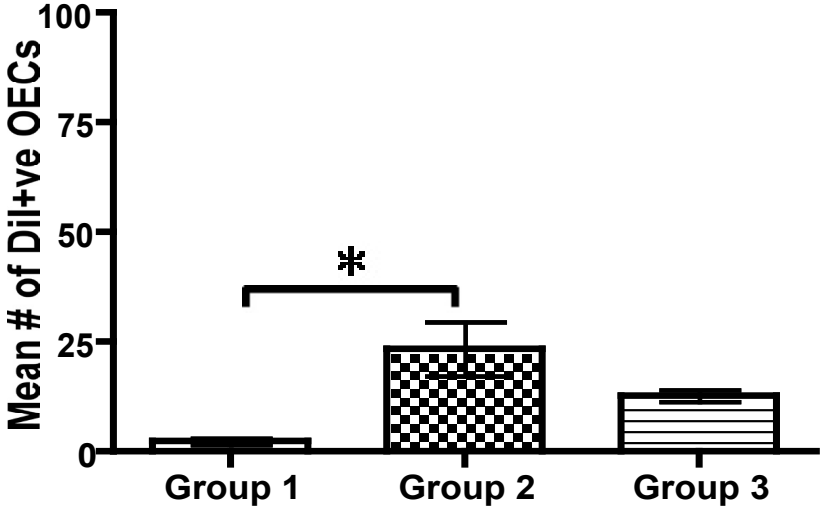
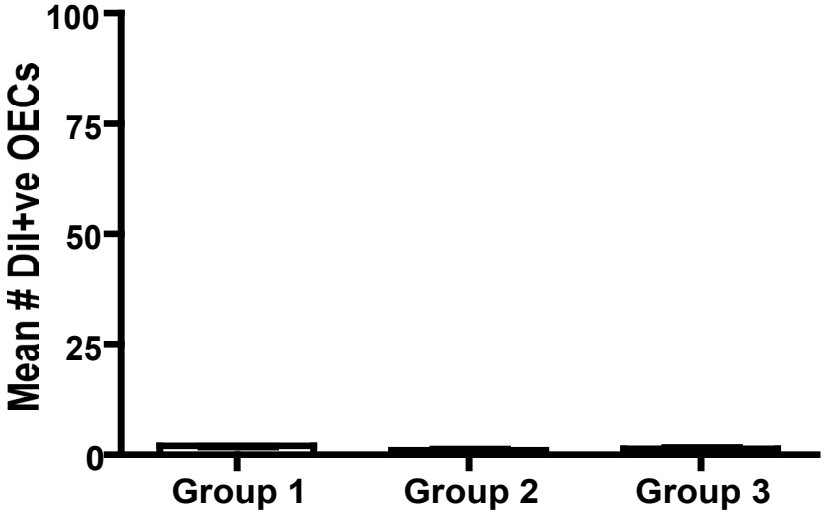
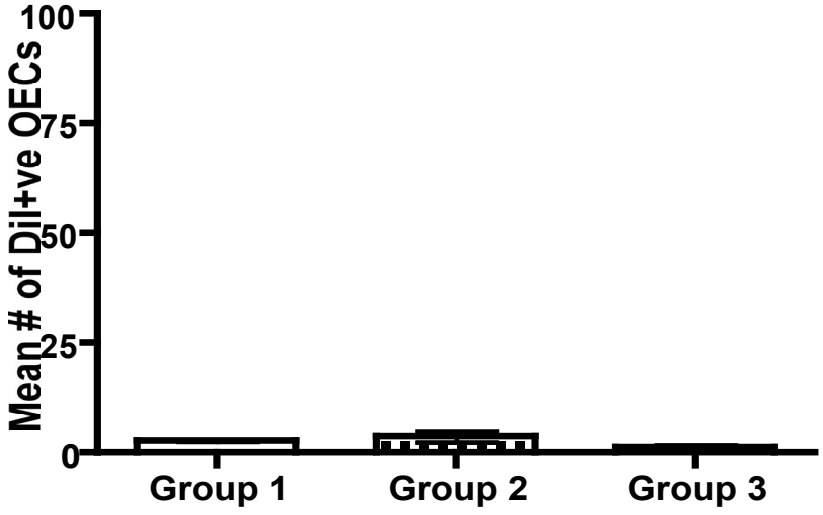

Mean # of DiI+ve OECs
100
75
50
25
0
*
Group 1
Group 2
Group 3

FIGURE 4.9: Number of DiI+ve OECs in the dorsal funiculus of T8 in Groups 1-3 rats.

Histograms depicting the mean number (+/- SEM) of DiI+ve Cells (Y-axis) in the dorsal funiculus of the T8 spinal cord segment of control rats (OECs@T12; Group 1) or rats injected with EtBr at either T8 (EtBr@T8/OECs@T12; Group 3) or T10 (EtBr@T10/OECs@T12; Group 2) at 2 (a), 4 (b) or 8 weeks (c) after grafting of OECs at T12 ($n = 3$ for each Group at each survival time). A one-way analysis of variance was applied to the data with the data for each survival time being analyzed separately for statistical significance. The resulting values of F (18.43) were statistically significant only at the 8 week time point ($p < 0.003$). Multiple post hoc comparisons between Groups 1-3 were performed with Bonferonni's multiple comparison post-test revealing a significant difference between Groups 1 and 2 ($p < 0.05$; 1 asterisk) and between Groups 1 and 3 ($p < 0.01$; 2 asterisks) at 8 weeks survival. However, the numerical difference in cell counts was so small as to make it questionable whether the observed differences were therapeutically of any interest.


Mean # Dil+ve OECs
100
75
50
25
0
Group 1
Group 2
Group 3


Mean # of Dil+ve OECs
100
75
50
25
0
Group 1
Group 2
Group 3

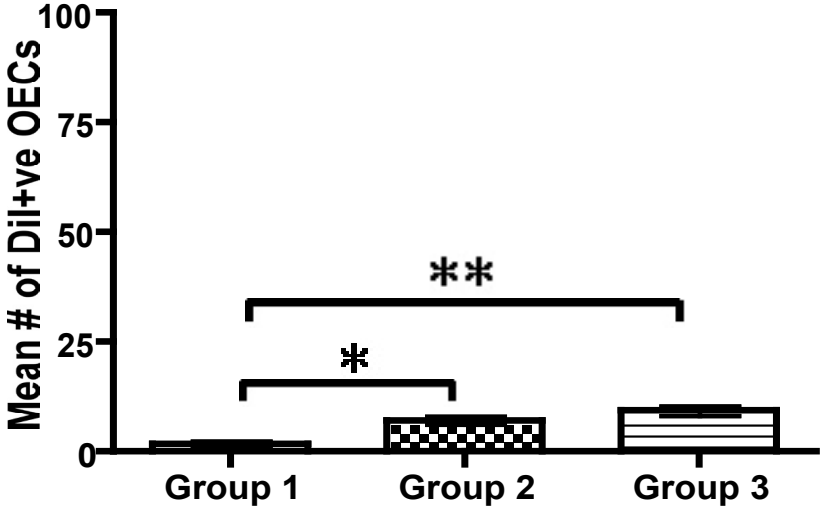
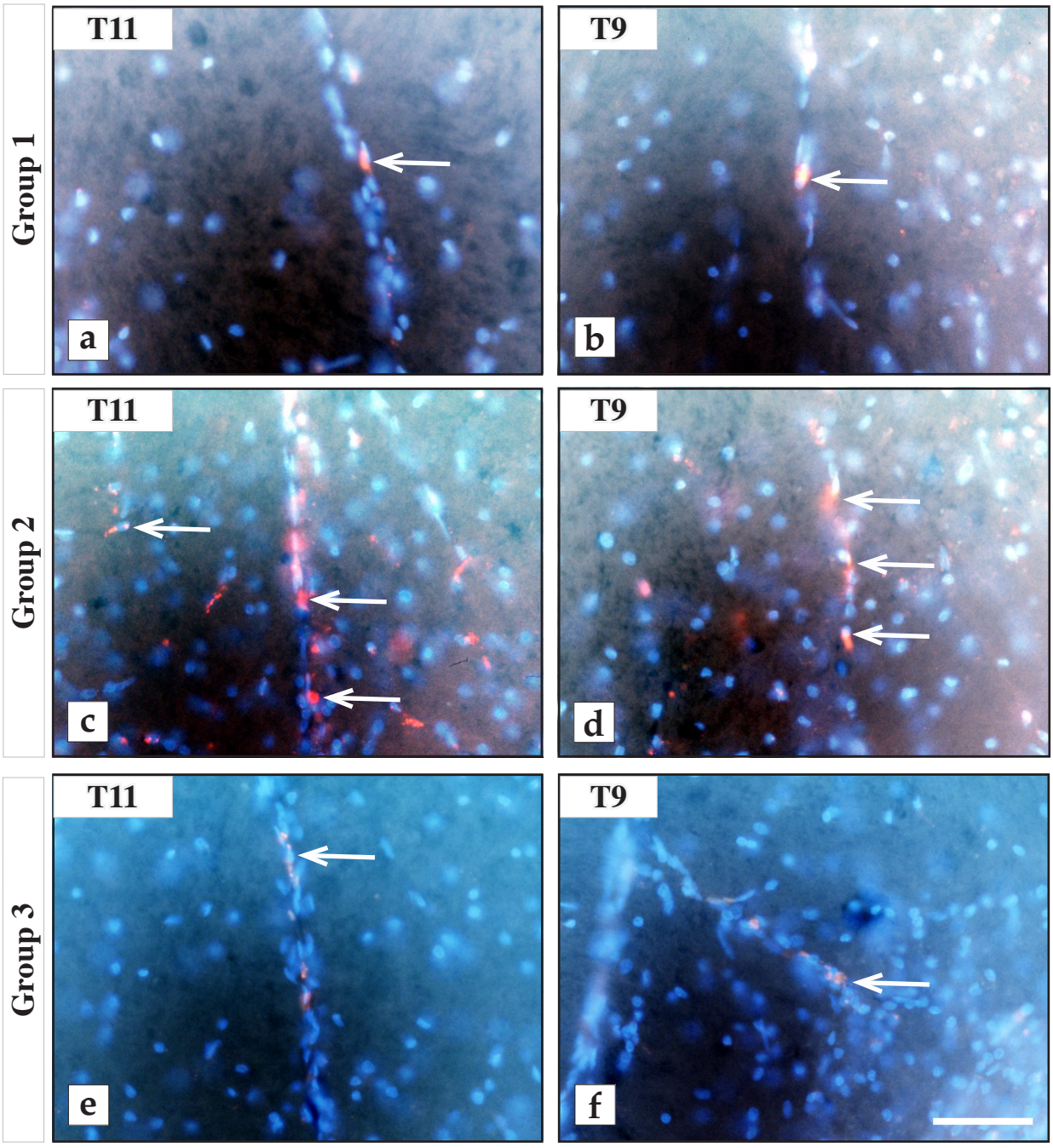

Mean # of Dil+ve OECs
100
75
50
25
0
**
*
Group 1
Group 2
Group 3

FIGURE 4.10: DiI+ve cells in the dorsal funiculus of T11 and T9 in Groups 1-3 rats.

Fluorescent images of the dorsal funiculus of the T11 and T9 spinal cord segments of control rats (Group 1) and of rats injected with EtBr at either T10 (Group 2) or T8 (Group 3). All rats survived for 8 weeks after grafting. a & b) In control rats, DiI+ve OECs (white arrow) were seen along the septa at T11 (a) and T9 (b). c & d) In rats injected with EtBr at T10, DiI+ve OECs (white arrows) were seen not only along the septa but also within the neuropil at both T11 (c) and T9 (d). e & f) In rats injected with EtBr at T8, DiI+ve OECs (white arrow) were also found along the septa and in neuropil at both T11 (e) and T9 (f), although they were much fewer in number than those in Group 2 rats. Bar = 100 μm.


Group 1
T11
a
T9
b
Group 2
T11
c
T9
d
Group 3
T11
e
T9
f

4.4.2 The Ethidium Bromide Lesion.

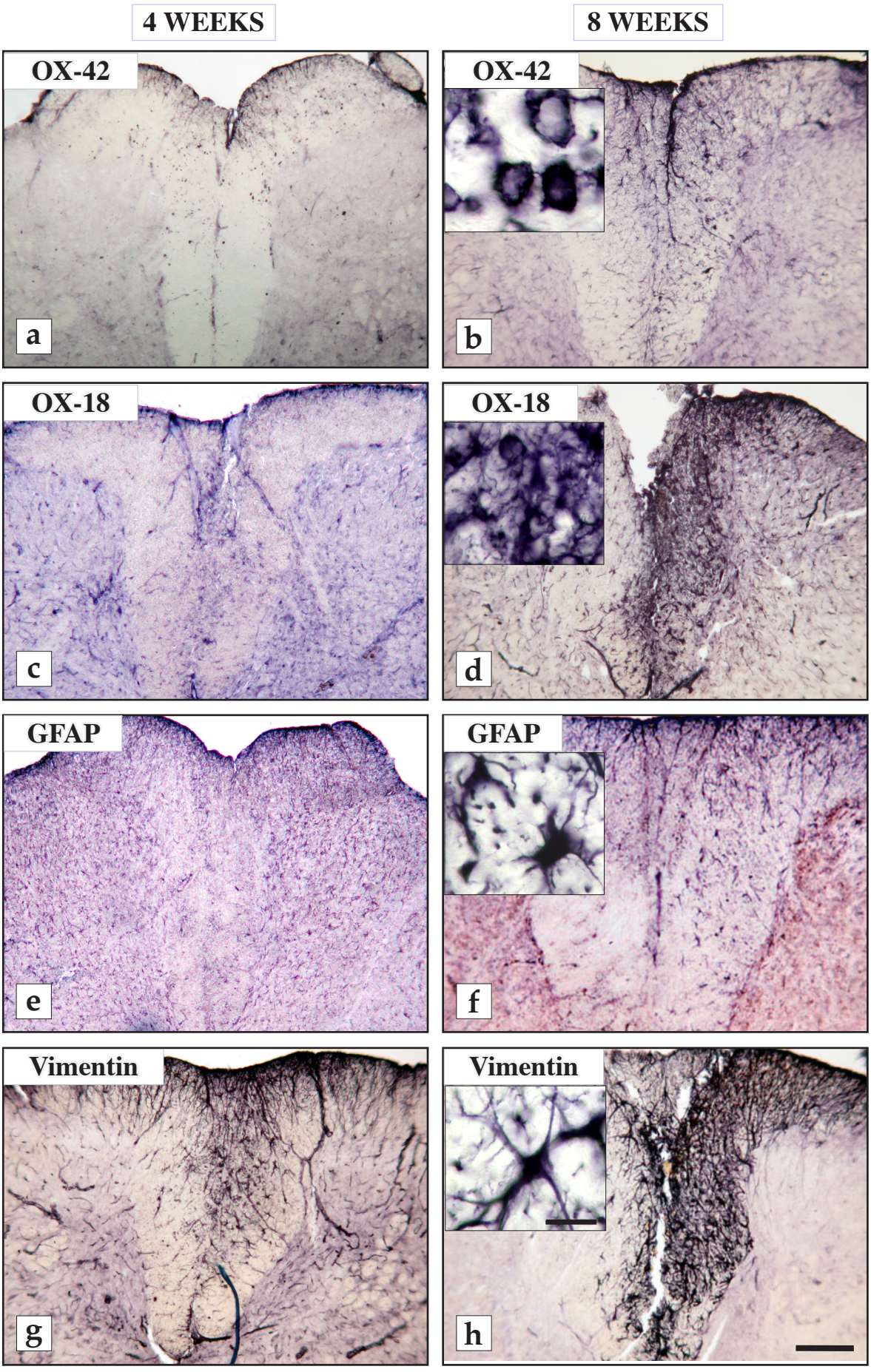
After injecting EtBr into the right dorsal funiculus, the area of the injection acquired yellow discoloration due to host tissue reaction to needle insertion and injection of the EtBr. Macroscopically, the EtBr lesion was identified by the yellow discoloration (see Section 4.2.1) of the dorsal funiculus immediately to the right of the dorsal spinal artery in tissue sections cut through the middle of either the tenth (T10) or the eighth (T8) spinal cord segments in Groups 2 and 3 rats, respectively. In the rats used in this study, the EtBr lesion was fusiform in shape and about 1 mm long, with the yellow discoloration being most intense at 2 weeks of survival. At 4 and 8 weeks after cell grafting, the intensity of yellow discoloration was less intense and harder to identify. The spinal cord segments with an EtBr injection were identified by comparing the location of the EtBr injection with the location of the laminectomy at the time of spinal cord dissection and spinal rootlet count prior spinal cord segment sectioning. For an entire spinal cord segment (e.g. T10), up to twenty 40 μm coronal tissue sections contained the demyelinated lesion, which was confined to the right dorsal funiculus without involvement of the corticospinal tract (Fig. 4.2e). By 8 weeks of survival, the right dorsal funiculus of T10 (Group 2 rats) and T8 (Group 3 rats) was close to being fully remyelinated, at least in terms of the histological appearance as seen in tissue sections stained with Luxol Fast Blue (Fig. 4.2f).

4.4.3 Host Glial Cell Reactivity.

Similar to what was seen in the T12 segment of the spinal cord in Group 1 rats, reactive microglia and reactive astrocytes were present in and around the cell graft in the right dorsal funiculus. Microglial and astrocytic reactivity was also observed within the ipsilateral dorsal funiculus of the T11 spinal cord segment and at the site of EtBr injection at T8 (Group 3 rats) and T10 (Group 2 rats). In the rats injected with EtBr at T10 and killed 4 weeks after receiving an OEC graft, the intensity of OX-42 (Fig. 4.11a) and OX-18 (Fig. 4.11c) expressed by microglia within the right dorsal funiculus of the T11 spinal cord segment was slightly higher than that of the opposite funiculus or adjacent right dorsal horn. At the same survival time, the level of GFAP expression by astrocytes within the right dorsal funiculus was also close to that of the

FIGURE 4.11: Microglial and astrocytic reactivity in the dorsal funiculus of T11 in Group 2 rats.

OX-42+ve (a, b) and OX-18+ve (c, d) microglia, and GFAP+ve (e, f) and vimentin+ve (g, h) astrocytes in the dorsal funiculus of T11 at 4 (a, c, e, g) and 8 weeks (b, d, f, h) after grafting OECs at T12 in Group 2 rats. Minimal microglial and astrocytic reactivity is seen ipsilaterally at T11 by 4 weeks (a, c, e & g) after grafting, but the reactivity as evidenced by OX-18 (d) and vimentin (h) immunostaining is much more intense by 8 weeks. Insets in b, d, f and h show the morphology of reactive microglia (b, d) and astrocytes (f, h). Bar in h = 400 μm and applies to a-h. Bar in inset in h = 20 μm and applies to all insets.


4 WEEKS
8 WEEKS
OX-42
a
OX-42
b
OX-18
c
OX-18
d
GFAP
e
GFAP
f
Vimentin
g
Vimentin
h

other parts of the section (Fig. 4.11e). Only vimentin expression by astrocytes localized within the right dorsal funiculus looked more intense in comparison with level of its expression by astrocytes in the left funiculus (Fig. 4.11g). Interestingly, the expression of OX-18 by reactive microglia (Fig. 4.11d) and vimentin by reactive astrocytes (Fig. 4.11h) was noticeably more intense in the rats surviving for 8 weeks to those at shorter survival time. Numerous intensely stained microglia and astrocytes were found throughout much of the extent of the right dorsal funiculus reaching but sparing the right corticospinal tract, left dorsal funiculus and right dorsal horn. Although, microglia and astrocytes expressed OX-42 (Fig. 4.11b) and GFAP (Fig. 4.11f), respectively, at 8 week survival time at more intense, these expressions were not as prominent as that of OX-18 (Fig. 4.11d) and vimentin (Fig. 4.11h).

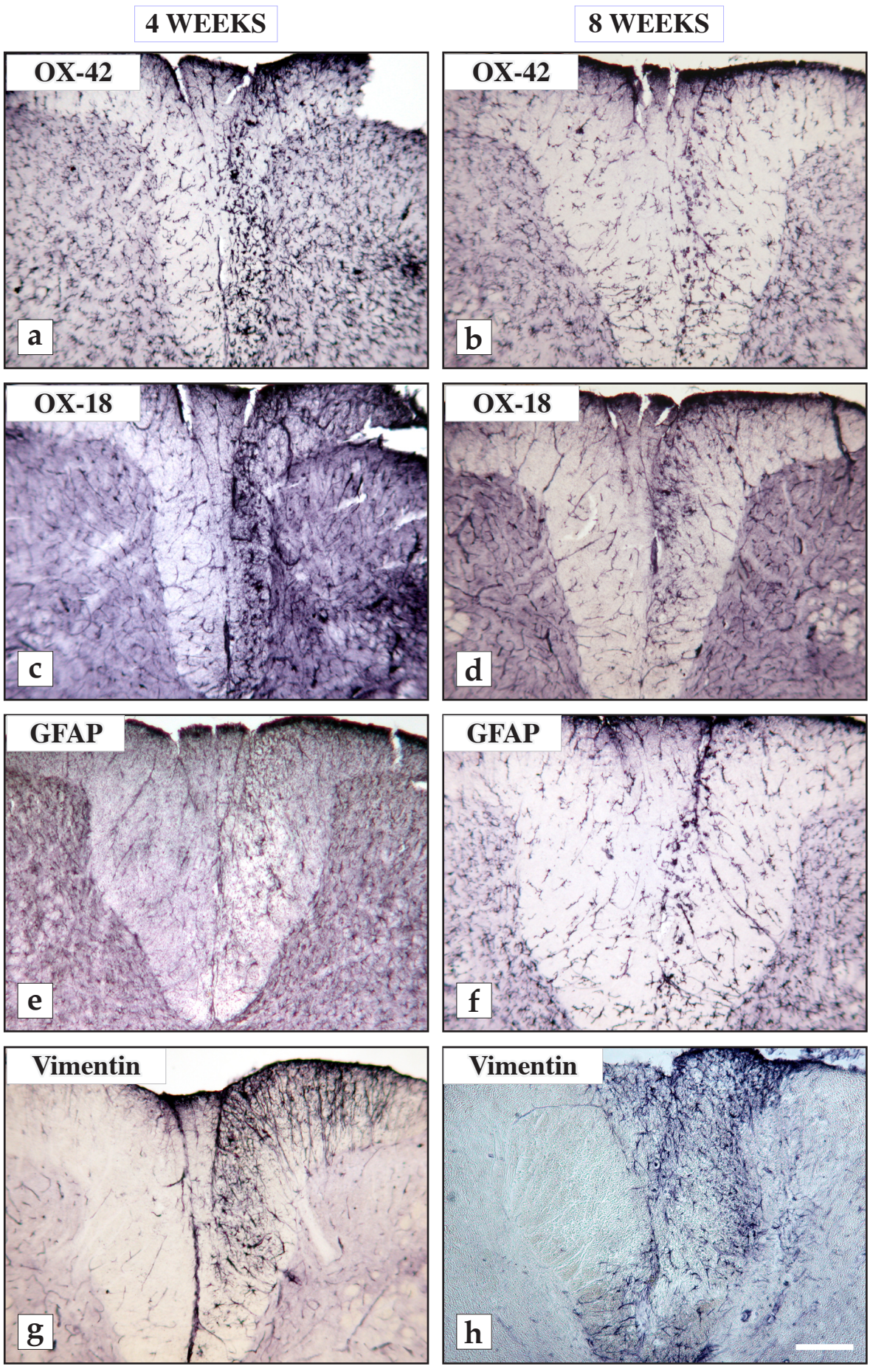
In the rats that received an EtBr injection at T8, OX-42- (Fig. 4.12a & b) and OX-18-expressing (Fig. 4.12c & d) microglia grouped predominantly within the right dorsal funiculus at both 4 and 8 weeks after grafting cells. GFAP+ve astrocytes (Fig. 4.12e & f) were seen in groups also throughout the extent of the right dorsal funiculus. Single OX-42- and OX-18-positive microglia as well as GFAP-expressing astrocytes lined along the numerous septi of both dorsal funiculi and scattered throughout dorsal horns. However, vimentin+ve astrocytes (Fig. 4.12g & h) were more prominent in the right dorsal funiculus than in adjacent structures at both 4 and 8 week survival period.

4.4.4 DiI+ve Cells Rostral to Cell Graft

DiI+ve OECs were present within the dorsal funiculus of T11, with the numbers of these migrating cells being quite similar in the experimental and control groups at two (Fig. 4.6a) and four (Fig. 4.6b) weeks after grafting. However, by 8 weeks after grafting there were several fold more DiI+ve OECs in the dorsal funiculus of T11 (Fig. 4.6c) and T9 (Fig. 4.8c) in those rats that had received an EtBr injection at T10, in comparison to that seen in either of the other two groups of rats. In contrast to that seen in control rats, the migrating DiI+ve OECs in rats in which EtBr had been injected at T10 were found not only along the septi of both dorsal funiculi but also within the neuropil of both right and left dorsal funiculi of T11 (Fig. 4.10c) and T9 (Fig. 4.10d). Although, the mean numbers of DiI+ve OECs in the dorsal funiculus of T8 at the 8

FIGURE 4.12: Microglial and astrocytic reactivity in the dorsal funiculus of T11 in Group 3 rats.

OX-42+ve (a, b) and OX-18+ve (c, d) microglia, and GFAP+ve (e, f) and vimentin+ve (g, h) astrocytes in the dorsal funiculus of T11 at 4 (a, c, e, g) and 8 weeks (b, d, f, h) after grafting OECs at T12 in Group 3 rats. Minimal microglial reactivity is seen ipsilaterally at T11 regardless of survival time (a-d). There was also no detectable difference in GFAP (e, f) or vimentin (g, h) immunostaining between the 4 and 8 week survival. In contrast to GFAP immunostaining, however, the vimentin immunostaining suggested there was astrocytic reactivity ipsilaterally at T11 at least as early as 4 weeks. Bar = 400 μm


4 WEEKS
8 WEEKS
OX-42
a
OX-42
b
OX-18
c
OX-18
d
GFAP
e
GFAP
f
Vimentin
g
Vimentin
h

week time point after grafting in Group 2 and 3 rats were both significantly higher than that of the control group (Fig. 4.9), the numerical difference in cell counts was so small it is questionable whether the difference is therapeutically of any interest.

4.5 Inducing OECs To Migrate Away From A Focal Demyelination

To address specific aim #2, DiI+ve OECs were stereotactically injected into the right dorsal funiculus of the T12 spinal cord segment of 9 rats (Group 4 rats; see Table 4.1) three days after an injection of 1μl of a 0.03% solution of EtBr into right dorsal funiculus of T12 (Fig. 4.4e). In order to address specific aim #3, an additional nine rats received injections of 1μl of a 0.03% solution of EtBr into right dorsal funiculus of both T10 and T12 (Group 5 rats; see Table 4.1). Three days later, these latter nine rats were also stereotactically injected with DiI+ve OECs into the right dorsal funiculus of the T12 spinal cord segment (Fig. 4.4f).

4.5.1 The Ethidium Bromide Lesions

The EtBr lesion was identified by a yellow-pink discoloration at the middle of the T12 spinal cord segment and on the right side immediately lateral to the dorsal spinal artery. The size of the discoloration was slightly bigger due to additional injection of DiI-labelled OECs but it never exceeded 1-1.2 mm in rostral-caudal extent. Approximately 30 spinal cord sections of the middle portion of T12 contained the lesion and occupied the entire right dorsal funiculus with minimal extension into adjacent structures. The EtBr lesion at T10 of the Group 5 rats was similar in its location and extent to that described in Section 4.4.2.

4.5.2 The Cell Graft.

DiI+ve OECs were grafted into a three day old EtBr lesion within the midportion of the T12 spinal cord segment. Similar to that previously described in Section 4.3.1 with respect to the appearance of the cell graft within undamaged CNS tissue, compact groups of 20 to 30 DiI+ve OECs were seen throughout the right dorsal funiculus, with the total number of grafted cells per tissue section averaging more than 200 at the rostral-caudal midpoint of the graft. However, due to grafting the OECs into an EtBr-induced demyelinated lesion, the OECs were scattered throughout the whole

right dorsal funiculus and within the entire area encompassed by the yellow-pink discoloration of the lesion.

4.5.3 Host Glial Cell Reactivity.

As expected, reactive microglia and reactive astrocytes were both found throughout the whole right dorsal funiculus of the T12 spinal cord segment where OECs had been grafted into an EtBr-induced demyelinated lesion in both Group 4 and 5 rats (data not shown). OX-18-expressing microglia and vimentin+ve astrocytes were also seen occupying the entire right dorsal funiculus of the T10 spinal cord segment in Group 5 rats, which had received a second EtBr injection at T10.

In the T11 spinal cord segment, microglia expressing OX-42 (Fig. 4.13a, c, e & g) and OX-18 (Fig. 4.13b, d, f & h) were single cells with small round cell bodies and elongated, branched processes evenly distributed throughout the dorsal horns and dorsal funiculi. However, OX-42+ve/OX-18+ve microglia with round cell bodies and short or no processes grouped together within the right dorsal funiculus of T11 regardless of the survival time (i.e. 2 or 8 weeks) and presence of one (Group 4 rats) or two (Group 5 rats) EtBr lesions. Astrocytes expressing GFAP (Fig. 4.14a, c, e &g) and vimentin (Fig. 4.14b, d, f & h) were less abundant than microglia in T11 spinal cord sections, but when present they were located mostly in the white matter adjacent to the pial surface. Although GFAP+ve astrocytes grouped together within the right dorsal funiculus of the T11 spinal cord segment of Group 4 and 5 rats after 2 and 8 weeks, the level of GFAP expression was more prominent in rats of both groups that survived 8 weeks. The level of vimentin expression was always high in astrocytes and it accumulated mostly along the septa/blood vessels within the T11 right dorsal funiculus of Group 4 and 5 rats.

4.5.4 DiI+ve Cells Rostral to Cell Graft.

In the rats with only a single EtBr lesion (Group 4) DiI+ve OECs were present in the dorsal funiculus of T11 (Fig. 4.15a, c & e), T9 (data not shown) and as

FIGURE 4.13: Microglial reactivity in the dorsal funiculus of T11 in Group 4 and 5 rats.

OX-42+ve (a, c, e & g) and OX-18+ve (b, d, f & h) microglia in the dorsal funiculus of T11 at 2 (a-d) and 8 weeks (e-h) after grafting OECs at T12 in Group 4 (a, b, e & f) and 5 (c, d, g, & h) rats. Microglial reactivity in the ipsilateral dorsal funiculus of T11 as evidenced by OX-42 immunostaining was noticeably greater in the double-lesioned Group 5 rats at both 2 (c) and 8 (g) weeks after OEC grafting as compared to that seen in Group 4 rats (a, e). The OX-18 immunostaining suggested a greater microglial reactivity at 8 weeks (f, h) in both groups as compared to the 2 week survival (b, d). Bar = 400 μm

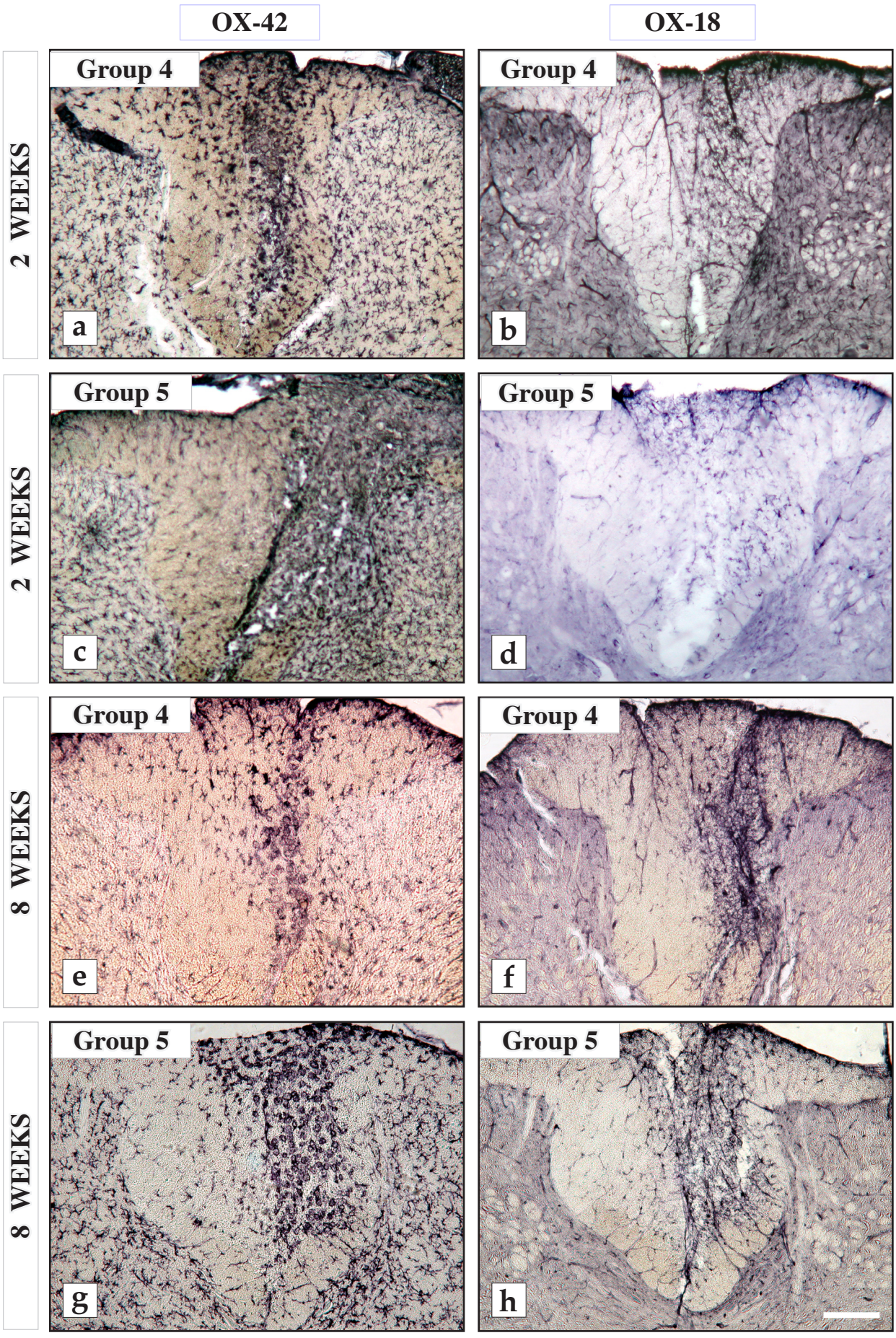

OX-42
OX-18
2 WEEKS
Group 4
a
Group 4
b
2 WEEKS
Group 5
c
Group 5
d
8 WEEKS
Group 4
e
Group 4
f
8 WEEKS
Group 5
g
Group 5
h

FIGURE 4.14: Astrocytic reactivity in the dorsal funiculus of T11 in Group 4 and 5 rats.

GFAP+ve (a, c, e & g) and vimentin+ve (b, d, f & h) astrocytes in the dorsal funiculus of T11 at 2 (a-d) and 8 weeks (e-h) after grafting OECs at T12 in Group 4 (a, b, e & f) and 5 (c, d, g, & h) rats. Astrocytic reactivity in the ipsilateral dorsal funiculus of T11 as evidenced by GFAP immunostaining was noticeably greater at 8 weeks survival in both Group 4 (a, e) and 5 (c, g) rats as compared to that seen at 2 weeks. In the double-lesioned Group 5 rats the vimentin immunostaining suggested a more intense astrocytic reactivity at 2 weeks (d) after grafting as compared to 8 weeks (h) and was also more intense than that seen in Group 4 rats (b) at 2 weeks survival. However, the astrocytic reactivity in Group 4 and 5 rats was comparable at 8 weeks survival as evidenced by vimentin immunostaining (f, h). Bar = 400 μm

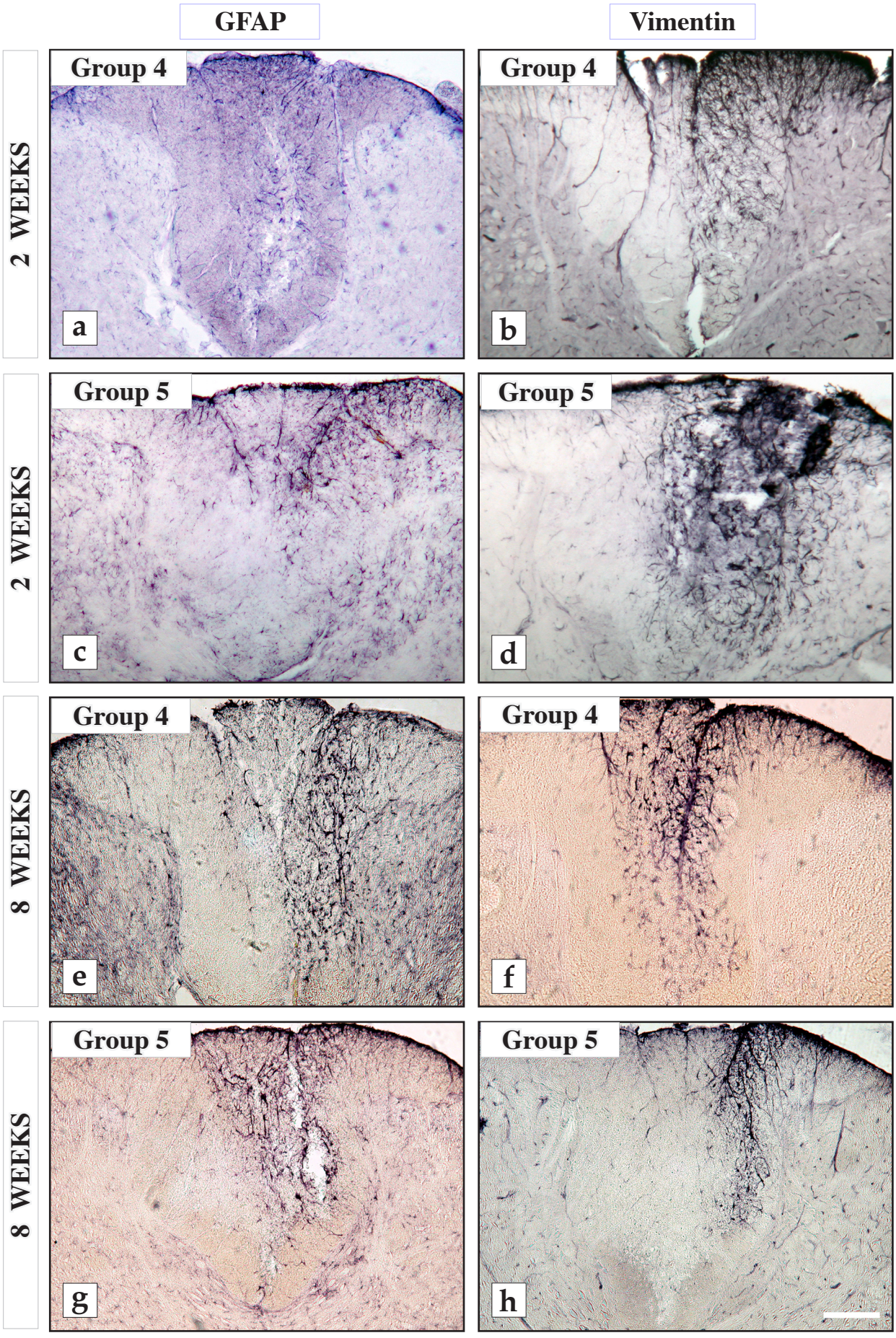
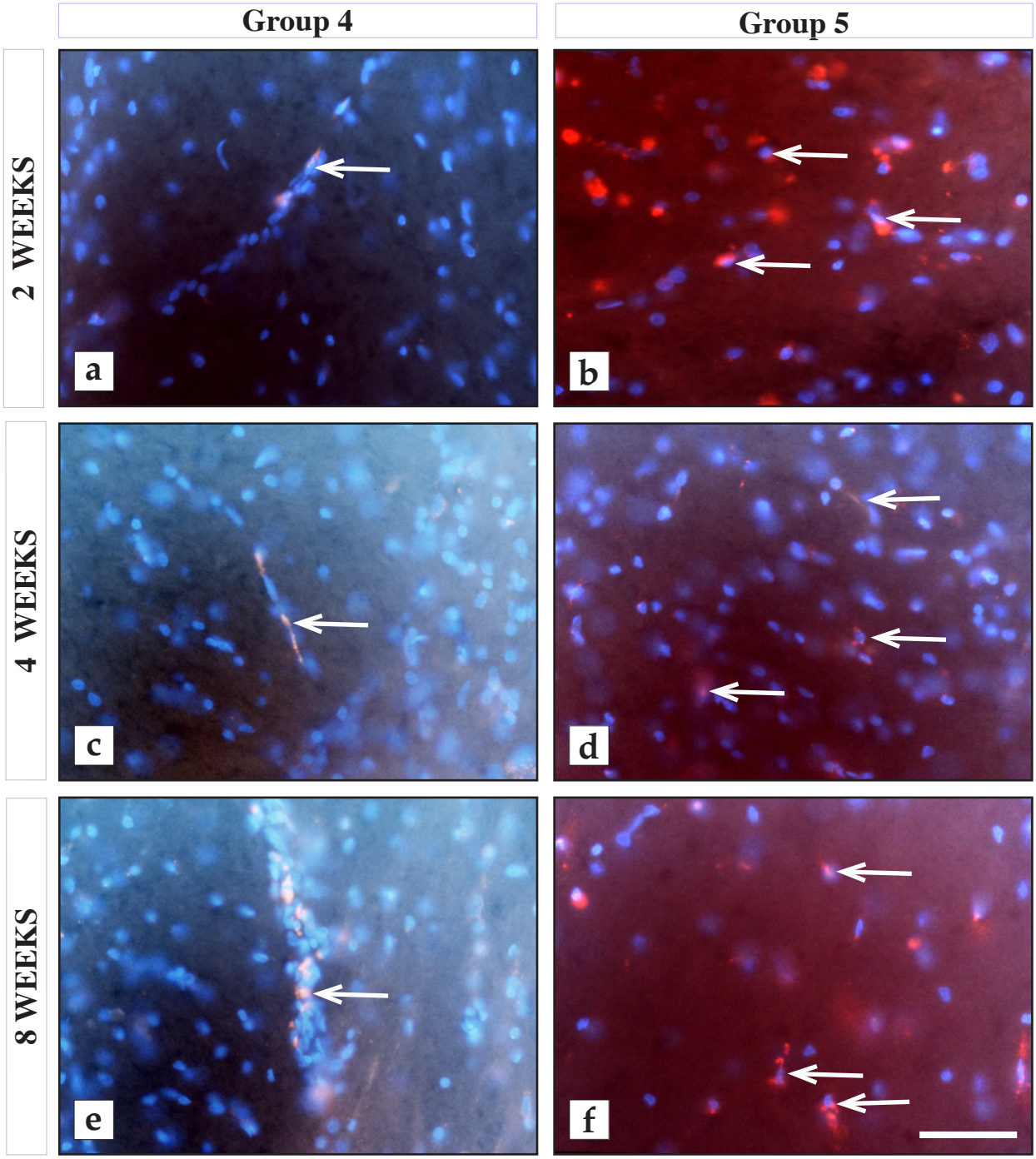

GFAP
Vimentin
2 WEEKS
Group 4
a
Group 4
b
2 WEEKS
Group 5
c
Group 5
d
8 WEEKS
Group 4
e
Group 4
f
8 WEEKS
Group 5
g
Group 5
h

FIGURE 4.15: DiI+ve cells in the dorsal funiculus of T11 in Group 4 and 5 rats.

Fluorescent images of the dorsal funiculus of the T11 spinal cord segment of rats that were injected with EtBr at either T12 (Group 4) or at both T10 and T12 (Group 5) and three days later received an OEC graft into the lesion at T12. The rats survived for 2 (a, b), 4 (c, d) and 8 (e, f) weeks after grafting. Consistently at each survival time there were several fold more DiI+ve cells (white arrows) in the dorsal funiculus of T11 in Group 5 rats (b, d, &f) as compared to that seen in Group 4 rats (a, c & e). Bar = 100 μm.

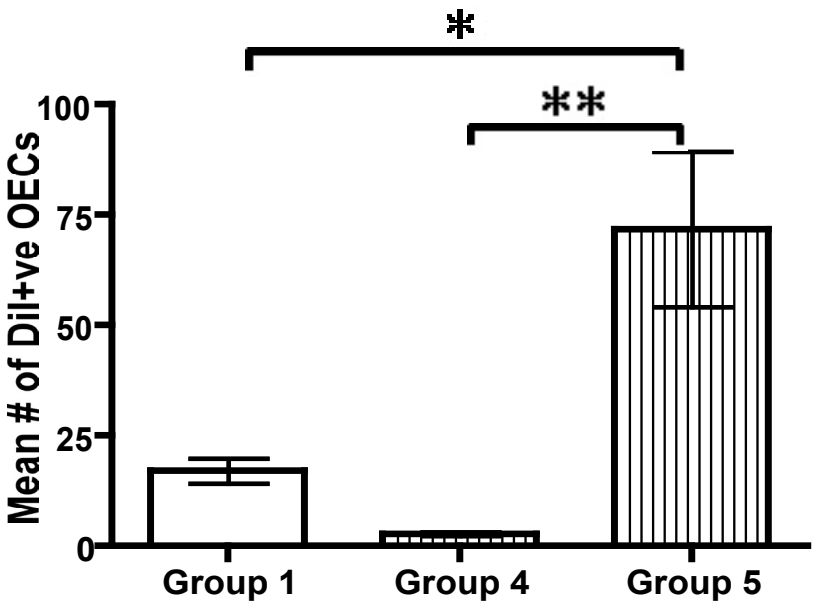

Group 4
Group 5
2 WEEKS
4 WEEKS
8 WEEKS
a
b
c
d
e
f

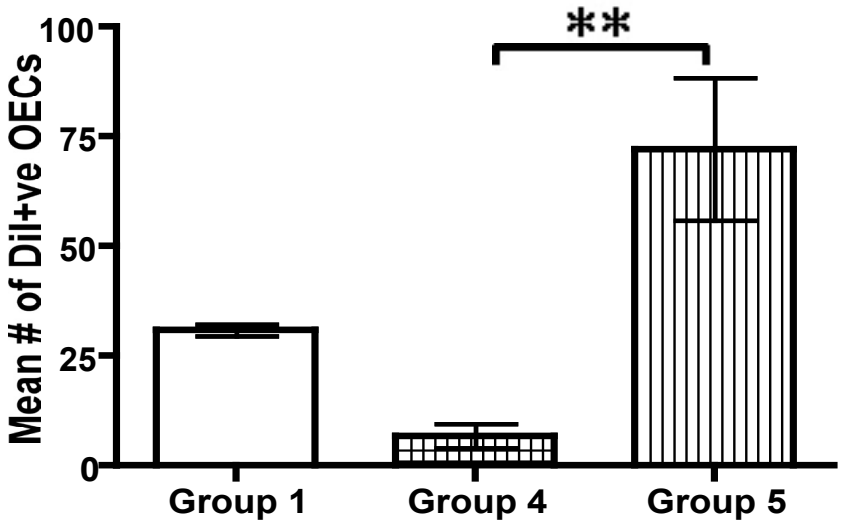
far rostrally as T8 (data not shown), which was the most rostral spinal cord segment examined. In the single lesioned rats (Group 4), however, the DiI+ve OECs had migrated singly or at most as pairs of cells. The number of such cells at the levels of T11 and T10 was consistently lower at two (Fig. 4.16a & 4.17a) and four (Fig. 4.16b & 4.17b) weeks postoperatively than had been observed in Groups 2 and 3 rats at equivalent survival times (1.7 – 6.8 fold fewer cells) and was similar to that seen in the latter two groups of rats at the T9 (Fig. 4.18) and T8 (Fig. 4.19) spinal cord segments at two and four weeks after grafting. Even by 8 weeks postoperatively, the average number of DiI+ve OECs in the dorsal funiculus of T11 (Fig. 4.16) to T8 (Fig. 4.19) remained relatively low.

However, the numbers of DiI+ve OECs were significantly higher in the spinal cord segments of rats with two EtBr lesions (i.e., at both T12 and T10) (Fig. 4.15b, d & f). The average number of DiI+ve OECs in the T11 spinal cord segment of Group 5 rats was 71.8 cells per tissue section as soon as 2 weeks after grafting (Fig. 4.16a). Mean numbers of DiI+ve OECs remained high in Group 5 rats even up to 8 weeks (50.4 cells) after grafting (Fig. 4.16c). These numbers were significantly higher than those rats that had received only a single EtBr injection at T12 (i.e. Group 4 rats) regardless of survival time (2, 4, or 8 weeks) and in comparison to those in control rats at both 2 and 8 weeks survival. Proceeding rostrally, there were also significantly more DiI+ve OECs in the dorsal funiculus of T10 at 4 (39.7 DiI+ve OECs) and 8 (20.9 DiI+ve cells) weeks after grafting in Group 5 rats as compared to Group 4 rats (Fig. 4.17). The average number of DiI+ve cells at T9 (5.5 – 12.8; Fig. 4.18) and at T8 (4.9 – 6.4; Fig. 4.19) in Group 5 rats was not significantly different from that of the other groups regardless of survival time, except for the cell counts at T8 for the 2 week time point (Fig. 4.19). However, the actual numerical difference between the cell counts for Groups 4 and 5 at T8 was so small it is questionable whether they are of any therapeutic interest. Either singly or in small groups the DiI+ve OECs were found along the septi of both dorsal funiculi and also within the neuropil of the right dorsal funiculus of T11-T10 in both Groups 4 and 5 rats.

FIGURE 4.16: Number of DiI+ve OECs in the dorsal funiculus of T11 in Groups 1, 4 and 5 rats.

Histograms depicting the mean number (+/- SEM) of DiI+ve Cells (Y-axis) in the dorsal funiculus of the T11 spinal cord segment of control rats (OECs@T12; Group 1) or rats injected with EtBr at either T12 (EtBr@T12/OECs@T12; Group 4) or T10 and T12 (EtBr@T10, T12/OECs@T12; Group 5) 2 (a), 4 (b) or 8 weeks (c) after grafting of OECs at T12 (n = 3 for each Group at each survival time). A one-way analysis of variance was applied to the data with the data for each survival time being analyzed separately for statistical significance. The resulting values of F were statistically significant at the 2 ($F = 12.51$; $p < 0.0075$), 4 ($F = 11.98$; 0.0085) and 8 ($F = 14.17$; $p < 0.006$) weeks time points. Multiple post hoc comparisons between Groups 1, 4 and 5 at each time point were performed with Bonferonni's multiple comparison post-test revealing a significant difference between Groups 4 and 5 at all survival times and Groups 1 and 5 at 2 and 8 weeks survival times. 1 asterisk = $p < 0.05$; 2 asterisks = $p < 0.01$.


*
**
100
75
50
25
0
Mean # of Dil+ve OECs
Group 1
Group 4
Group 5


**
100
75
50
25
0
Mean # of Dil+ve OECs
Group 1
Group 4
Group 5

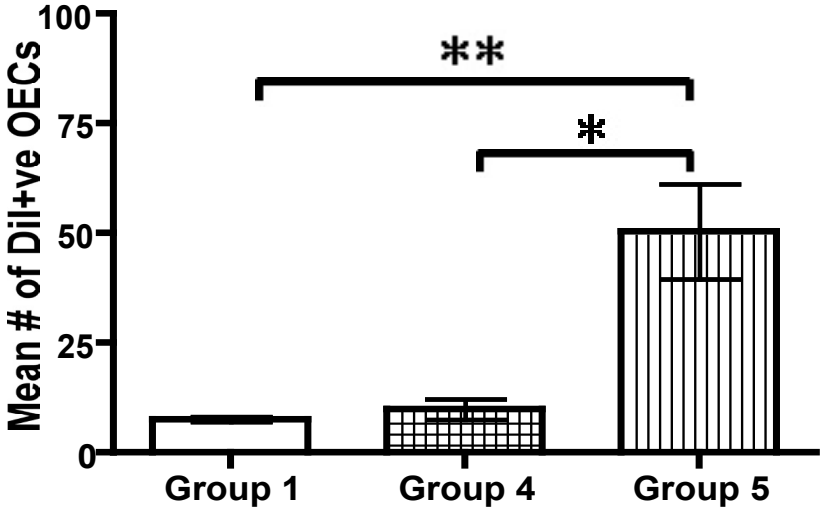
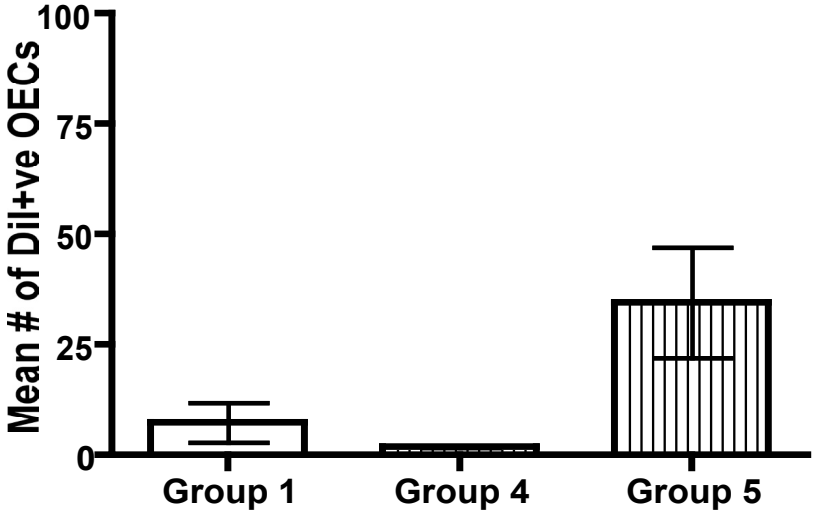
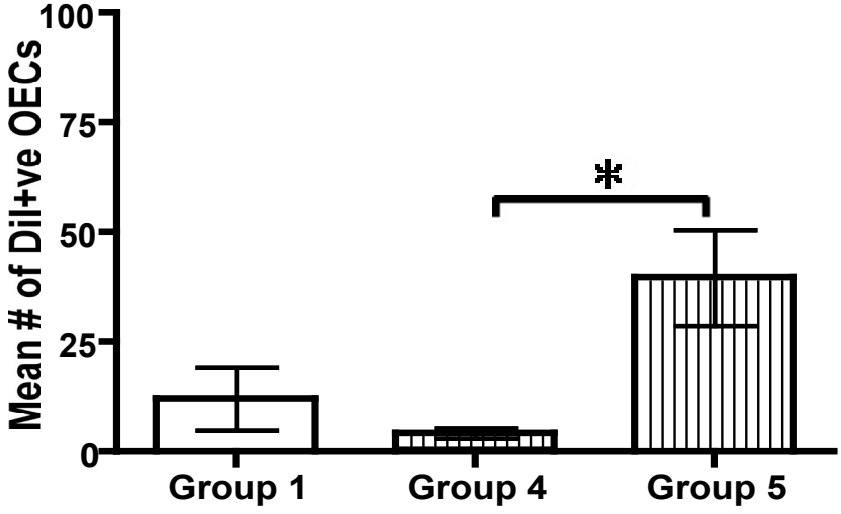

**
*
100
75
50
25
0
Mean # of Dil+ve OECs
Group 1
Group 4
Group 5

FIGURE 4.17: Number of DiI+ve OECs in the dorsal funiculus of T10 in Groups 1, 4 and 5 rats.

Histograms depicting the mean number (+/- SEM) of DiI+ve Cells (Y-axis) in the dorsal funiculus of the T10 spinal cord segment of control rats (OECs@T12; Group 1) or rats injected with EtBr at either T12 (EtBr@T12/OECs@T12; Group 4) or T10 and T12 (EtBr@T10, T12/OECs@T12; Group 5) 2 (a), 4 (b) or 8 weeks (c) after grafting of OECs at T12 (n = 3 for each Group at each survival time). A one-way analysis of variance was applied to the data with the data for each survival time being analyzed separately for statistical significance. The resulting values of F were statistically significant at the 4 ($F = 6.025$; $p < 0.04$) and 8 ($F = 22.02$; $p < 0.002$) weeks time points. Multiple post hoc comparisons between Groups 1, 4 and 5 were performed with Bonferonni's multiple comparison post-test revealing a significant difference between Groups 4 and 5 at 4 and 8 weeks survival times, as well as between Groups 1 and 5 at only the 2 week time point. 1 asterisk = $p < 0.05$; 2 asterisks = $p < 0.01$.


Mean # of DiI+ve OECs
100
75
50
25
0
Group 1
Group 4
Group 5


Mean # of DiI+ve OECs
100
75
50
25
0
*
Group 1
Group 4
Group 5

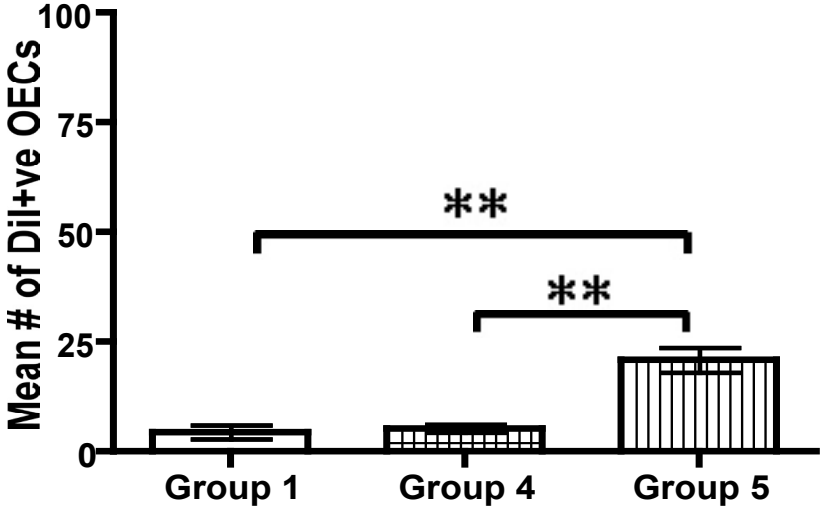
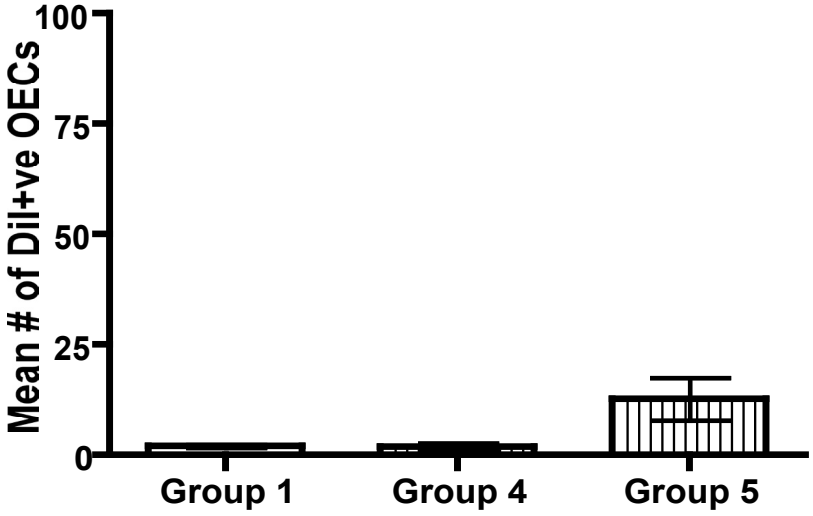
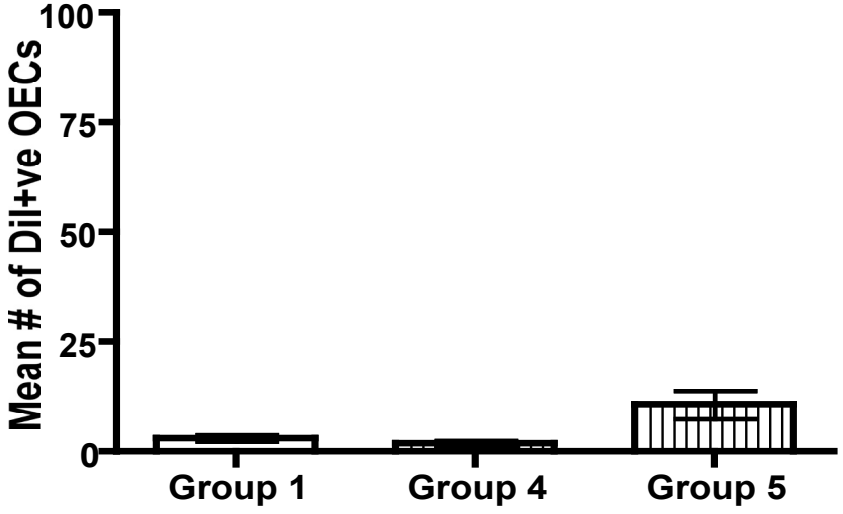

Mean # of DiI+ve OECs
100
75
50
25
0
**
**
Group 1
Group 4
Group 5

FIGURE 4.18: Number of DiI+ve OECs in the dorsal funiculus of T9 in Groups 1, 4 and 5 rats.

Histograms depicting the mean number (+/- SEM) of DiI+ve Cells (Y-axis) in the dorsal funiculus of the T9 spinal cord segment of control rats (OECs@T12; Group 1) or rats injected with EtBr at either T12 (EtBr@T12/OECs@T12; Group 4) or T10 and T12 (EtBr@T10, T12/OECs@T12; Group 5) 2 (a), 4 (b) or 8 weeks (c) after grafting of OECs at T12 (n = 3 for each Group at each survival time). A one-way analysis of variance was applied to the data with the data for each survival time being analyzed separately for statistical significance. The resulting values of F were not significantly different at any survival time.


Mean # of DiI+ve OECs
100
75
50
25
0
Group 1
Group 4
Group 5


Mean # of DiI+ve OECs
100
75
50
25
0
Group 1
Group 4
Group 5

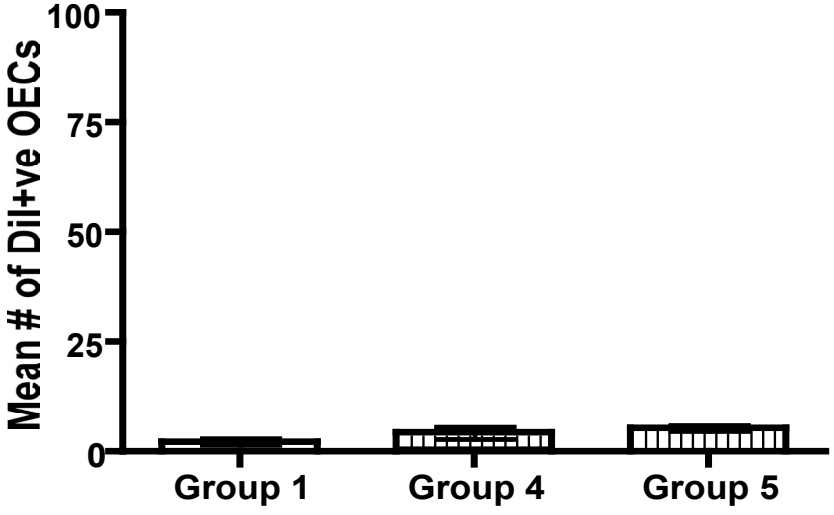
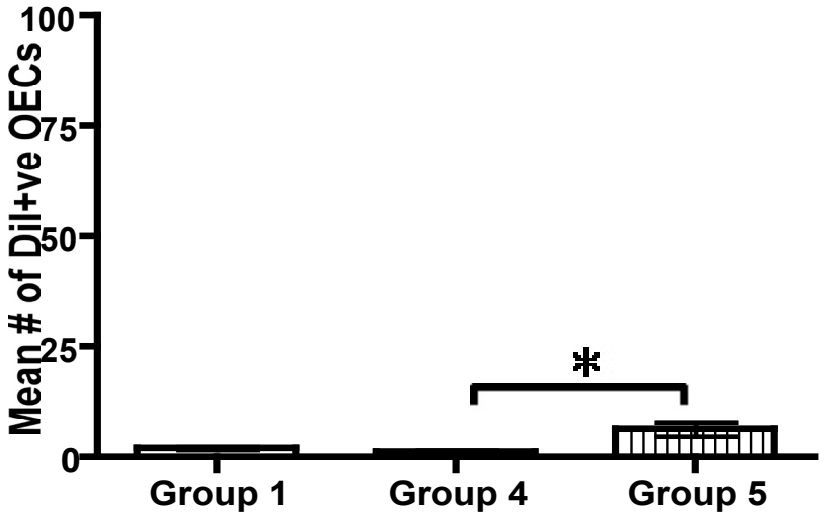
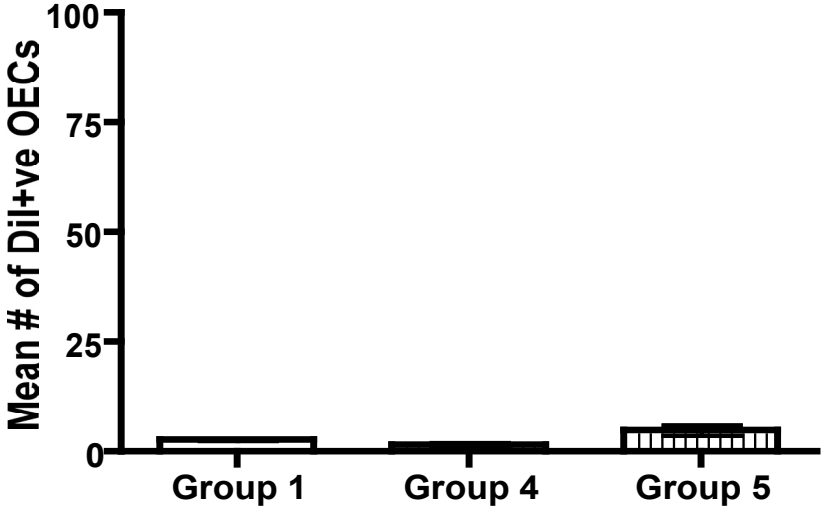
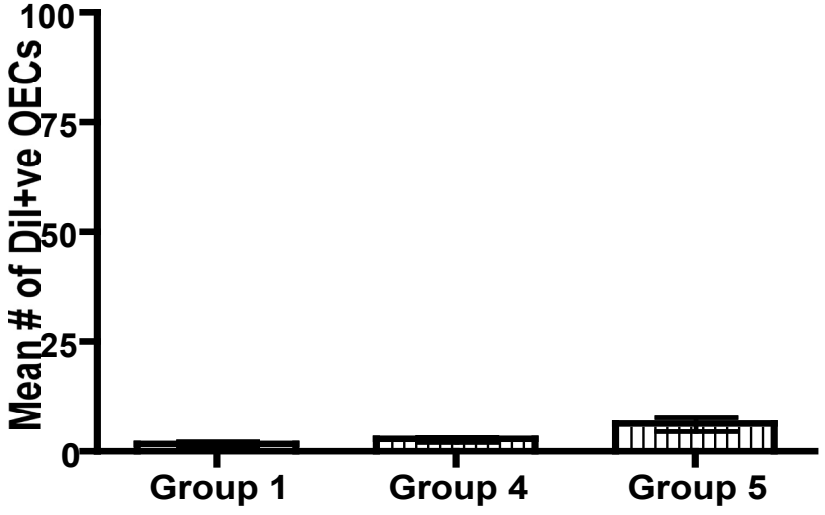

Mean # of DiI+ve OECs
100
75
50
25
0
Group 1
Group 4
Group 5

FIGURE 4.19: Number of DiI+ve OECs in the dorsal funiculus of T8 in Groups 1, 4 and 5 rats.

Histograms depicting the mean number (+/- SEM) of DiI+ve Cells (Y-axis) in the dorsal funiculus of the T8 spinal cord segment of control rats (OECs@T12; Group 1) or rats injected with EtBr at either T12 (EtBr@T12/OECs@T12; Group 4) or T10 and T12 (EtBr@T10, T12/OECs@T12; Group 5) 2 (a), 4 (b) or 8 weeks (c) after grafting of OECs at T12 (n = 3 for each Group at each survival time). A one-way analysis of variance was applied to the data with the data for each survival time being analyzed separately for statistical significance. The resulting values of F (8.429) were statistically significant ($p < 0.02$) only at the 2 week time point. Multiple post hoc comparisons between Groups 1, 4 and 5 at the 2 week time point were performed with Bonferonni's multiple comparison post-test revealing a significant difference between Groups 4 and 5 ($p < 0.05$; 1 asterisk). However, the numerical difference in cell counts was so small as to make it questionable whether the observed differences were therapeutically of any interest.


Mean # of DiI+ve OECs
100
75
50
25
0
*
Group 1
Group 4
Group 5


Mean # of DiI+ve OECs
100
75
50
25
0
Group 1
Group 4
Group 5


Mean # of DiI+ve OECs
100
75
50
25
0
Group 1
Group 4
Group 5

4.6 Microglial Reactivity Contributes To The Migratory Signal

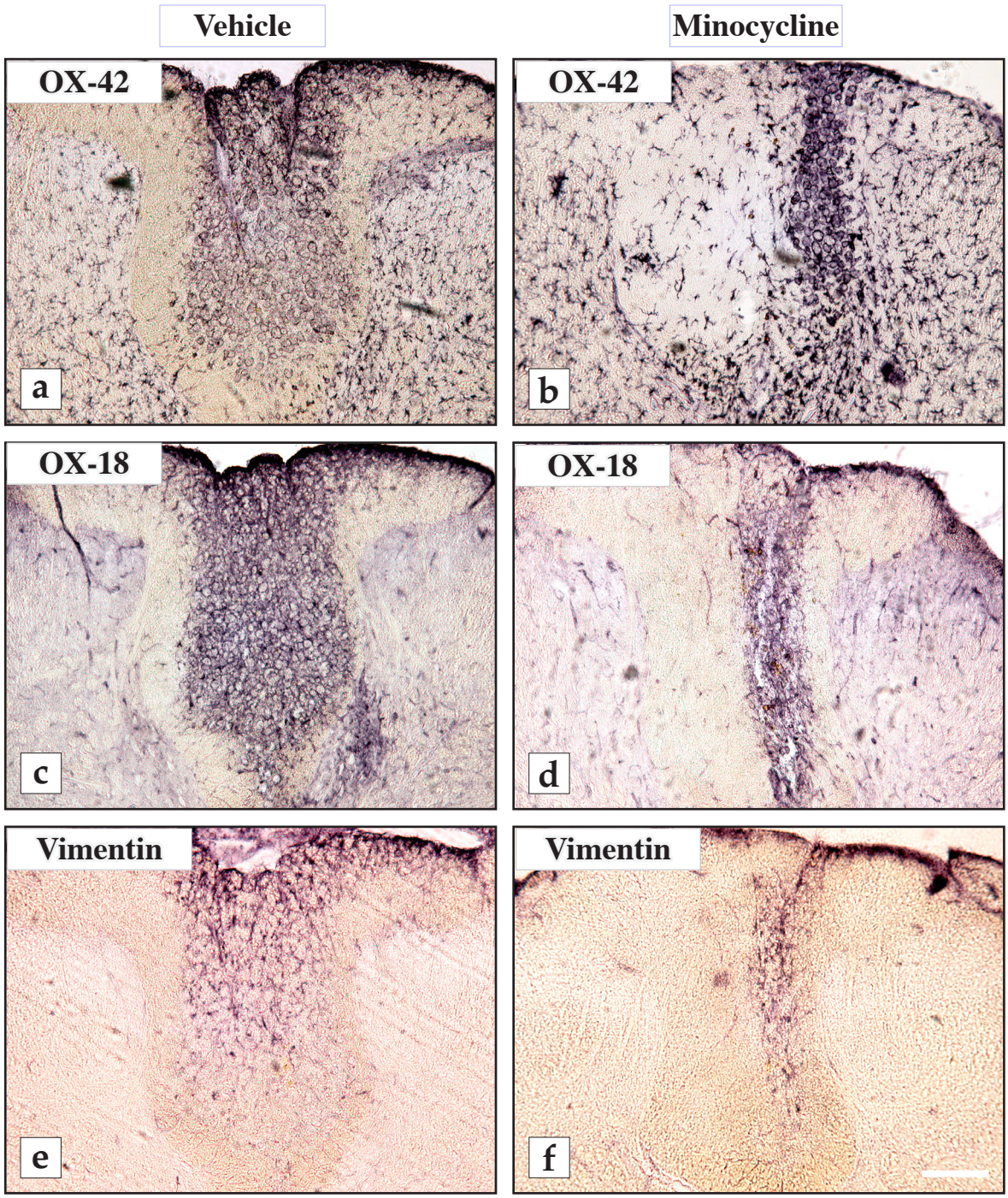
To address specific aim #4, systemically administered minocycline was used to reduce the activation of microglia both at the demyelinating lesion(s) and along the white matter extending throughout the ipsilateral dorsal funiculus of T11. Six rats (Group 6; see Table 4.1) received an injection of 1 μl of 0.03% EtBr in the right dorsal funiculus of the T10 spinal cord, followed three days later by an injection of DiI-labelled OECs into the right dorsal funiculus of the T12 spinal cord segment (Fig. 4.4g). An additional six rats (Group 7; see Table 4.1) received an injection of 1 μl of 0.03% EtBr in the dorsal funiculus of both the T10 and the T12 spinal cord segments, followed three days later by an injection of DiI-+ve OECs into the right dorsal funiculus of the T12 spinal cord segment (Fig. 4.4h). Half of the rats in each group received daily intraperitoneal injections of minocycline while the remainder rats received vehicle (see Section 2.4.7) for the first four weeks after grafting the cells. This four week treatment schedule was chosen to ensure the anti-inflammatory actions of minocycline would be active for at least the first four weeks since it was during this time that the first signs of lesion-induced migration of OECs was seen in the experiment of Section 4.4. All rats in both Groups 6 and 7 were killed 8 weeks after receiving an OEC graft.

4.6.1 Ethidium Bromide Lesion at T10 (Group 6 rats)

The spinal cord tissues of Group 6 rats were stained with antibodies that identified reactive microglia (OX42 and OX18 monoclonal antibodies) and reactive astrocytes (anti-GFAP and anti-vimentin antisera) to confirm that the minocycline treatment had been effective in preventing the activation of microglia and in assessing the treatment's effect on astrogliosis. In the saline-treated, OX-42 (Fig 4.20a) and OX-18-expressing (Fig. 4.20c) microglia were located in groups occupying the entire right dorsal funiculus of T11. Visibly fewer OX-42+ve (Fig. 4.20b) and small OX-18+ve (Fig. 4.20d) microglia with short processes were identified within the T11 right dorsal funiculus of Group 6 rats treated with minocycline. No cell counts of microglia or other tests to determine the level of OX-42 and OX-18 expression were performed on spinal cord tissue of Group 6 rats treated with either saline or

FIGURE 4.20: Microglial and astrocytic reactivity in the dorsal funiculus of T11 in Group 6 rats.

OX-42+ve (a, b) and OX-18+ve (c, d) microglia, and vimentin+ve (e, f) astrocytes in the dorsal funiculus of T11 at 8 weeks after grafting OECs at T12 in Group 6 rats receiving minocycline (b, d & f) or vehicle (a, c & e) for the first 4 weeks after EtBr injection at T10. The minocycline treatment reduced the microglial reactivity as evidenced by both OX-42 (b) and OX-18 (d) immunostaining as compared to vehicle controls (a, c). There was no noticeable difference in the density of vimentin+ve astrocytes between the vehicle and minocycline treated groups, although the medial-lateral extent of the astrocytic reactivity was smaller after minocycline treatment. Bar = 400 μm


Vehicle
Minocycline
OX-42
a
OX-42
b
OX-18
c
OX-18
d
Vimentin
e
Vimentin
f

minocycline. Vimentin+ve astrocytes were primarily identified in the spinal cord tissue adjacent to the pial surface and within the right dorsal funiculus of the Group 6 rats regardless of treatment with saline (Fig. 4.20e) or minocycline (Fig. 4.20f).

Tissue sections from the T11 spinal cord segment of each Group 6 rat were selected from the rostral, middle and caudal thirds of the segment and examined to count the number of DiI+ve cells. In Group 6 rats treated with saline, the numbers of DiI+ve OECs found within the T11 dorsal funiculus averaged 49.3 per 40 μm tissue section (Fig. 4.21a), values that were similar to those of Group 2 rats that survived for 8 weeks. However, the average number of DiI+ve OECs found in the same location in Group 6 rats treated with minocycline for the first 4 weeks after EtBr injection was only 6.8 per 40 μm tissue section, which was more than 7 fold less than that observed in the vehicle control rats. Mostly single DiI+ve OECs were localized to septi/blood vessels of the right dorsal funiculus of Group 6 rats after minocycline treatment (Fig. 4.21b). Similar to that described for Group 2 rats, single and small groups of DiI+ve OECs were found throughout the neuropil as well as along the septi predominantly of the right dorsal funiculus of the saline-treated rats of Group 6 (Fig. 4.21c).

4.6.2 Ethidium Bromide Lesions at T10 and T12 (Group 7 rats)

Tissue sections from the rostral, middle and caudal thirds of T11 in Group 7 rats were examined for the presence and the location of reactive microglia and reactive astrocytes. Similar to that seen in Group 2 and 6 rats after 8 week survival, OX-42-expressing microglia scattered throughout the grey and white matter as single cells with small round cell body and short, branched processes (Fig. 4.22a & b). Under light microscopy, there were visibly more OX-42 (Fig. 4.22a) and OX-18+ve (Fig. 4.22c) microglia present within the right dorsal funiculus of Group 7 rats treated with saline than that observed in corresponding spinal cord area of Group 7 rats treated with minocycline (Fig. 4.22b &d). Neither cell counts of microglia nor other tests to determine the level of OX-42 and OX-18 expression were performed on spinal cord tissue of Group 7 rats treated with either saline or minocycline. GFAP+ve astrocytes were distributed throughout the grey and white matter of T11 spinal cord sections with

FIGURE 4.21: DiI+ve OECs in the dorsal funiculus of T11 in Group 6 rats.

a) A histogram depicting the mean number (+/- SEM) of DiI+ve cells (Y-axis) in the dorsal funiculus of the T11 spinal cord segment of rats injected with EtBr at T10 (EtBr@T10/OECs@T12; Group 6) and OECs at T12 and treated with either saline or with minocycline for the first 4 weeks after EtBr injection (n = 3 for each Group). The Mann-Whitney U test was applied to the data. The resulting value of U (9.0) was statistically significant ($p < 0.05$; 1 asterisk) thus supporting the hypothesis of the importance of the microglial reactivity in contributing to the strength of the migratory signal(s). b & c) Fluorescent images of the dorsal funiculus of the T11 spinal cord segment of Group 6 rats. All rats survived for 8 weeks after grafting at which time the saline treated rats (c) had several fold more DiI+ve cells (white arrows) in the dorsal funiculus of T11 as compared to that of minocycline treated rats (b). Bar = 100 μm and applies to both 'b' and 'c'.

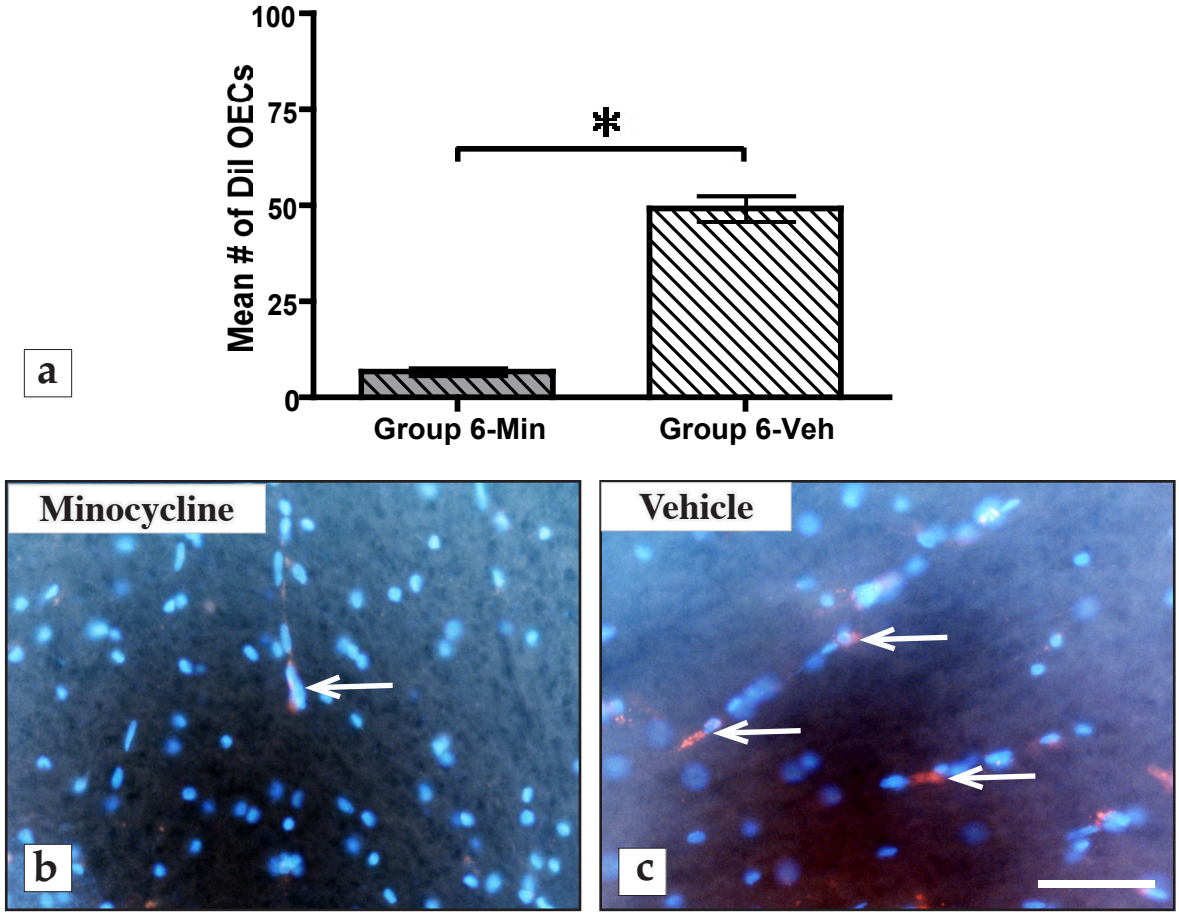
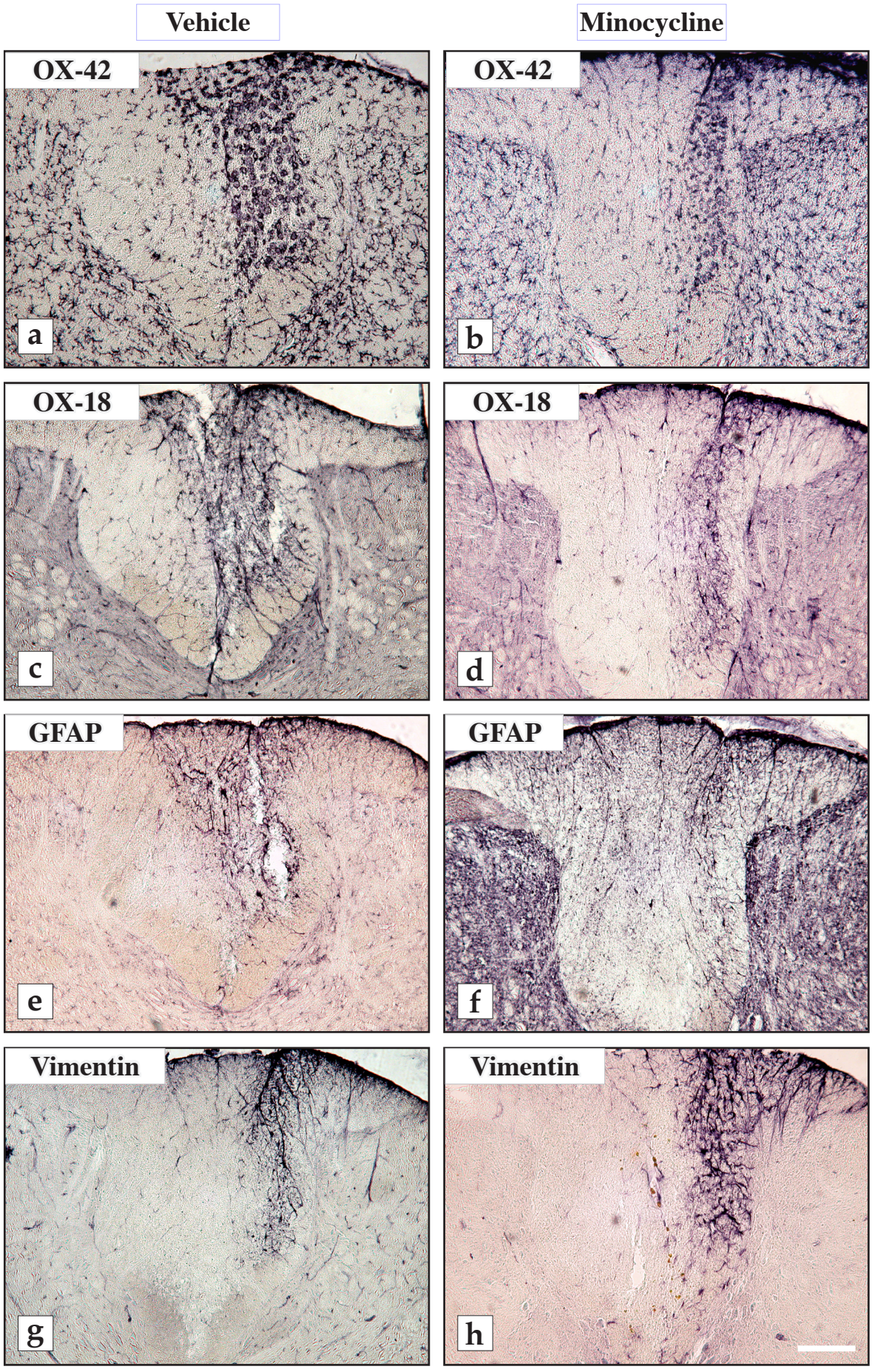

a
100
75
50
25
0
Mean # of DiI OECs
*
Group 6-Min
Group 6-Veh
Minocycline
b
Vehicle
c

FIGURE 4.22: Microglial and astrocytic reactivity in the dorsal funiculus of T11 in Group 7 rats.

OX-42+ve (a, b) and OX-18+ve (c, d) microglia, and GFAP+ve (e, f) and vimentin+ve (g, h) astrocytes in the dorsal funiculus of T11 at 8 weeks after grafting OECs at T12 in Group 7 rats. The minocycline treatment dramatically reduced the microglial reactivity as evidenced by both OX-42 (b) and OX-18 (d) immunostaining as compared to vehicle controls (a, c). There was no significant difference in the density of GFAP+ve (e, f) or vimentin+ve (g, h) astrocytes between the vehicle and minocycline treated groups. Bar = 400 μm


Vehicle
Minocycline
OX-42
a
OX-42
b
OX-18
c
OX-18
d
GFAP
e
GFAP
f
Vimentin
g
Vimentin
h

some accumulating in groups within the right dorsal funiculus of Group 7 rats treated with either saline (Fig. 4.22e) or minocycline (Fig. 4.22f). There was an accumulation of vimentin-expressing astrocytes in the right dorsal funiculus and at the pial surface of T11 of all Group 7 rats regardless of treatment (Fig. 4.22g & h). In contrast to observed differences in microglia distribution, there was no such effect of minocycline on either GFAP- or vimentin-expressing astrocytes in comparison to that after saline treatment.

The location and the numbers of DiI+ve OECs were also determined in the rostral, middle and caudal thirds of the T11 spinal cord segment in Group 7 rats. In the three saline-treated rats of this group, the number of DiI+ve OECs averaged 60.4 per 40 μm tissue section, which was similar to that observed in Group 5 rats at 8 weeks survival (Fig. 4.23a). However, the average numbers of DiI+ve OECs found in the right dorsal funiculus of T11 in minocycline-treated Group 7 rats was significantly lower, being only 19.9 DiI+ve OECs per 40 μm tissue section, which was 3 fold less than that observed in saline-treated rats. Single DiI+ve OECs were found migrating along septi of T11 dorsal funiculi in Group 7 rats treated with minocycline (Fig. 4.23b). However, migrating DiI+ve OECs were found throughout the neuropil and along septi of the T11 spinal cord segment of the Group 7 rats injected with saline instead (Fig. 4.23c).

4.7 Grafting Retroviral-Infected OECs

To address specific aim #5, OECs were infected with a replication-deficient retrovirus (RV) containing the Lac-Z gene and a neomycin-resistance gene (Fig. **4.**24). Geneticin-selected LacZ-expressing OECs were either injected into the right dorsal funiculus of the T12 spinal cord segment or grafted onto the pial surface at the level of the T12 spinal cord segment.

4.7.1 Retroviral-Infected OECs *In Vitro*

OEC cultures were obtained from the olfactory bulbs of E18 rat embryos as described in Section 2.1.1. After 10 days in mG5 medium (Fig. 4.24a), the cells were exposed to the RV (Fig. 4.24b). One control flask from each set of cultures was fed with DMEM/F12/10%FBS medium containing no RV (Fig. 4.24c). After the two week

FIGURE 4.23: DiI+ve OECs in the dorsal funiculus of T11 in Group 7 rats.

a) A histogram depicting the mean number (+/- SEM) of DiI+ve cells (Y-axis) in the dorsal funiculus of the T11 spinal cord segment of rats injected with EtBr at both T10 and T12 (EtBr@T10, T12/OECs@T12; Group 7) and OECs at T12 and treated with either saline or with minocycline for the first 4 weeks after EtBr injections (n = 3 for each Group). The Mann-Whitney U test was applied to the data. The resulting value of U (9.0) was statistically significant ($p < 0.05$; 1 asterisk) thus supporting the hypothesis of the importance of the microglial reactivity in contributing to the strength of the migratory signal(s). b & c) Fluorescent images of the dorsal funiculus of the T11 spinal cord segment of Group 7 rats. All rats survived for 8 weeks after grafting at which time the saline treated rats (c) had several fold more DiI+ve cells (white arrows) in the dorsal funiculus of T11 as compared to that of minocycline treated rats (b). Bar = 100 μm and applies to both 'b' and 'c'.

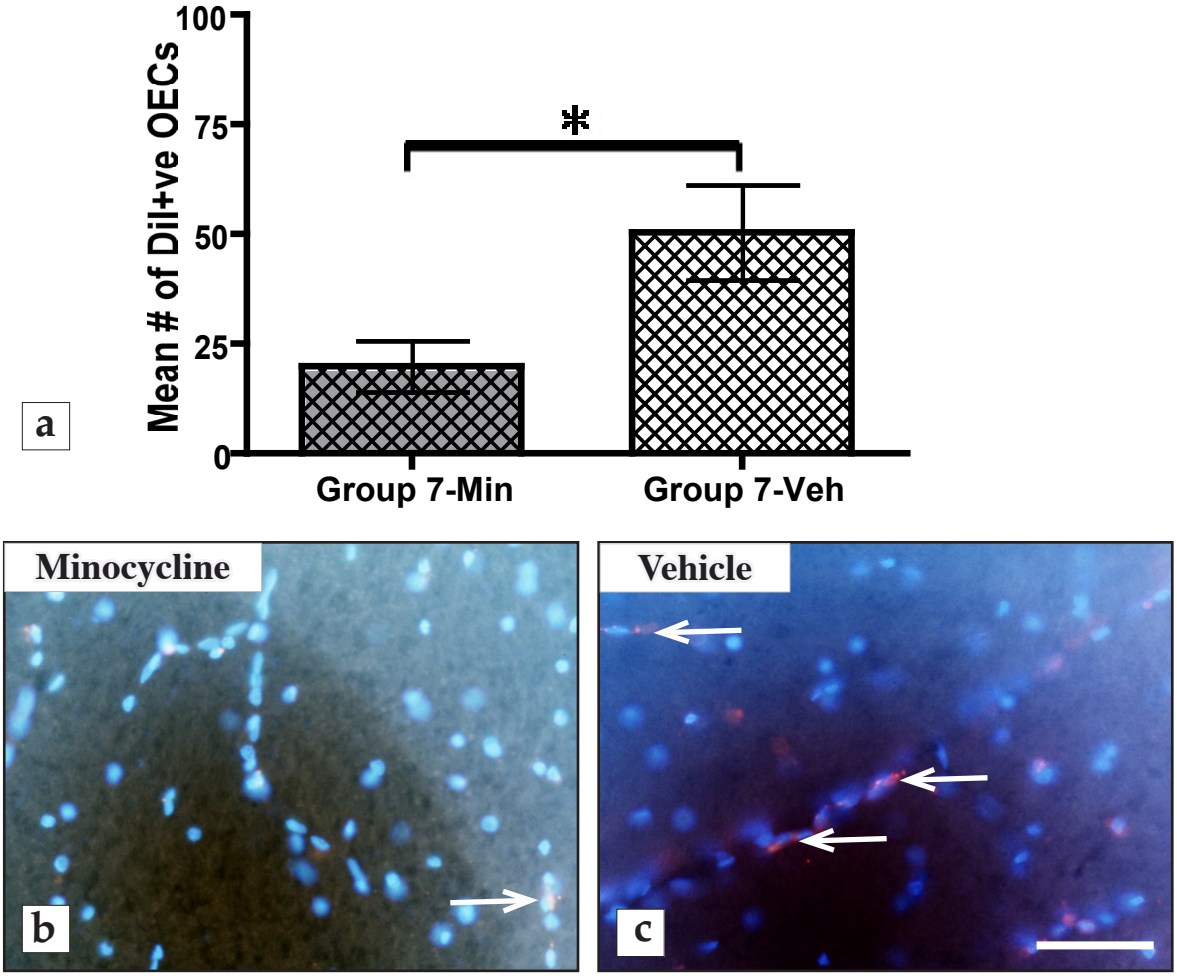
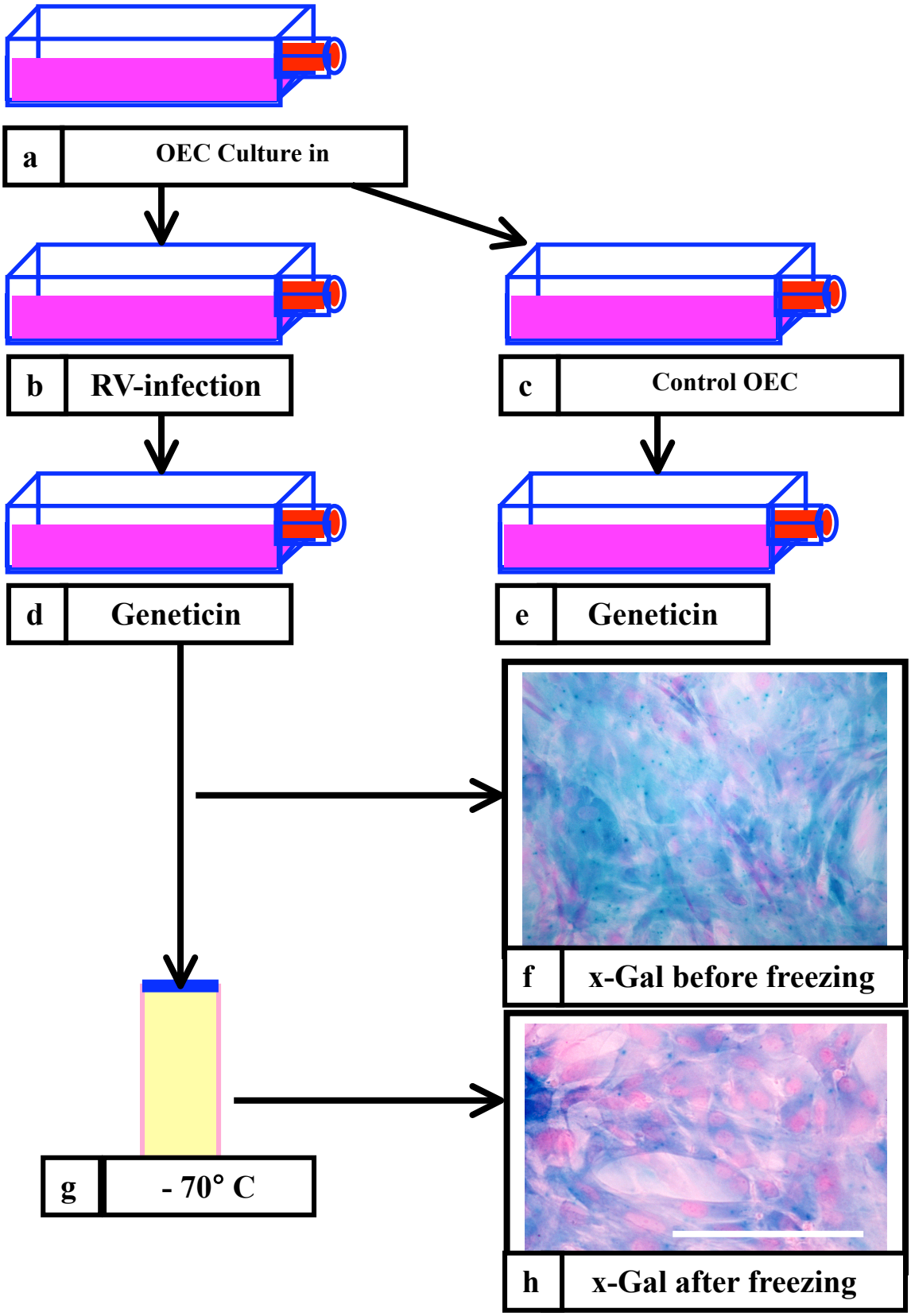

a
Mean # of DiI+ve OECs
100
75
50
25
0
*
Group 7-Min
Group 7-Veh
Minocycline
b
Vehicle
c

FIGURE 4.24: Schematic description of retroviral infection of OEC cultures.

(a) OEC cultures in serum-free medium (mG5). b) RV-infection starts when the OEC cultures are switched from mG5 to serum-containing medium. c) As a control, selected flasks are fed with medium containing no RV supernatant. d & e) Both control and RV-infected flasks are fed with medium containing geneticin for the duration of time *in vitro*. Cells in the control flasks were killed by the geneticin treatment within 4-5 days *in vitro*. g) RV-infected and geneticin-selected OECs are frozen for long term storage in the vapor phase of liquid nitrogen. f & h) x-Gal staining of representative OECs from an RV-infected cell culture at the time they were harvested for freezing (f) and in a cell culture initiated from a cell suspension of OECs that had been grafted onto the pial surface of the spinal cord (h). Bar = 200 μm


a
OEC Culture in
b
RV-infection
c
Control OEC
d
Geneticin
e
Geneticin
f
x-Gal before freezing
g
- 70° C
h
x-Gal after freezing

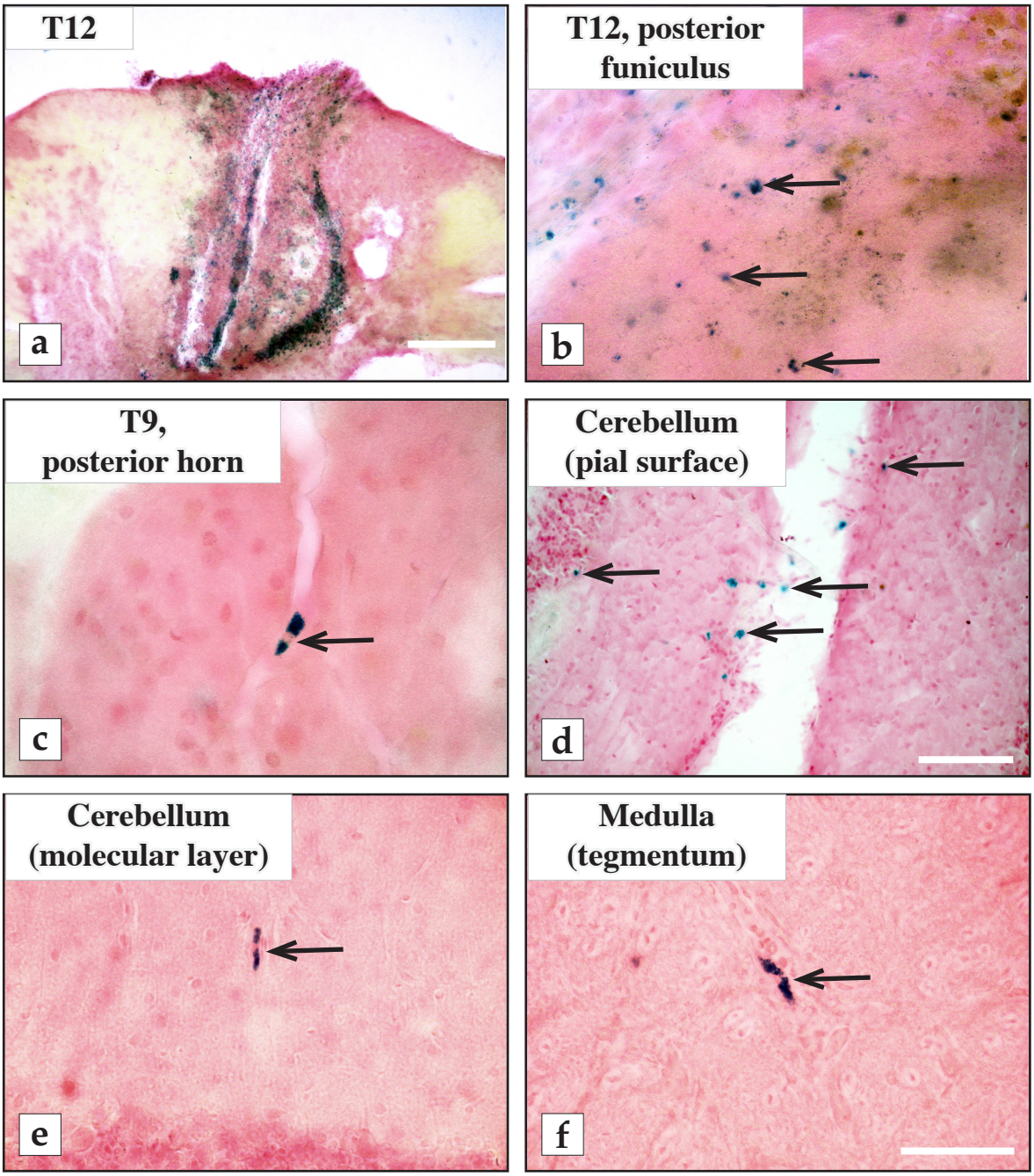
period, both infected (Fig. 4.24d) and uninfected control (Fig. 4.24e) cells were exposed to geneticin. Close monitoring of control cultures determined that treatment with geneticin for 4-5 days killed 99% cells in uninfected cultures. At this time, 50-70% of the cells in cultures infected with RV survived the geneticin treatment. Then, OEC cultures were expanded with the DMEM/F12/10%FBS medium with no geneticin. Cell cultures approached confluency within one month. In order to eliminate persisting uninfected cells if present, OEC cultures were exposed to geneticin for 2 additional days at this time. After the second treatment with geneticin, OEC cultures from each set were harvested to assay the cells for β-galactosidase expression. Every single cell in all OEC cultures with a fusiform cell body and flat processes contained blue particles, the protein products of β-galactosidase activity, which densely packed the entire cell cytoplasm but not cell nuclei (Fig. 4.24f). The remaining OEC cultures were then harvested for storage at -70°C (Fig. 4.24g). RV-infected OECs were used later for implantation in experiments described in Sections 4.7.2 and 4.7.3. After grafting, the remaining cell suspensions were plated onto glass coverslips and grown in vitro for 48 hours to assay the Lac-Z-expression in OECs after freezing and thawing. As prior to freezing, all OECs expressed β-galactosidase as seen by the presence of blue particles throughout the cell cytoplasm but not in cell nuclei (Fig. 4.24h).

4.7.2 OEC Grafts Into Dorsal Funiculus

Prior to addressing the Specific Aim #5, RV-infected OECs were grafted into the lower thoracic spinal segment in order to confirm that β-Gal-expressing OECs could be detected *in vivo*. RV-infected OECs were stereotactically injected into the right dorsal funiculus of the T12 spinal cord segment of one rat. This rat did not receive any other experimental manipulations and was sacrificed 4 days after grafting. The rat was perfused with 4% paraformaldehyde and free-floating 40 μm thick coronal sections were cut through the spinal cord. The tissue sections were stained with x-Gal (to identify β-galactosidase+ve cells) and counterstained with Fast Red (to identify all cells in the tissue section) (see Section 2.5.8). The grafted OECs occupied the entire right dorsal funiculus at the grafting site and were easily identified by virtue of their blue, x-Gal-staining (Fig. 4.25a). Within the graft, OECs were closely packed in groups of 20-

FIGURE 4.25: β-Gal+ve OECs in the spinal cord, brainstem and cerebellum

a & b) These micrographs are of the T12 segment of the spinal cord in a rat that had received an injection of OECs expressing β-Gal into the dorsal funiculus of this segment 4 days previously. Numerous β-Gal+ve cells (blue staining) can be seen (arrows in 'b'). c-f) After being grafted onto the pial surface at the level of T12, β-Gal+ve cells could be seen in close association with blood vessels in rostral spinal cord segments (e.g. the dorsal horn of T9 as shown in 'c'). These β-Gal+ve were also found along the pial surface of the cerebellum (d), which presumably represents one possible route by which the cells could gain access to the neuropil of the CNS such as the molecular layer of the cerebellum (e). The β-Gal+ve cells were also seen associated with blood vessels in the tegmentum of the brainstem (f). All of these sections were counter stained with Neutral fast Red. Bar = 400 μm (a), 400 μm (b, d), and 200 μm (c, e & f).


T12
a
T12, posterior funiculus
b
T9, posterior horn
c
Cerebellum (pial surface)
d
Cerebellum (molecular layer)
e
Medulla (tegmentum)
f

30. X-Gal stained OECs had round cell bodies with short processes (Fig. 4.25b).

4.7.3 OEC Grafts Onto Pial Surface

RV-infected OECs were also grafted onto the pial surface of the right dorsal funiculus of the T12 spinal cord segment of three rats, with one rat being killed at each of 11 days, 1 month and 2 months after grafting. These rats received no other experimental manipulation aside from the OEC graft. In the spinal cord tissue obtained from the cervical and thoracic levels of the rat killed at 11 days after grafting, β-Gal+ve cells were distributed across the coronal plane of the spinal cord sections with no particular pattern. These cells were lying along the septi/blood vessels of the dorsal, lateral and anterior funiculi, but were never seen lying close to the pial surface of the spinal cord. The β-Gal+ve cells were found singly or in small groups of 2-4 cells. Within the white matter, β-Gal+ve cells were sometimes found within the perivascular space in close apposition to the endothelial lining of blood vessels. Some β-Gal+ve cells were also found in the grey matter, including the dorsal and anterior horns and Clark's nucleus. Within the grey matter the β-Gal+ve cells were found in close apposition to blood vessels whereas others were closely apposed to neuronal cell bodies. The RV-infected OECs very likely had floated into the CSF shortly after the graft had been placed on the pial surface, since no cells were found on the pial surface of the spinal cord of any tissue section examined. The number of migrating cells varied from 40 to 50 cells per section at the rostral part of T12 to 20-30 per section at the midportion of T8 to 5-10 per section in the lower medulla. Cell counts for sections of rostral part of T12 and for sections from the middle part of T8 are presented in Table 4.2. The medulla was the most rostral part of the CNS examined in this rat. No cell count was performed on tissue sections from cervical and lumbar segments of this rat.

In the remaining rats, one killed one month after grafting and the second rat at two months, β-Gal+ve cells were found within the white and grey matter of the cervical, thoracic and lumbar spinal cord segments in a similar distribution to that described above for the rat killed 11 days after receiving a pial surface graft. In addition, the brain stem, cerebellum, cerebral hemispheres and olfactory bulbs were also examined to determine whether they contained any β-Gal+ve cells. β-Gal+ve OECs were found in

the white and grey matter of the brain stem where they were found along septi/blood vessels or located close to neuronal cell bodies. A few single β-Gal+ve cells were also found within the olfactory bulbs and on the pial surface of the cerebellum as well as within the Purkinje cell layer (Fig. 4.25d). β-Gal+ve OECs were not seen in the cortex or white matter of the frontal, parietal and temporal lobes of either rat brain in coronal sections collected from the anterior pole of the frontal lobe back to the level of the optic chiasm. β-Gal+ve OECs were also absent from the basal ganglia and diencephalon.

Table 4.2 Number of β-Gal-expressing OECs found in the T12 and T8 spinal cord segments after a pial surface graft

Spinal Cord Segment	T12, rostral part			T8, middle part		
Section:	1st	2nd	3rd	1st	2nd	3rd
WHITE MATTER						
Anterior funiculus	4	3	5	3	3	3
Right lateral funiculus	3	0	1	3	2	1
Left lateral funiculus	2	0	1	2	1	3
Dorsal funiculi	3	0	1	5	5	0
GREY MATTER						
Right anterior horn	9	7	4	4	6	7
Left anterior horn	8	7	8	6	4	6
Right dorsal horn including Clark's nucleus	5	8	8	4	3	2
Left dorsal horn including Clark's nucleus	8	3	5	2	1	3

5 DISCUSSION.

5.1 Animal Models of CNS Demyelination for Studying the *In Vivo* Migratory Ability of Myelinating Glia

Several animal models have been developed to study the morphological, cellular and molecular mechanisms regulating demyelination and remyelination of CNS axons. Each model is designed to mimic some, but not all, of the pathological and neurological sequelae of MS. The most commonly used animal models (see Section 1.6) are: a) toxin-induced demyelination, such as with EtBr (Yajima and Suzuki, 1979), lysolecithin (Hall, 1972) or cuprizone (Blakemore, 1972, 1973; Matsushima and Morell, 2001); b) autoimmune-induced demyelination, the most common being experimental autoimmune encephalomyelitis (EAE) (Lyman *et al.,* 1985; Traugott *et al.*, 1985); and c) viral-induced demyelination [e.g., hepatitis virus (Herndon *et al.*, 1977) or murine viral encephalomyelitis (Lang *et al.*, 1984)]. Although each of these models are useful for addressing some of the questions concerning the mechanisms driving the demyelination and remyelination of CNS axons, most are of limited use in identifying factors important for inducing migration of the cells needed to assist in neural repair. Cell migration towards the site of injury is an important area of concern as part of any therapeutic approach to treating hypomyelinating or demyelinating disorders that involve glial cell transplantation. The grafted cells must be able to respond to migratory signals directing them to migrate towards focal areas of demyelination, such as the isolated plaques in the spinal cord of an MS patient. For this research project, the EtBr model of CNS demyelination was chosen because with the concentration (0.03%) and volume (1 μl) of injected neurotoxicant chosen both the location and the size of the lesion could be controlled.

The EtBr model used in this research project was modified in two major ways from that used by other investigators (Yajima and Suzuki, 1979; Blakemore, 1984;

Iwashita and Blakemore, 2000). Firstly, the neurotoxicant was injected at a concentration [i.e., 0.03% as opposed to 0.1% (Yajima and Suzuki, 1979; Franklin *et al*., 1996; Iwashita and Blakemore, 2000) or 0.3% (Honmou *et al.,* 1996)] that demyelinated only one half of the dorsal funiculus over a rostral-caudal length of less than 1 mm. Secondly, x-irradiation was not used thus permitting host glial cells to respond properly to the lesion and initiate neural repair. Previous investigators have made much larger EtBr-demyelinated lesions (7-8 mm in length) in the spinal cord of adult rats in combination with x-irradiation (Akiyama *et al*., 2001, 2002a, 2002b; Blakemore and Crang, 1985; Franklin *et al*., 1996; Honmou *et al*., 1996) to study the migratory ability of oligodendrocyte progenitor cells (Duncan *et al.,* 1992; Franklin and Blakemore, 1997; Groves *et al.*, 1993; Nait-Oumesmar *et al.*, 1999; O'Leary and Blakemore, 1997; Reynolds *et al.*, 2001; Warrington *et al*., 1993) and Schwann cells (Baron-Van Evercooren *et al*., 1992; Blakemore and Crang, 1985, 1989; Blakemore *et al.*, 1995; Franklin et al., 1997; Gilson and Blakemore, 2002). In spite of the larger size of these lesions, there was no detectable Schwann cell migration nor did the oligodendrocyte progenitor cells migrate more than a couple of mm towards the lesion (Baron-Van Evercooren *et al*., 1992; Blakemore *et al.,* 2002; Chari *et al*., 2003; Franklin *et al*., 1996, 1997; Franklin and Blakemore, 1997). Other than these few studies, there has been no systematic examination of the factors inducing or guiding the migration of myelinating glia using an *in vivo* model in the CNS of adult mammals. The EtBr model used in the present research project can be used to study the migratory ability of myelinating glia in response to single or multiple small, focal (<1 mm long) gliotoxin-induced demyelinated lesions in the dorsal funiculus (unilaterally) of the adult rat spinal cord. Due to the small size of the lesions, the EtBr can even be injected at variable distances from the site of the graft. With this EtBr model it will be possible to identify factors that induce or guide the migration of myelinating glia, as was done in the present project where microglial reactivity was identified as one potential source of a migratory signal inducing OECs to migrate over distances of up to 20 mm towards a more rostrally located focal demyelination.

This research project focused on the migratory abilities of OECs grafted into the adult rat spinal cord in either the presence or absence of a focal demyelinated lesion(s). One μl of a 0.03% EtBr solution was injected at different levels of the lower thoracic spinal cord (i.e., at T8, T10 or T12, or at both T10 and T12) in separate groups of rats. Three days after the EtBr injection, OECs were grafted into the right dorsal funiculus of the T12 spinal cord segment. The experimental design gave control over both the location and the size of the demyelinated lesion as well as the distance of the lesion from the OEC graft. This EtBr model was used to study: a) the migration of OECs towards a single demyelinated lesion located either two or four spinal cord segments away from the cell graft; b) the migration of OECs away from a focal EtBr-demyelinated area of CNS white matter in the presence and absence of a second EtBr lesion located two spinal cord segments away; c) the contribution of microglial reactivity to the migratory signal(s) inducing OECs to migrate when EtBr was used to make a focal demyelinated lesion in CNS white matter.

In order to study the migratory ability of any type of myelinating glia grafted into an animal's CNS, it is important to have a model in which the number, location and size of the demyelinated lesions can be reproducibly controlled. The EtBr model used in this research project has several advantages over the models used by other investigators to study demyelination and remyelination of CNS axons. First, the precise location of the EtBr injection can be controlled as well as the size of the demyelinated area. Second, by eliminating x-irradiation from the experimental design, host glia can contribute to neural repair at the site of the EtBr injection thus contributing to the generation of a more natural response by cells of the host animal. By allowing the host glial cells to contribute to neural repair, the dorsal funiculus at the site of EtBr injection appeared to be almost completely repaired by 8 weeks, as judged by Luxol Fast Blue staining (Fig. 4.2f). With these modifications included in the EtBr model used in this research project, reactive microglia were identified as one potential source of a migratory signal inducing OECs to migrate towards a focal EtBr-induced demyelination.

In the autoimmune- or viral-induced *in vivo* animal models of CNS demyelination the lesions are typically induced by injections of MBP or MOG (i.e., the EAE model; Raine *et al*., 1974, 1977, Raine and Traugott, 1983, 1984; Steinman, 1999) or by infecting the animal with Theiler's virus (murine encephalomyelitis model; Dal Canto *et al*., 1996; Lipton and Jelachich, 1997; Monteyne *et al*., 1997; Oleszak *et al*., 1995; Tsunoda and Fujinami, 1996), respectively. The demyelinated lesions in these animal models are found scattered throughout the CNS white matter with no consistent location or size. It would be extremely difficult to identify factors regulating the migration of any of the myelinating glia when both the location and the size of the demyelinated lesion cannot be consistently controlled. Therefore, a direct injection of a chemical agent (e.g., EtBr) into the CNS white matter is much more appropriate for creating a focal demyelination in each animal that is in a precise location and is of a predetermined size. If these criteria are met, then the myelinating glia can be injected at variable distances from the focal demyelination to study their migratory abilities and the signal(s) regulating their migration.

5.2 Migration of Myelinating Glia in the CNS

Remyelination of CNS axons remains an important goal of researchers who are working towards developing therapeutic approaches to treating neurological diseases such as MS and spinal cord injury. With respect to demyelinating diseases, any therapeutic approach to treating hypomyelinating or demyelinating disorders that involve the migration of myelinating glia must take into account factors responsible for inducing or guiding not only the migration of grafted cells but also that of the host myelinating glia (i.e. oligodendrocyte progenitors and Schwann cells) whose remyelinating ability is known to decrease as one gets older (Ando *et al*., 2003; Berlet and Volk, 1980; Chari *et al.,* 2003; Franklin *et al.,* 2002c). Any therapeutic approach must also take into account that in demyelinating diseases such as MS the endogenous migratory signals, which direct the migration of host and grafted myelinating glia towards focal areas of demyelination, are actually arising from numerous spatially separated lesions, such as for example the isolated plaques in the spinal cord of an MS

patient. It is important to have a better understanding of these migratory signals and/or be able to induce them so appropriate therapeutic approaches can be developed to enhance the migration of myelinating glia without impeding the process of neural repair.

5.2.1 Host Myelinating Glia

In order for spontaneous remyelination to occur either adjacent oligodendrocytes have to remyelinate the axons or oligodendrocyte progenitor cells must migrate into the lesion. Within the oligodendrocyte lineage, oligodendrocyte progenitors have the highest migratory abilities (Gansmuller *et al.,* 1991; Small *et al.,* 1987). Endogenous oligodendrocyte progenitor cells located in the subventricular zone respond to a lysolecithin-induced demyelination in the corpus callosum by migrating towards the lesion and expressing PSA-NCAM all the way to the lesion (Nait-Oumesmar *et al*., 1999). The migration of the oligodendrocyte progenitors can be facilitated by FGF-2 and PDGF (Lachapelle *et al*., 2002) and neutrin-1 (Spassky *et al.,* 2002). On the other hand, oligodendrocyte progenitor migration can be inhibited by the chemokine CXCL1 (Tsai *et al*., 2002), by apotransferrin (Paez *et al*., 2002) and by class 3 semaphorins (Spassky *et al.,* 2002). Several studies indicate that oligodendrocyte progenitors are present in the demyelinating lesions of an MS patient's CNS (Chang *et al*. 2002; Wolswijk, 2002) indicating that in spite of the slower recruitment of progenitors that occurs as one gets older this is not the sole obstacle impeding remyelination in the plaques in MS.

However, there is an age-related decline in the rate of recruitment and differentiation of oligodendrocyte progenitor cells in the repair of a demyelinated lesion (Ando *et al.,* 2003; Berlet and Volk, 1980; Chari *et al*., 2003; Franklin *et al*., 2002), which is of obvious relevance to MS patients who may suffer for several decades. Franklin *et al.* (2002) and Sim *et al*. (2002) suggested that impairment both of the recruitment of oligodendrocyte progenitors cells into the lesion as well as the differentiation of these cells into myelinating glia recently observed in the CNS of aged animals collectively are primarily responsible for the age-associated decline in remyelination efficiency. The expression of growth factors, including PDGF, IGF and

TGF-β, has been shown to be important for the development, migration and maturation of oligodendrocytes (Milner *et al*., 1997). A temporal delay in the expression profiles of these growth factors was observed in old animals in comparison to that observed in younger animals (Franklin *et al.,* 2002; Hinks and Franklin, 2000). The decreased expression of myelin genes (i.e. MBP and PLP) was also shown in old rats (>12 months of age; Ibanez *et al.,* 2003). Therefore, the recruitment and differentiation of oligodendrocyte progenitors in older people likely occurs more slowly than in younger individuals.

A number of developmental and pathological studies have shown the ability of endogenous Schwann cells to migrate into the CNS toward a chemically induced (EtBr, lysolecithin) demyelinated lesion and to remyelinate CNS axons (Blakemore, 1975, 1976, 1979; Graca and Blakemore, 1986). The exposure of the neonatal spinal cord to x-irradiation results in prevention not only of the generation of myelin-forming oligodendrocytes just prior to myelination (Gnatack *et al*., 1973) but also compromises the generation of astrocytes needed to populate the developmentally increasing size of the spinal cord. In an environment where astrocytes fail to maintain the CNS-PNS border, Schwann cells now can enter the CNS, passing through the incomplete glia limitans to myelinate axons (Blakemore and Patterson, 1978; Sims and Gilmore, 1983; Sims *et al*., 1985). In myelin-deficient rats myelinating oligodendrocytes degenerate leading to a failure in myelination (Duncan *et al.,* 1987). However, astrocytes in myelin-deficient rats are not compromised and thus maintain an intact glia limitans throughout development and into adulthood. In contrast to what is seen with x-irradiation of the neonatal spinal cord of wild-type rats, no Schwann cell invasion is detected in myelin-deficient rats. However, Schwann cells will migrate into the CNS and myelinate axons if the spinal cord of neonatal myelin-deficient rats is x-irradiated neonatally. This illustrates the importance of an intact glia limitans, which is formed by astrocytes, in preventing Schwann cell invasion into the CNS (Duncan *et al.,* 1988).

In a different animal model, where gliotoxins like EtBr (see Section 1.6.1) or lysolecithin (see Section 1.6.2) are injected into white matter tracts of the spinal cord, Schwann cells can be observed migrating into the CNS and contributing to

remyelination of the axons (Blakemore, 1975, 1976, 1979; Graca and Blakemore, 1986). EtBr kills oligodendrocytes, microglia and astrocytes resulting in demyelination as well as breaking down the glia limitans that had been formed by the astrocytes, whereas lysolecithin is more selectively toxic to oligodendrocytes, thus largely sparing astrocytes. Therefore, Schwann cells extensively repopulate EtBr-induced demyelinated areas (e.g. caudal cerebellar peduncle) and remyelinate CNS axons in these lesions (Woodruff and Franklin, 1999), but in lysolecithin demyelinated lesions Schwann cell invasion is restricted only to the central, astrocyte-poor core of the area of demyelination (Blakemore, 1976). Itoyama *et al*. (1983) showed that Schwann cells could be identified inside the astrocyte-poor central area of MS plaques. Thus, Schwann cell migration into the CNS and their remyelination of CNS axons are related to the presence or absence of an intact astrocytic environment.

Schwann cell migration *in vivo* is likely to involve several cellular interactions acting in concert, even though each interaction alone may promote sufficient migration *in vitro*. For example, molecules such as laminin, fibronectin and N-cadherin (Itoyama *et al*., 1983; Iwashita *et al.,* 1983; Jasmin *et al*., 2000), the CD9 glycoprotein (Anton *et al*., 1995), the NG2 proteoglycan (Schneider *et al.,* 2001), and the p75NGFR (Anton *et al.,* 1994) have all been implicated in the migratory ability of these glial cells. Anton *et al.* (1994) demonstrated that the CD9 glycoprotein enhances Schwann cell migration both on rat pup DRG neurites and on rat pup sciatic nerve pieces *in vitro.* The CD9-induced increase in migratory ability of Schwann cells was correlated with elevation of intracellular calcium level and tyrosine phosphorylation (Anton *et al.*, 1995). Studies on the development of peripheral nerves (Heumann *et al*., 1987; Yan and Johnson, 1988; Di Stefano and Johnson, 1988; Rush, 1984) and on peripheral nerve injury (Fields *et al*., 1989; Williams *et al*., 1983) have demonstrated an increased NGF and p75NGFR expression by migrating Schwann cells. Although the mechanisms by which NGF binding to p75NGFR activates Schwann cell motility are not known, NGF was reported to increase the expression of the L1 cell adhesion molecule by Schwann cells *in vitro* and it was suggested that this neurotrophin may control cell migration (Seilheimer and Schacher, 1987). Which interaction or combination of interactions regulates Schwann

cell migration *in vivo* may depend on the immediate environment and the molecular characteristics of the migrating cells. For example, in contrast to oligodendrocyte progenitor cells, the intense inflammation and glial reactivity that accompanies infection with Theiler's virus in highly susceptible strains of mice appears to prevent Schwann cells from migrating into the CNS (Ozden *et al*., 1993).

Although not the focus of this study, there was a contribution of host myelinating glia (i.e. oligodendrocytes and Schwann cells) to the repair of the EtBr lesion induced in the model used in this research project. At 2 weeks after injection of EtBr into the right dorsal funiculus of the adult rat spinal cord, demyelination was still evident at the lesion site (Fig. 2e) but by 8 weeks this region of white matter was almost entirely remyelinated (Fig. 2f). Based on studies by Yajima and Suzuki (1979), Blakemore (1984) and Felts *et al.* (1997), this remyelination would be by both oligodendrocyte progenitors and Schwann cells that had migrated into the EtBr-induced lesion. Whether the grafted OECs also contributed to this neural repair, since we now know they will migrate to an EtBr-induced lesion, is a topic for future investigations; the focus of the present study was on the migration of the OECs, not what the cells did upon reaching the lesion.

5.2.2 Donor-Derived Myelinating Glia

The transplantation of myelin forming cells including oligodendrocytes and their progenitors, Schwann cells, OECs, neuronal precursor cells or bone marrow cells has shown therapeutic potential in promoting CNS repair (see Section 1.7). The transplanted cells have been examined for their ability to remyelinate CNS axons, to survive within damaged CNS environment, and to migrate in the presence or absence of reactive astrocytes and microglia. The ability of donor-derived myelinating glial cells to respond to migratory signals arising from focal areas of demyelination has to be carefully investigated in order to identify the ideal cell candidate for therapeutic transplantation.

In the EtBr model used in this study, OECs were grafted at T12 and the EtBr was injected either at T8, T10, or T12 to induce demyelination at various distances (0 mm to > 15 mm) from the cell graft. With an average length of each spinal cord

segment in the lower thoracic area of 5 mm, OECs were able to migrate through the dorsal funiculus at least as far as the T8 spinal cord segment (most rostral segment examined), which was located as far as 15 - 20 mm from the grafting site. When the OECs were injected directly into an EtBr-induced demyelinated lesion at T12 there was limited cell migration. Perhaps in the latter instance the majority of OECs stayed within the lesion vicinity to help in neural repair, maybe even being "instructed" to provide such assistance by activated microglia. OECs can, however, be induced to migrate away from such a demyelinated lesion provided a second lesion is made a short distance away from the first.

This is not the first study to find that OECs can migrate after grafting into the CNS of an adult rat. Using other models, OECs were shown to migrate from the dorsal root entry zone of the spinal cord into dorsal horn and were thought to promote axonal regeneration through laminae 1 to 5 of the grey matter (Ramon-Cueto and Nieto-Sampedro, 1994). The farthest distance to which OECs are thought to have migrated after being grafted at T9 in the proximal stump of the transected spinal cord of an adult rat is as far as the caudal cervical level (i.e. C7; Ramon-Cueto *et al*., 1998). However, a recent report suggested that OECs, derived from either the lamina propria of the mouse olfactory mucosa (Ramer *et al.* (2004) or neonatal rat olfactory bulb (Riddell et al., 2004), grafted directly into the DRG failed to migrate across the DREZ and also failed to promote the regeneration of dorsal root axons across the DREZ.

Other myelinating glial cells (e.g. Schwann cells and oligodendrocyte progenitor cells) have been shown to migrate for only limited distances after being grafted into the CNS (Baron-Van Evercooren *et al*., 1992; Blakemore *et al.,* 1986; Fawcett and Asher, 1999). Transplanted into the cerebral hemisphere of newborn shiverer mice, Schwann cells stayed within the vicinity of the graft and eventually disappeared from the CNS parenchyma. The migration and survival of CG4 cells (an oligodendrocyte progenitor cell line) was found to be limited to areas of the injection site after transplantation into the normal rat spinal cord (Franklin *et al.,* 1996). Host oligodendrocyte progenitors were also shown to be unable to migrate over distances greater than 2 mm into the areas of demyelination (Franklin *et al.,* 1997). Reactive astrocytes appear to impede the

migration of both oligodendrocyte progenitor cells and Schwann cells (Fawcett and Asher, 1999; Lakatos *et al*., 2003; Shields *et al*., 2000). In contrast, OECs in our experiments were able to migrate through areas containing reactive astrocytes provided reactive microglia were also present along the migratory pathway.

Our data indicate that reactive microglia enhance OEC migration. The distance over which migratory signals were distributed in most of the experiments performed using this *in vivo* model was only one spinal cord segment (~ 5 mm). This distance was still 2-3 times further than oligodendrocyte progenitors (~ 2 mm; Nait-Oumesmar *et al.*, 1995; Franklin and Blakemore, 1997) or Schwann cells (around the implantation site; Baron-Van Evercooren et al., 1992; Blakemore et al., 1986) migrated in response to a demyelinated lesion even in the presence of activated host glia in the intervening CNS tissue. Thus, not only do oligodendrocyte progenitor cells and Schwann cells very likely differ in their ability to respond to migratory signals emanating from an area of tissue injury, but the migratory ability of both of these cell types within the injured CNS of adult mammals is also far less that of OECs.

5.3 Contribution of Reactive Microglial Cells to Migration of Myelinating Glia

Microglia are extraordinarily sensitive to any changes within the CNS microenvironment (Barron, 1995; Gabicke-Haerter *et al*., 1996; Kreuzberg, 1996; Moore and Thanos, 1996). In response to CNS injury (i.e., trauma, inflammation, etc.) microglial reactivity is sometimes detrimental (Piani *et al.*, 1991; Chao *et al.,* 1992; Giulian *et al*., 1993) and sometimes beneficial (Mallat *et al*., 1989; Elkabes *et al*., 1996; Rabchewsky and Streit, 1998) in terms of contributing to the repair of neural tissue. They play a major role in orchestrating the contribution of multiple cell types to immune reactivity, inflammation, and demyelination within the CNS (Chao *et al.,* 1995; Giulian and Lachman, 1985; Giulian *et al.,* 1986; Lee *et al*., 1993; Righi *et al.,* 1989; Selmaj *et al.,* 1990), and thus in many instances exacerbate the inflammatory response associated with CNS injury (Benveniste, 1997). Microglia have been shown to release cytokines, including IL-1, TNF-α, IL-6 (Dickson *et al.,* 1993; Hetier *et al.*, 1988; Lee *et al.,* 1993), excitatory amino acids (i.e. glutamate), oxidative radicals, and nitric oxide

(reviewed in Banati *et al*., 1994; Gordon, 1995; Gehrmann *et al*., 1995), all of which contribute to the inflammatory reaction in the damaged CNS. The proinflammatory cytokines (i.e. IL-1, TNF-α, IL-6) are secreted early in the glial cell response to brain injury (Eddleston and Mucke, 1993; Hebert *et al*., 2003; Ridet *et al*., 1997) and in addition to promoting the inflammatory response also stimulate astrogliosis (Tetzlaff *et al.,* 1988). The inflammatory environment of the damaged CNS, where activated microglia and astrocytes are present, has been suggested to limit the migration of myelinating glia including oligodendrocyte progenitors (Fawcett and Asher, 1999) and Schwann cells (Baron-Van Evercooren *et al*., 1992; Fawcett and Asher, 1999; Shields *et al.,* 2000).

Activated microglia in MS also express proteinases such as MMPs (Colton *et al.,* 1993; Gehrmann *et al.,* 1995; Gottschall *et al.*, 1995). MMPs represent a family of 23 zinc-containing endopeptidases characterized in mammals (reviewed in Yong *et al*., 2001), which are known to degrade proteins of the extracellular matrix during embryogenesis and wound healing (Basbaum and Web, 1996; Werb, 1997). Increased expression of MMPs has been implicated in several neurological diseases such as MS and ischemic stroke, in which inflammation occurs (reviewed in Yong *et al.*, 2001). Pro-inflammatory cytokines (i.e. IL-1, TNF-α,) can up-regulate MMP expression, in part through NFkB transcription activity (Lee *et al.,* 2001). MMPs, in turn, can proteolytically cleave precursor molecules such as pre-IL-6, pre-TNF-α, pre-TGF-α (Black *et al.,* 1997; Yu and Stamenkovic, 2000), and thus significantly promote inflammation in the CNS. In further contribution to the pathogenesis of MS, MMPs cause the disruption of blood-brain barrier and facilitate leukocyte invasion into the CNS parenchyma (Yong *et al*., 1998), where activated leukocytes make their contribution to the inflammatory response by secreting proinflammatory cytokines (i.e. INF-γ, IL-6, TNF-α, TGF-α; Merrill and Benveniste, 1996; Selmaj and Raine, 1988). Gelatinase B (MMP9), in particular, was shown to degrade MBP in the myelin sheath *in vitro* (Gijbelts *et al.*, 1993; Chandler *et al*., 1995) and this action of MMP9 could be an important contributor to demyelination in neuroinflammatory disease. Breakage of cell-extracellular matrix contacts by MMPs leads to cell apoptosis as a result of cell

detachment and loss of integrin signaling (Ruoslanti and Reed, 1994; Chen and Strichland, 1997). Production of these cytokines and MMPs by host glia and infiltrating inflammatory cells plays therefore a detrimental role in MS.

How increased secretion of proinflammatory cytokines and MMPs might influence the migration of myelinating glia remains to be elucidated. Tourbah *et al.* (1997) noted that migrating CG4 cells were often associated with reactive astrocytes. However, the migration of these cells was limited possibly due to an inhibitory effect of the CCL3 chemokine (i.e. macrophage inflammatory protein-1α; Nguyen *et al*., 2003) via CCR1, which is the the CCL3 chemokine receptor that is expressed by CG4 cells (Nguyen and Stangel, 2001; Stangel *et al*., 1999). Filipovic *et al.* (2003) observed the expression of one chemokine, growth-related oncogene-alpha (GRO-alpha) and its receptor CXCR2 by oligodendrocyte progenitors in the highly proliferative subventricular zone of the telencephalon of human fetuses. However, neither GRO-alpha nor CXCR2 were present in oligodendrocyte progenitors found on autopsy in the brain sections of MS patients. Instead, activated microglia localized on the border of demyelinated plaques expressed both GRO-alpha and CXCR2. Thus, the change in the chemokine receptor profile of oligodendrocyte progenitors, for example downregulating the expression of CXCR2 and possibly up-regulating the expression of CCR1, may partially explain the limited migration of oligodendrocyte progenitors within areas of inflammation in the CNS and why remyelination is not more efficient in MS.

There is very little data on how and whether microglial reactivity can influence the migration of oligodendrocytes and Schwann cells during the remyelination phase of neural repair. Among the cells of the oligodendrocyte lineage, O4+ve/GalC-ve oligodendrocyte progenitor cells possess the best migratory ability (Gansmuller *et al*., 1991; Small *et al.,* 1987). The presence of EAE was reported to enhance CG4 cell migration (Tourbah *et al*., 1997). Although Tourbah *et al*. (1997) did not mention the association of migrating CG4 cells with reactive microglia, one could assume that microglia would be an important player within the inflammatory environment of the CNS in EAE. Microglial reactivity in response to trauma can also be beneficial to neural repair and facilitate axonal regeneration through areas of injured CNS tissue

(Prewitt *et al*., 1997; Rabchevsky and Streit, 1997; Lazarov-Spiegler *et al*., 1996; Stichel *et al*., 1999). Schwann cells migrate into such areas of injury, which may be under both the facilitatory control of microglia (Zeev-Brann *et al.,* 1998; Dezawa *et al*., 1999) and the inhibitory influence of astrocytes (Fawcett and Asher, 1999). On the other hand, the depletion of microglia and macrophages by clodronate in young adult female rats that have a lysolecithin-induced demyelinated lesion in their spinal cord had no effect on Schwann cell remyelination but resulted in a decreased remyelination by oligodendrocytes (Kotter *et al.*, 2001). The specific response of microglia to CNS damage may well depend on the type of injury and, depending on how the demyelination was induced, could potentially change the profile of migratory signals expressed by these glial cells and to which myelinating glia respond.

The contribution of reactive microglia to the generation of migratory signals for cells of the blood has been known for some time now (Babcock *et al.*, 2003; Gehrmann *et al.*, 1993), but only recently has it been suggested that these chemotactic signals likely also induce the migration of other cell types (Aarum *et al.*, 2003; Bettinger et al. 2002). Aaron *et al*. (2003) have shown that EGF released from mouse microglial cells influence the directional migration of neural precursor cells of the CNS and hypothesized that activated microglia play a role in directing the replacement of cells damaged or lost from injury to the brain or spinal cord. Bettinger *et al*. (2002) reported a similar chemotactic effect on GL261 mouse glioma cells and suggested microglial reactivity may promote the invasive phenotype of astrocytoma cells.

The findings reported in this thesis also implicate microglial cells in contributing to the generation of a migratory signal for cells other than lymphocytes, monocytes, neural precursor cells or glioma cells. The numbers of OECs that migrated into the T11 segment of the spinal cord after grafting into the dorsal funiculus of T12 varied depending on the presence, location, and number of small, focal, EtBr-induced demyelinated lesions. It was also observed that as increased numbers of OECs entered the T11 segment they were found within the area of astrocytic and microglial reactivity in the ipsilateral dorsal funiculus. In contrast to other studies concentrating on the association between reactive astrocytes and migrating oligodendrocyte progenitors

(Armstrong *et al*., 1990; Fruttiger *et al*., 1999; Milner *et al*., 1997; Osterhout *et al*., 1999; Tourbah *et al*., 1998) or Schwann cells (Fawcett and Asher, 1999; Franklin and Blakemore, 1993; Harrison, 1985; Itoyama *et al.*, 1983), the migration of OECs in the animal model used in this research project seemed to be dependent on the presence of reactive microglia since it was significantly diminished when the microglial response was dampened by a 4 week exposure to daily injections of minocycline. The minocycline had no apparent effect on the astrocytic reactivity, at least as evidenced by staining with vimentin and GFAP antisera, thus implicating reactive microglia as one potential source, either directly or indirectly, of the migratory signal(s) driving OEC migration in this animal model.

Microglial activity observed at the EtBr-induced lesion (i.e. at T10) and along the migratory path (i.e. T11) may well differentially contribute to the generation of a migratory signal for myelinating glia. Similar to that reported in previous studies (Yajima and Suzuki, 1979), an EtBr injection in our experiments resulted in killing all glial cells including oligodendrocytes, microglia, and astrocytes in the vicinity of the injection site with the demyelination of the right dorsal funiculus still being evident at 2 weeks survival (Fig. 4.2e). At the lesion site, dying cells release various signals including growth factors, cytokines, etc., that activate microglia, macrophages, and astrocytes to begin the process of neural repair. These cells, as well as oligodendrocyte progenitor cells and Schwann cells, migrated into the lesion and contributed to the injury repair that was observed by 8 weeks survival (Fig. 4.2f). Microglia and astrocytes of the adjacent spinal cord segments also become activated under signals (e.g. cytokines, MMPs, etc.) released from the cells of the damaged area and in turn also secrete cytokines and MMPs. Along with those factors secreted at the site of injury, these additional induced signals may facilitate not only the migration of host myelinating glia but also that of grafted OECs. The specific signals generated by this glial reactivity and that contributed to OEC migration in the present study require additional investigation. It is possible OECs express cytokine receptors that can bind microglial-derived cytokines, which in turn could facilitate their migration chemotactically through areas of neuropil containing reactive microglia. OECs are

known to express the IL-6 receptor (Nan *et al*., 2001), but it is not known which additional cytokine receptors they might also express. In addition, MMPs released by microglia in response to injury may have also contributed to inducing migration of OECs along the path of glial reactivity by reconstructing the extracellular matrix, breaking cell-matrix contacts and activating cytokine precursors (e.g., pre-IL-6), all of which may contribute to generating a chemotactic gradient for the migration of OECs.

Why does it take more than 4 weeks for a significant number of OECs to migrate into T11 when a single lesion is made at T10 even though microglia in the T11 segment react much sooner than this? The glial reactivity detected at T11 appeared to be more intense in rats surviving for 8 weeks in comparison to those at shorter survival times (fig. 4). Whatever the reason is, it was found in this study that a 4 week treatment with minocycline diminished microglia activity and resulted in decreased OEC migration, thus implicating the microglial response during the first 4 weeks after injury in contributing to the generation of a migratory signal. In contrast, precocious (at 2 weeks) migration of OECs away from a demyelinated lesion was noted in rats in which a second lesion had been made 2 segments away. This may indicate that two EtBr-induced lesions result in the migratory signal(s) being generated faster and/or more intensely when there are 2 lesions rather than one, even though the OX-42 and OX-18 immunostaining could not detect a difference in the microglial response. To further complicate matters, OECs did not migrate away when injected directly into the EtBr lesion of a single lesioned rat even though the intensity of the microglial reactivity detected by staining with anti-OX-18 antibody in the adjacent segment (i.e. T11) was similar to those of rats that had received an EtBr injection at T10 and an OEC graft at T12. Clearly, these experimental manipulations reveal variations in the generation of a migratory signal(s) that may be of use in identifying the molecules involved.

5.4 Relevance of Migratory Ability of OECs to Neural Repair

MS pathology is characterized by the presence of multiple plaques of demyelination scattered throughout the CNS. In order to contribute to neural repair in these demyelinated lesions, myelinating glia transplanted into the CNS of an MS patient

as part of a therapeutic approach to repairing the damage would have to migrate through both normal and inflammed areas of neuropil to reach the numerous isolated plaques. Upon reaching each demyelinated lesion the myelinating glia will then have to contribute to remyelination of the axons in the presence of myelin debris and of reactive astrocytes and microglia. In addition to remyelination, the ability to promote the regeneration of transected axons that are often found in MS plaques (Bitsch *et al*., 2000; Davie *et al.,* 1995; De Stefano *et al.,* 1998; Ferguson *et al.,* 1997; Kornek *et al.,* 2000; Trapp *et al*., 1998) would be an additional benefit of myelinating glia following transplantation. OECs have a greater likelihood of being able to perform the migratory, remyelination, and axonal growth promoting tasks than either oligodendrocyte progenitors or Schwann cells, which is why they are being considered as potential candidates as part of a therapeutic approach to treating neurological disorders including MS and spinal cord injury (Barnett and Chang, 2004; Barnett and Riddell, 2004; Boyd *et al*., 2003, 2004; Doucette, 1995, 2001; Franklin and Barnett, 2000; Li *et al*., 1997, 1998; Nieto-Sampedro, 2003; Ramon-Cueto *et al.,* 1998, 2000).

The beneficial contribution of OECs to neural repair include their abilities: 1) to facilitate remyelination of CNS axons *in vivo* in animal models with demyelination and spinal cord injury, as well as to remyelinate DRG axons *in vitro* (see Section 1.2.5); 2) to promote the regeneration of not only olfactory axons but also of other PNS and CNS axons, with the axon regeneration leading to functional recovery (see Section 1.2.5); 3) to integrate into the neuropil of the CNS, coming into close contact with astrocytes with no intervening basal lamina (see Section 1.2.5); 4) to secrete growth factors such as NGF, express cell adhesion molecules such as L1, N-CAM, PSA-N-CAM, and express extracellular matrix molecules such as laminin and fibronectin, which collectively play key roles in neural repair (see Section 1.2); 5) to migrate through CNS neuropil containing reactive astrocytes and microglia (see Section 1.8.3); and 6) to respond to migratory signal(s) arising as a result of a focal demyelination and move towards the demyelinated lesion, as described in this research project.

The main goal of transplanting myelinating glia as part of a therapeutic approach to treating neurological disorders such as MS and spinal cord injury is to facilitate host

tissue repair processes including remyelination and axonal regeneration. There is a great possibility that implanted OECs will facilitate the remyelination of CNS axons after they have migrated some distance from the grafting site. OEC remyelination of CNS axons *in vivo* has been reported in animals with EtBr-induced demyelination (Akiyama *et al*., 2004; Franklin *et al.,* 1996; Imaizumi *et al.,* 1998, 2000; Kato *et al*., 2000; Smith *et al*., 2002) and after spinal cord injury when the OECs were grafted into the lesion (Li *et al.*, 1997, 1998). The *in vitro* remyelination of DRG neurites by OECs was also previously shown (Devon and Doucette, 1992; 1995). In addition to their remyelinating ability, OECs have been shown to enhance axonal conduction through previously demyelinated areas of the spinal cord (Imaizumi *et al*., 1998, 2000). However, the ability to remyelinate either CNS (Boyd *et al.,* 2004; Takami *et al.,* 2002) or PNS (Plant *et al.,* 2002) axons by OECs has been questioned.

Although Boyd *et al.* (2004) recently reported that Lac-Z expressing OECs implanted into a cystic cavity of the adult rat spinal cord facilitated axonal regeneration and remyelination, these OECs did not associate directly with the remyelinated CNS axons. Peripheral type remyelination of the regenerating axons was observed, however, and was found to be due to Schwann cell migration into the lesion in the presence of transplanted OECs. This indicates that OECs may facilitate axonal regeneration in the damaged spinal cord by providing a microenvironment conducive to Schwann cell migration into the site of injury, which in turn provides support for axonal growth and results in remyelination of the axons. When grafted into the cystic cavity of the adult rat spinal cord, OECs may not only be "instructed" by reactive glia, and perhaps also by other cells comprising the inflammatory response, to stay in the vicinity of the lesion but they themselves may also be playing an "instructive" role in attracting host Schwann cells to migrate into the lesion. In addition, NGF secreted in part by OECs (Boruch *et al.,* 2001; Ramon-Cueto *et al*., 1993) may bind to the p75NGFR on Schwann cells, possibly facilitating their migration chemotactically as has been shown *in vitro* (Anton *et al*., 1994). The OECs in the lesion cavity may also shelter the Schwann cells that migrate into the lesion, effectively allowing them to function as axonal growth promoting and remyelinating glia in an area of extensive astrocytic and microglial

reactivity. Such sheltering may especially protect these Schwann cells from activated astrocytes previously shown to inhibit Schwann cell migration into the CNS (Fawcett and Asher, 1999).

The very first clinical signs of disability experienced by MS patients are due to axonal injury that occurs inside the earliest forming plaques (Bitsch *et al*., 2000; Davie *et al.,* 1995; De Stefano *et al.,* 1998; Ferguson *et al.,* 1997; Kornek *et al.,* 2000; Trapp *et al*., 1998). Therefore, transplantation of myelinating glia for the treatment of demyelinating diseases should also address the regeneration of these transected axons to restore function and reverse the disability. OECs have been shown to promote the regeneration of not only olfactory axons (Graziadei and Monti Graziadei, 1978, 1979; Barber and Lindsay, 1982; Doucette *et al*., 1983; Doucette, 1990) but also of other PNS (Guntinas-Lichius *et al*., 2001, 2002; Ramon-Cueto and Nieto Sampedro, 1994) and CNS (Li *et al*., 1997, 1998; Nash *et al*., 2002; Ramon-Cueto *et al*., 1998; Smale *et al*., 1996) axons. Transplantation of OECs into animal models of spinal cord injury enhances functional recovery of spinal reflexes (Navarro *et al.,* 1999), locomotion (Keyvan-Fouladi *et al.,* 2003; Li *et al*., 2003; Lu *et al*., 2001, 2002; Plant *et al*., 2003; Ramon-Cueto *et al*., 2000), forepaw reaching (Nash *et al.*, 2002), and breathing (Li *et al*., 2003). However, recent reports have tempered the excitement surrounding the therapeutic potential of OECs in promoting the regeneration (Gomez *et al*., 2003; Riddell *et al*., 2004; Ramer *et al*., 2004; Takami *et al*., 2002) and remyelination (Takami *et al*., 2002; Boyd *et al*., 2004) of damaged CNS axons. Possible failure of OECs to survive after being grafted into the cystic cavity of a compression injury in the adult rat spinal cord in a study by Takami *et al*. (2002) was associated with far less axonal regeneration and locomotor recovery in comparison to that of grafted Schwann cells OECs derived from either the lamina propria of the mouse olfactory mucosa (Ramer *et al*., 2004) or neonatal rat olfactory bulb (Riddell *et al*., 2004) also failed to promote the regeneration of dorsal root axons across the DREZ. The type of injury and the complexity of the reparative processes due to the host cell response to tissue damage, as well as the origin of OECs used for grafting, may all potentially influence

the survival and subsequent response of the grafted OECs to neural repair of the CNS tissue.

In order for myelinating glia to perform the migratory, remyelination, and axonal growth promoting tasks discussed above, these cells must integrate into the CNS neuropil thus coming into close contact with host glial cells, especially reactive astrocytes. Reactive astrocytes impede the migration of both oligodendrocyte progenitor cells and Schwann cells (Fawcett and Asher, 1999; Lakatos *et al*., 2003; Shields *et al*., 2000). In contrast, OECs in the present research project migrated through areas containing both reactive astrocytes and reactive microglia. Grafting of OECs into the spinal cord of Group 2-7 rats (Table 4.1) resulted in activation of microglia and astrocytes at the site of the graft as well as in adjacent spinal cord segments, as detected with OX-18 and vimentin immunostaining, respectively. The migration of OECs into the neuropil of the lower thoracic spinal cord segments rostral to the site of injection (i.e., T11-T8) at all survival times (2-8 weeks) in these experimental groups shows that OECs can coexist with reactive astrocytes and microglia within the CNS. Lakatos *et al.* (2003) reported lessening of the response of host astrocytes and of the expression of chondroitin sulphate proteoglycans at the site of injection after grafting neonatally-derived OECs into the non-lesioned dorsal funiculus of the adult rat spinal cord in comparison to that seen after grafting Schwann cells. In addition, OECs can intermingle with astrocytes in co-culture without causing astrocytes to undergo hypertrophy and to increase their expression of chondroitin sulphate proteoglycan in comparison to that of Schwann cells and astrocytes (Lakatos *et al.,* 2000). Collectively, these data suggest the OECs have little difficulty integrating into the neuropil of damaged CNS tissue primarily because they can co-exist, unlike Schwann cells, with astrocytes without being rejected, much as occurs normally in the CNS portion of the primary olfactory pathway (see Section 1.2.3).

Originally identified as critical mediators of neuronal survival and nerve fiber outgrowth during development, neurotrophic factors have been shown to protect neurons and facilitate repair in areas of CNS injury in adult animals (Campenot *et al.,* 2004; D'Ambrosi *et al.,* 2004; Jones *et al*., 2001; Pierchala et al., 2004; Tuszynski *et al*.,

1999). The infusion of neurotrophic factors such as BDNF and NT-3 into Schwann cell-containing guidance channels connecting the proximal and distal stumps of the transected spinal cord promoted regeneration of axons in descending pathways from the brainstem that grew into these guidance channels (Xu *et al*., 1995). OECs have been shown to produce PDGF (Kott *et al.,* 1994), NGF (Boruch *et al*., 2001; Guthrie and Gall, 1991; Ramon-Cueto *et al*., 1993), neuropeptide Y (Ubnik *et al*., 1994), and S100 (Barnett *et al*., 1993; Cummings and Brunjes, 1995; Doucette and Devon, 1995; Franceschini and Barnett, 1996; Gong *et al*., 1994). Furthermore, Ramon-Cueto and Avila (1994) suggested that OECs might secrete additional growth factors including BDNF, neurotrophin-3 and neurotrophin-4 based on evidence that these cells express the mRNAs for these growth factors. The action of secreted neurotrophins on neurons and Schwann cells might be facilitated by the OEC expression of the p75NGFR with which OECs might use to present the specific neurotrophins, such as NGF, to these other cells types (Barnett *et al*., 1993; Franceschini and Barnett, 1996; Goodman *et al*., 1993; Ramon-Cueto and Nieto Sampedro, 1992; Turner and Perez-Polo, 1993, 1994; Vickland *et al*., 1991). This close contact putative chemotactic effect of the OEC expression of the p75NGFR and NGF secretion would be in addition to a possible longer range migratory signal(s) that the OECs may provide to induce p75NGFR+ve Schwann cells to migrate into the lesion. Migration of Schwann cells *in vitro* has been shown to be influenced by levels of NGF (Anton *et al.,* 1994). OECs also express several adhesion molecules including N-CAM (Key and Akeson, 1990), PSA-N-CAM (Franceschini and Barnett, 1996; Miragall *et al*., 1988), and L1 (Doucette, 1995; Miragall *et al*., 1988; Ramon-Cueto and Avila, 1994), as well as extracellular matrix molecules such as laminin (Doucette, 1996; Kafitz and Greer, 1997; Liesi, 1985), and other growth promoting molecules such as glia-derived nexin (Reinhardt *et al*., 1988). All of these molecules are known to facilitate axonal growth both *in vivo* and *in vitro* (Bixby *et al.,* 1988; Boyd and Gordon, 2003; Fu and Gordon, 1997; Kleitman *et al.,* 1988).

Transplantation of myelinating glia and the administration of growth factors to areas of CNS injury have helped to overcome the normally abortive regeneration of

CNS axons and to facilitate their remyelination within the injured CNS. Researchers have used various molecular genetic techniques to transfer selected growth factor genes into cells that will then be used to deliver growth factors constitutively to all cells at the site of injury. Both retroviral and adenoviral vectors have been utilized to introduce the genes of interest (Owens, 1991). Menei *et al.* (1998) transfected Schwann cells *in vitro* with a replication deficient Moloney murine leukaemia retrovirus carrying the human preproBDNF cDNA. These BDNF-secreting Schwann cells were transplanted into the transected adult rat spinal cord and were found to enhance axonal regeneration across the injury in comparison to wild-type Schwann cells. OECs transfected with the gene for glial cell line-derived neurotrophic factor (GDNF) were also found to promote CNS repair after being grafted into the transected adult rat spinal cord (Cao *et al.*, 2004). The expression of either BDNF by Schwann cells or GDNF by OECs enhanced the growth-promoting properties of these cells within the microenvironment of the lesion site during recovery period that followed the spinal cord injury.

The migration of OECs towards a focal demyelination after being grafted into normal or demyelinated areas of the adult rat spinal cord, as found in this thesis, is a very important ability of these cells. Several previous studies have reported circumstantial evidence that OECs can migrate within the microenvironment of the injured spinal cord when Hoechst prelabelled OECs were grafted very close (1 mm) to the site of injury (Navarro *et al*., 1999; Ramon-Cueto and Nieto-Sampedro, 1994; Ramon-Cueto *et al.,* 1998, 2000), or when unlabelled OECs are injected directly into a very small lesion (Li *et al*., 1997, 1998). An important finding arising from the present study is the ability of OECs to respond to migratory signals arising as a result of making a small, focal demyelination two to four spinal segments rostral to the site of grafting. This migratory ability of OECs, coupled with their potential ability to respond to endogenous migratory signal(s) arising from the numerous isolated plaques in the spinal cord of an MS patient, are invaluable qualities that make OECs very attractive as part of a therapeutic approach to treating the pathology of MS.

An additional important finding of this research project is that the migration of OECs within the spinal cord is in response to a signal(s) associated with the activation

of microglia, as opposed to activation of astrocytes, at the lesion site and/or along the migratory path. The injection of EtBr into the spinal cord at different distances from the site of an OEC graft in Group 2, 3 and 5 rats (see Table 4.1) resulted in an activation of astrocytes and microglia in the right dorsal funiculus at the site of the EtBr injection as well as along the migratory path between the cell graft and the lesion. In these rats, an increased number of OECs migrated preferentially through the neuropil of the ipsilateral dorsal funiculus, which was highly populated by reactive astrocytes and microglia. In addition to their being microglial-derived migratory signal(s) generated in the chemically induced demyelinated area, one has to also consider the possibility that there are concentration gradients of the migratory signal(s) along the migratory path between the cell graft and the lesion. In contrast to the migration of OECs when they were grafted at a distance from the EtBr lesion in Group 2 and 3 rats, when they were grafted directly into a small, focal demyelinated lesion (i.e., Group 4 rats) far fewer OECs migrated away from the grafting site even though reactive microglia were present in the adjacent spinal cord segments, as has been also reported by other neuroscientists (Boyd *et al.*, 2004; Plant *et al*., 2003; Takami *et al*., 2002). However, when two EtBr lesions were made 2 spinal cord segments apart (i.e., Group 5 rats) and the OECs were grafted directly into one of these lesions the OECs were found to migrate precociously towards the second lesion. Activated microglia at the lesion site in the Group 4 rats might have "instructed" grafted OECs to contribute to the repair process of demyelinated lesion, perhaps by creating a microenvironment conducive to Schwann cell migration.

The minocycline data reported in this thesis implicate microglial reactivity as a major contributor to the generation of the migratory signal(s) driving the migration of OECs in the EtBr model used in this study (see Section 5.3). Specifically, decreasing the reactivity of microglia by a four week daily exposure to minocycline resulted in a corresponding decrease in the number of OECs that migrated into the T11 segment of the spinal cord. There was no indication, as judged by GFAP and vimentin immunostaining, that the minocycline treatment reduced the astrocytic reactivity. Thus, this research project did not identify astrocytes as contributors of any potential signals

inducing OECs to migrate away from the site of a cell graft towards a small, focal demyelinated lesion. Although, the minocycline experiments identified the microglial reactivity as contributing to the EtBr lesion-induced OEC migration, it is possible the astrocytic reactivity may also have played a role in generating a migratory signal(s) but that the generation and secretion of such a signal(s) might be dependent on the presence of a microglial response. Therefore, even if the reactive astrocytes in the T11 segment of Group 2, 3 and 5 rats may have contributed to the migration of OECs away from the grafting site, the signal obviously was not powerful enough in rats in which the microglial reactivity was impeded by minocycline treatment (i.e., Group 6 and 7 rats). It is also possible that the presence of an astrocyte-derived signal(s) is totally dependent on the presence of reactive microglia.

The present study focused on the ability of OECs to respond to migratory signal(s) arising as a result of a small, focal demyelination in the spinal cord of adult rats and on the ability of these grafted cells to migrate through areas of neuropil containing reactive microglia and astrocytes. Both reactive astrocytes and microglia are important players in the complex repair processes that must occur to repair the demyelinated lesions in MS as well as during the regeneration and remyelination of damaged axons following spinal cord trauma. Because OECs are not rejected from the neuropil of the CNS, this enables them to migrate amongst reactive microglia and reactive astrocytes without obstructing the ability of these host glial cells to do the job of neural repair. Whether the OECs that migrate towards the demyelinated lesion contribute to neural repair upon reaching the lesion is an area for future study. Based on what is known about the growth promoting ability of OECs (see Section 1.2.5), however, it can be hypothesized that upon reaching the lesion the OECs will create a more favorable microenvironment for axonal regeneration and remyelination by host-derived myelinating glia, including even host Schwann cells.

5.5 Prelabelling Cells Prior to Grafting

Transplantation of myelinating glia for the purpose of neural repair is a promising approach in the treatment of demyelinating diseases and spinal cord injury. It

is critically important to determine the efficacy of grafting the different types of myelinating glia using animal models before the approach is translated to humans. A proper evaluation of the contribution of grafted oligodendrocyte progenitor cells, Schwann cells or OECs to neural repair cannot be made unless the cells are prelabelled prior to grafting. Otherwise there is no way to tell whether the oligodendrocyte remyelination is due to host oligodendrocytes or ones that arose from the grafted cells. Likewise with OECs and Schwann cells, which both remyelinate axons with peripheral type myelin (see Section 1.7), it is essential for the experimenter to determine the source of the remyelinating cells; i.e. are they host or donor in origin. For example, following intraspinal implantation, OECs are believed to transform into Schwann cell-like cells that are thought to be the ones facilitating the axonal regrowth and responsible also for remyelinating the axons (see Section 1.2.5). One fact often overlooked in these studies is that Schwann cells, which share several phenotypic features with OECs (see Section 1.2), migrate into the spinal cord after injury (Beattie *et al*., 1997; Brook *et al*., 1998; Namiki *et al*., 2000; West *et al.,* 2001; Takami *et al*., 2002). Given that the invasion of Schwann cells likely coincides with the retention of grafted OECs at sites of spinal cord injury, new questions are raised as to which glial cell type is responsible for supporting the regeneration of central axons. The study by Boyd *et al.* (2004) was the first ultrastructural study to use prelabelled OECs in a compression injury model and provided direct evidence that OECs do not associate with axons nor do they form myelin or basal lamina after implantation into the injured spinal cord of adult rats. None of the previous studies provided ultrastructural evidence of the fate of prelabelled OECs after intraspinal implantation into or adjacent to the site of injury, even though they attempted to describe in detail the interactions of these grafted cells with central axons and other neural elements (e.g., invading Schwann cells) (Li *et al*., 1998, 2003; Plant *et al.,* 2003; Takami *et al.,* 2002). Instead, the researchers assumed the Schwann cell-like cells seen at the site of injury arose from the implanted OECs. Because Boyd *et al.* (2004) used prelabelled OECs in their grafting experiments, they could determine that grafts of OECs are indeed beneficial, but not necessarily for the reasons proposed previously in the literature.

5.5.1 Fluorescent dyes

Fluorescent dyes such as DiI and Hoechst, as well as transfections with a viral vector containing the reporter gene for green fluorescent protein (discussed in Section 5.5.2), have all been widely used to label cells before grafting into host CNS tissue and to visualize the transplanted cells using fluorescent microscopy (Baron-Van Evercooren *et al*., 1996; Gansmuller *et al*., 1992; Ramon-Cueto and Nieto-Sampedro, 1994; Ramon-Cueto *et al*., 2000; Verdu *et al*., 2001). Prelabeling cells before grafting into the CNS of experimental animals is necessary to determine whether the cells survive grafting into an area of CNS injury, to track their migration (or lack thereof), their migratory routes, and the cells with which they come into intimate contact.

DiI, a carbocianone, is a lipophilic dye that has been utilized in some studies to prelabel cells (e.g. Schwann cells, oligodendrocyte progenitors, astrocytes) prior to grafting (Baron-Van Evercooren *et al*., 1996; Gansmuller *et al*., 1992). Soluble in 100% ethanol but not in aqueous solution (Honig and Hume, 1986), DiI is inserted into the plasma membrane and diffuses within the plane of the membrane to label the entire cell surface. As noted by Gansmuller *et al*. (1992), DiI labeling does not affect the survival, migratory ability of astrocytes, oligodendrocyte progenitors and the Schwann cell line MSC80, transplanted into the newborn Shiverer and normal mouse brain. Baron-Van Evercooren *et al*. (1996) also showed that DiI-labeling did not impinge on the migration of oligodendrocyte progenitors and Schwann cells in response to migratory signals arising from a lysolecithin induced demyelinated lesion in the adult wild type and Shiverer mouse spinal cord.

In this research project, DiI-labelled OECs were frozen and stored at -70°C until needed for grafting in one of the cell migration experiments (see Section 2.4.4). Similar to that of unlabelled OECs, the viability of the cells after thawing was approximately 75%. Furthermore, some animals included in Group 1 to 5 rats and all animals in Group 6 and 7 rats survived for 8 weeks, at which time they were perfused, their spinal cords were dissected and then processed for fluorescent microscopy. After all this time, DiI-labelled OECs were easily detected within the rat's spinal cord rostral to the cell

graft. Therefore, DiI is a reliable marker, which can help identify grafted cells within host animal tissue, without compromising the viability of the grafted cells.

One disadvantage to the use of DiI for prelabelling cells is the possibility that the dye may leak out of the cell. If leakage does occur, then cells of the host animal may incorporate the dye into their plasma membrane and be mistakenly identified as grafted cells. In the present study, DiI+ve particles were found intra- and extracellularly in spinal cord sections obtained from experimental animals. Spinal cord tissue in our experiments was additionally stained with Hoechst dye in order to label cell nuclei. Each DiI+ve/Hoechst+ve cell had an elongated cell body and unbranched cytoplasmic processes and these were the cells counted for statistical analysis (Fig. 4.2). Several spinal cord sections chosen for staining with the microglial markers OX-18 and OX-42 were also studied using fluorescent microscopy and in none of these sections were microglia seen to be DiI+ve, nor did any of the DiI+ve cells have a multipolar morphology characteristic of microglia or astrocytes. Furthermore, leakage of DiI from the grafting site into the adjacent spinal cord segment is extremely unlikely since there was no staining of axons in the dorsal funiculi of any of the rats in this study.

Hoechst (2'-(4-Ethoxyphenyl)-5-(4-methyl-1-piperazinyl)-2,5'-bi-1*H*-benzimidazole trihydrochloride, bisBenzimide Hoechst 33342) is membrane-permeable fluorescent dye, which intercalates in A-T regions of DNA and stains nuclei with low cytotoxicity. Labeling with Hoechst was used to identify cells within host tissue after transplantation of either Schwann cells in animal models with lysolecithin–induced demyelination (Baron-Van Evercooren *et al*., 1992, 1993) or OECs in animals with spinal cord injury (Ramon-Cueto and Nieto-Sampedro, 1994; Ramon-Cueto *et al*., 2000; Verdu *et al*., 2001). Both Schwann cells and OECs were visualized by virtue of their blue stained nuclei. Baron-Van Evercooren *et al*. (1992) also demonstrated that the Hoechst-labelled Schwann cells, transplanted into or at a short distance (2-8 mm) from a demyelinated lesion in Shiverer mouse spinal cord, were able to migrate into the lesion and remyelinate CNS axons. Limited migration of both Hoechst-labelled Schwann cells and a Schwann cell line MSC80 was observed in wild type (2-3 mm) or shiverer (8 mm) mouse spinal cord towards a focal demyelination, with some of these

cells contributing to myelin repair upon reaching the lesion and expressing the peripheral myelin protein, P0. Hoechst labeling did not impede the expression of O4, Gal-C or MBP by oligodendrocyte progenitors and their progeny after being transplanted into the newborn shiverer mouse brain. However, tissue from animals that received Hoechst-labelled cells has to be processed very carefully as Ramon-Cueto and Nieto-Sampedro (1994) reported that a long paraformaldehyde incubation results in the loss of Hoechst flourescence. In addition, it has been suggested that phagocytosis of cellular debris by microglia and/or macrophages at the site of implantation can result in an overestimation of the survival and migration of transplanted cells because the phagocytosed Hoechst dye and the nuclear staining do not reveal the morphology of the cells and therefore it is impossible to exclude reactive glia or macrophages from the quantification (Ruitenberg *et al.*, 2002).

Labelling cells with fluorescent dyes (e.g. DiI, Hoechst) have advantages over other prelabelling methods. Firstly, this method consumes less time and secondly, it is more cost effective in comparison to other methods such as viral infection. Prelabelling of cells with DiI prior to grafting does not impede the phenotype or the function of the cells after transplantation. In the present study the DiI could be detected for at least 8 weeks after grafting, although there was a tendency for the fluorescence to fade after a 2-3 minute exposure to light while performing the cell counts on the fluorescent microscope. However, DiI is easiest to use when fluorescent microscopy is being used to detect the presence of prelabelled cells.

5.5.2 Viral Infection

Advances in molecular biology allow scientists to transfer a foreign gene(s) into different cells. Transfer of such a foreign gene into a cell results in the production of the foreign gene's protein, which in turn serves as a "reporter" protein or marker of transfected cells. Viral vectors such as RV (Langford and Owens, 1990; Homnou *et al*., 1996), the Meloney Murine Leukemia Virus packaging cell line PT67 (Mosahebi *et al.,* 2001), adenovirus (Cot *et al*., 1998; Ruitenberg *et al*., 2002; Shy *et al.*, 1995), and HIV-1-derived lentivirus (Kosaka *et al*., 2004; Ruitenberg *et al*., 2002) have been utilized to transfect cells designated for transplantation. Genes encoding chloramphenicol

acetyltransferase, luciferase, and β-galactosidase have been widely as reporter genes in viral vectors (reviewed by Kosaras and Snyder, 2002). For example, the *Lac-Z* gene encodes the "reporter" enzyme protein, β-galactosidase. RV vectors harboring the *Lac-Z* gene have been utilized for infection of Schwann cells (Langford and Owens, 1990; Homnou *et al*., 1996) and OECs (Boyd *et al*., 2004; Ruitenberg *et al*., 2002) before transplanting these cells into the adult rat spinal cord.

In the experiments reported in this thesis, OEC cultures were exposed to the BAG replication-deficient RV harboring *Lac-Z* gene. *Lac-Z*-expression by OECs did not alter the morphology of these cells either *in vitro* or *in vivo*. After grafting onto the pial surface of the lower thoracic segment of the adult rat spinal cord, *Lac-Z* expressing OECs were detected within the cerebellum, brainstem and spinal cord 4 weeks after transplantation in Group 8 rats. The morphology of these cells was similar to that of the DiI-labelled OECs found in the spinal cord of Group 1-7 rats, being elongated cells with unbranched cytoplasmic processes. The expression of β-galactosidase by OECs transplanted into a one-week-old cystic cavity in the adult rat spinal cord was also detected three weeks after transplantation (Boyd *et al*., 2004). The expression of *Lac-Z* gene did not appear to affect the phenotype, survival or migratory ability of the OECs in either the present study or the study of Boyd *et al.* (2004). Moreover, Arroyo *et al.* (1998) reported that *Lac-Z* expression does not hinder the ability of Schwann cells to produce myelin or associate with unmyelinated axons *in vivo*. Remyelination of spinal cord axons was also observed by either Schwann cells or OECs derived from a transgenic rat expressing human placental alkaline phosphatase (Akiyama *et al*., 2004).

A fluorescent marker, GFP (Clontech, Palo alto, CA), can be also incorporated into a cell by transfecting it with adenovirus (Li *et al*., 2003). GFP expressing mice bred in the Jackson Laboratory (Bar Harbor, ME, USA) have been used as a source for obtaining various GFP expressing cells, e.g. bone marrow cells (Akiyama *et al*., 2002a). In addition, GFP can be delivered into cells (e.g. OECs) *in vitro* by means of infection with either adenovirus or lentivirus harboring the GFP gene. GFP-expression by OECs, which were transplanted into an adult rat spinal cord, did not alter the phenotype of grafted cells, which displayed a bipolar morphology similar to that seen in the present

study with DiI+ve cells and β-Gal+ve cells, and also expressed the p75NGFR (Ruitenberg *et al*., 2002). In contrast to DiI, GFP does not leak out of a cell. However, Ruitenberg *et al*. (2002) found that only approximately 50 % of the OECs expressed GFP *in vitro* after the infection of cultures with either adenovirus or lentivirus. In addition, the expression of GFP by OECs was detected for only up to 12-14 days after cell transplantation into an adult rat spinal cord (Ruitenberg *et al*., 2002), in contrast to being able to see DiI+ve cells and β-Gal+ve cells as long as 2 months after grafting into the dorsal funiculus or onto the pial surface of the spinal cord, respectively (present study).

It is still unclear whether viral transfection has any long-term consequences on the phenotype of infected cells. Based on the data presented in this research project and that reported by others it would appear that transfection with a viral vector has no cytopathic effect on the cells (Akiyama *et al*., 2004; Boyd *et al*., 2004; Homnou *et al*., 1996; Mosahebi *et al*., 2001; Shy *et al*., 1995). Genetic labeling is therefore a stable and reliable method for prelabeling cells prior to grafting (Mosahebi, *et al*., 2001) provided the viral vector has a selectable marker gene for purifying the cell culture.

5.6 Methods of Delivery of Cells to Injured CNS

Manipulation of the cellular environment within and adjacent to injured CNS tissue through the use of cell transplantation is being studied in animal models of demyelinating disease (e.g., EtBr or lysolecithin) and of spinal cord injury (e.g., spinal compression). These manipulations have included the transplantation of Schwann cells (Neuberger *et al.*, 1992; Ramon-Cueto *et al.*, 1998; Xu *et al.*, 1997), OECs (Imaizumi *et al*., 1998; Li *et al.*, 1997; Navarro *et al.*, 1999; Ramon-Cueto *et al.*, 2000; Smale *et al.*, 1996), immature astrocytes (Silver, 1998; Smith *et al.*, 1986), oligodendrocyte progenitor cells (Franklin, 1996; Lachapelle et al., 1994; Milward et al., 2000; O'Leary and Blakemore, 1997), and bone marrow progenitor cells (Akiyama et al., 2002a; Sasaki *et al*., 2001). Cells that have been genetically modified to secrete large amounts of specific growth factors have also been used to make the microenvironment more conducive to axonal growth (Cunningham *et al.*, 1991; Kawaja *et al.*, 1991; Kawaja *et*

al., 1992). Although the usual method of delivery of such cells to the damaged neural tissue is by injection, a procedure that by its very nature is traumatic to the brain and spinal cord, there are other less invasive options that have also been explored.

5.6.1 Grafting Cells Directly into CNS Tissue

Bony structures form a protective shield around the fragile brain and spinal cord. Therefore, in order for the cells to be grafted directly into the CNS tissue the investigator must inflict various degrees of destruction on either the bones of the skull or on the vertebrae, unless perhaps these bones have already been broken as the intial injury was induced. In most of the animal models, however, the cells have been injected into the neuropil of the spinal cord or brain only after mechanical removal of part of the bony protection. Directly injecting cells into an EtBr-induced (Akiyama et al., 2001, 2002a, 2004; Barnett et al., 2000; Blakemore and Crang, 1989; Franklin et al., 1996, 1997; Jeffery et al., 1999; Sasaki *et al*., 2001) or lysolecithin-induced (Baron-Van Evercooren et al., 1992, 1996; Duncan et al., 1981; Vignais et al., 1993) demyelinated lesion, into the cystic cavity that forms after spinal injury (Boyd et al., 2003; Lu et al., 2002; Resnick et al., 2003; Takami et al., 2002), or into the proximal and/or distal stumps of the transected spinal cord (Lu et al., 2001; Ramon-Cueto et al., 1998, 2000), has been one of the favoured approaches for delivering cells of interest to injured neural tissue. In many of these cell grafting experiments a stereotaxic apparatus was used to deliver the cells to a precise location, which was especially important when the migratory abilities of the transplanted cells were being studied. The cell suspensions were mostly injected into the neural tissue using either 5 or 10 µl Hamilton syringes with an attached glass or metal (30-32 G) needle. Compared to cell injection done by hand, a syringe pump like the one used in the present experiments makes cell grafting far more accurate by being able to control both the flow rate and the volume injected. There is no way to avoid some mechanical damage to the neural tissue when a needle, no matter how small, is inserted into the brain or spinal cord. Moreover, it would be inappropriate, as part of a therapeutic approach to neural repair in humans, to inflict yet additional damage on the patient's nervous tissue during the delivery of cells meant to facilitate the tissue repair.

Most of the experimental cell therapies being developed in animal models for delivering myelinating glia to the site of demyelination have used a cell grafting approach (see Section 1.7). Although this approach shows promise in terms of neural repair, facilitating functional recovery, and restoring axonal conduction, the limited migration of oligodendrocyte progenitor cells and Schwann cells through the CNS parenchyma restricts the beneficial effect of transplanting these two cell types to the immediate vicinity of the site of injury (Baron-Van Evercooren *et al*., 1992; Blakemore *et al.,* 1986; Fawcett and Asher, 1999; Franklin and Blakemore, 1993, 1997). The therapeutic usefulness of focal cell grafts, therefore, is of limited benefit in the treatment of diseases such as MS in which multifocal demyelination is the main pathological feature. Thus, in an MS patient there would have to be multiple such injections of myelinating glia, even taking into account the ability of OECs to respond to a migratory signal(s) arising as a result of the multiple lesions of focal demyelination.

5.6.2 The Vascular Route

The intravenous route of administration is widely used in modern medicine to deliver various drugs to target tissues and has also been used to deliver cells to an EtBr-induced and x-irradiated demyelinated lesion (Akiyama *et al.*, 2002; Inoue *et al.*, 2003) or to an ischemic area of the cerebral hemisphere induced by occlusion of the middle cerebral artery (Chen *et al.*, 2001). Bone marrow cells (Akiyama *et al.*, 2002; Li *et al.*, 2001; Inoue *et al.*, 2003), Schwann cells and OECs (Akiyama *et al.*, 2002) are the cell types that have been delivered via this route. Intravenous administration of bone marrow cells in animals with demyelinated lesions resulted in varying degrees of remyelination, with the myelin formed being of both peripheral (i.e., Schwann cell-like) and central (i.e., oligodendrocyte-like) type myelin, as well as improving the conduction velocity of the remyelinated axons (Akiyama *et al.*, 2002; Inoue *et al.*, 2003). An improvement on the adhesive-removal behavioral test and the modified neurologic severity scores in adult rats with cerebral ischemia were also reported subsequent to an intravenous infusion of bone marrow cells (Li *et al.*, 2001).

Adverse effects, if any, on non-neural organs such as the liver, spleen, etc through administration of cells derived from bone marrow via the intravenous route has

yet to be determined, and especially whether there are effects that may arise in animals after long survival times. Inoue *et al.* (2003) reported that they had to inject 100 times more bone marrow cells via the femoral vein to achieve a comparable density of remyelination relative to that achieved after direct intraspinal injection of these cells. However, although a relatively large number of axons were remyelinated in such a lesion subsequent to transplantation of Lac-Z expressing bone marrow cells directly into the demyelinated lesion, Akiyama *et al.* (2002) reported that only a small percentage of the myelinating cells were β-galactosidase positive. Further studies are needed to determine whether intravenously injected bone marrow cells do actually give rise to myelinating glia or whether the tissue repair is due to the facilitation of an endogenous repair mechanism. Furthermore, in contrast to that seen with the bone marrow cells, no remyelination was seen in areas of x-irradiation/EtBr-induced demyelination after intravenous delivery of either Schwann cells or OECs (Akiyama *et al.*, 2002).

Akiyama *et al.* (2002) suggested that intravenously delivered cells (e.g., bone marrow cells) are unlikely to migrate across the blood brain barrier into normal spinal cord or brain tissue, because this barrier would continue to function as it normally does in restricting access to the nervous system parenchyma. In support of this suggestion they reported that no intravenously delivered bone marrow cells were observed in CNS areas other than in the lesion. The focused entry of prelabeled bone marrow cells into sites of CNS injury in experiments where they were delivered intravenously is likely due to the ability of the cells to respond to chemotactic factors (e.g., monocyte chemoattractant protein 1), the expression of which is increased in damaged CNS tissue (Kim, 1996). In addition, the expression of cell adhesion molecules, such as intercellular adhesion molecule (Zhang *et al.*, 1995), vascular adhesion molecule 1, and E-selectin (Blann *et al.*, 1990; Haraldsen *et al.*, 1996) may also contribute to the migration of the prelabeled bone marrow cells across the blood brain barrier and into the damaged neuropil. Therefore, the vascular route is an approach that may prove benefical in the treatment of neurological diseases like MS and spinal cord injury where complex neurosurgical interventions can and should be avoided.

5.6.3 Injection of Cells Into CSF

There have been a few studies using an animal model of CNS demyelination in which progenitor cells have been delivered intracisternally (Einstein *et al.*, 2003; Learish *et al.*, 1999; Pluchino *et al.*, 2003). For example, neuronal progenitor cells prelabelled with bromodeoxyuridine have been injected into the lateral ventricle of C57BL/6 mice that are suffering from EAE (Einstein et al., 2003). Transplantation of these neuronal progenitor cells resulted in attenuation of clinical severity of EAE and brain inflammation of animals, indicated by reduction in perivascular infiltrates. Pluchino *et al.* (2003) injected neuronal progenitor cells infected with lentivirus harboring a *Lac-Z* gene into the lateral ventricle of mice with EAE and observed an increased number of oligodendrocyte progenitors and remyelinated axons within demyelinated areas. Learish *et al.* (1999) injected Lac-Z-expressing oligodendrocyte progenitor cells into the cerebral ventricles of embryonic myelin-deficient mice. The donor progenitor cells migrated into the CNS parenchyma including the corpus callosum, inferior hippocampus, thalamus, and inferior colliculus and facilitated neural repair. Learish *et al.* (1999) suggested that an intraventricular injection is a feasible approach to cell delivery in patients with demyelinating disorders, particularly for the demyelinated lesions located in periventricular white mater. However, an intraventricular injection of cells still involves some degree of mechanical injury to the cerebral hemisphere because the injection needle must pass through normal brain tissue before the tip enters the ventricular system.

In clinical practice and in experimental studies that involve the use of large animals, lumbar puncture is a widely used technique for collecting CSF or for delivery of drugs. An alternative approach for cell delivery is an intrathecal injection into the subarachnoid space, which is located between the arachnoid and pia mater and is filled with CSF. Since the pia mater intimately adheres to the surface of the brain and spinal cord, the subarachnoid space widens where the brain surface exhibits a deep sulcus, for example between the cerebellum and medulla, thus forming a cistern (e.g., cisterna magna). The lumbar cistern is formed as a pouch by the extension of the arachnoid mater caudally into the lumbar and sacral portions of the vertebral column leaving

behind the pia mater covering the conus medularis and fillum terminalis, as well as the lumbar and sacral rootlets. In adult humans, the spinal cord ends at the level of the first lumbar vertebra although it is much lower in the vertebral column in children. A needle insertion into the lumbar cistern is called a lumbar puncture and is considered a relatively safe procedure in comparison to needle insertion into the cisterna magna.

Only one study so far has reported on an intrathecal injection of neuronal progenitor cells in an EAE animal model (Ben-Hur et al., 2003). Injected into the CSF, neuronal precursor cells were found exclusively within demyelinated areas of brain and spinal cord white matter. Recently, De la Calle and Paino (2002) described a detailed procedure for direct lumbar puncture in rats, which was modified from that described previously by Hylden and Wilcox *et al.* (1980). Under isofluorane anesthesia, a 2 cm longitudinal percutaneous incision was made with a scalpel at the lower lumbar level and a 25G neonatal lumbar puncture needle was inserted through the intervertebral space between the L5 and L6 vertebrae. De la Calle *et al.* (2002) used this lumbar puncture technique to inject a cell suspension of Hoechst-prelabelled NB69 neuroblastoma cells, which are known to synthesize and secrete monoamines into the subarachnoid space of the rat with chronic contusion injury. Intrathecally grafted NB69 neuroblastoma cells survived and maintained their ability to secrete monoamines, thus abolishing chronic contusion injury-induced heat hyperalgesia.

The use of a direct lumbar puncture rather than injecting the cells into the cisterna magna or through brain tissue into the lateral ventricles is of particular clinical relevance. A lumbar puncture can be done relatively quickly, on the order of 10 minutes or less, and in comparison to needle insertion into the cisterna magna or the ventricles is far less traumatic for the patient. An additional advantage of injecting the cells directly into the CSF is that instead of the grafted cells immediately encountering the aggressive, inflammatory environment of the lesion epicenter, and thus possibly compromising the survival of the grafted cells, the myelinating glia could potentially benefit from the CSF in terms of being a source of nutrients. Admittedly, there is also a concern with possible damage to the spinal cord and/or the rootlets at the level of the lumbar puncture. Other possible complications that might arise after lumbar puncture

include headache, CNS infection (especially in immunocompromised patients), and intraspinal hemorrhage in patients receiving anticoagulation therapy. However, these risks are relatively low. In their study, De la Calle and Paino (2002) detected no signs of motor or sensory deficit in the rats subjected to lumbar puncture and saw no grossly detectable lesions in or on the lumbar spinal cord on post mortem examination.

5.7 General Conclusions

An *in vivo* model for studying the migratory ability of OECs within the adult rat spinal cord was developed for the present research study. A small focal (< 1 mm long) demyelination was induced by injecting 1µl of a 0.03% EtBr solution into the right dorsal funiculus of the spinal cord. This lesion was made at variable distances from the site of an OEC graft. In this model, the strength of the migratory signal(s) inducing OECs to migrate increased as the demyelinated lesion was located closer to the grafting site, with a significant increase in the number of OECs in the dorsal funiculus of the intervening segments being first apparent by 8 weeks after grafting. OECs grafted at T12 migrated as far as four spinal cord segments to reach a focal EtBr-induced demyelinated lesion located at T8. There was minimal migration of OECs when the cells were grafted directly into a demyelinated lesion unless a second demyelinated lesion was made two spinal cord segments away from the first, in which case there was precocious migration of OECs. The OEC migration occurred along a migratory path containing many reactive astrocytes and microglia and this migration of OECs was significantly reduced when the microglial reactivity was dampened using minocycline. OECs also survived grafting into cerebrospinal fluid (i.e. subarachnoid space) and migrated into the neuropil of the brain and spinal cord. The major conclusions are that OECs can respond to migratory signal(s) arising as a result of a focal EtBr-induced demyelination and that microglia are one potential source of these migratory signal(s).

5.8 Future Directions

The EtBr-induced demyelination animal model used in the present research project will be extremely useful for determining:

1) the time frame of microglial response contributing to the generation of the migratory signal(s) that induces OECs to migrate towards the lesion;
2) the molecular mechanisms facilitating and impeding the migration of OECs, for example:
    a. whether proinflammatory cytokines contribute to a migratory signal inducing OECs to migrate;
    b. whether anti-inflammatory cytokines attenuate the migratory signal;
    c. whether OECs express specific cytokine receptors to facilitate or impede their migration in response to proinflammatory or anti-inflammatory cytokines.
3) the contribution of grafted OECs to neural repair (including remyelination) once they have migrated into the neuropil of a focal demyelination lesion;
4) the ability of OECs to migrate towards a focal demyelination of spinal cord white matter and contribute to neural repair when the cells are grafted into the CSF via a lumbar puncture.

Before adopting a therapeutic approach that involves glial cell transplantations in treating demyelinated diseases, it is important to get a better understanding of host cell-grafted cell interactions in order to maximize the beneficial consequences of myelinating glial cell grafting. In MS multiple demyelinated lesions are not only scattered throughout the CNS neuropil, but the pathogenesis of each plaque develops with its own time frame with each new relapse adding to the pathology and creating new lesions. Thus, at any point during the pathogenesis of the disease in the same MS patient plaques can be found at different stages of progression. The host glial cell response within the demyelinated area and within the surrounding tissue depends on the stage of plaque progression with the highest level of glial reactivity being seen at the early, acute stages and lower reactivity at later, chronic stages. As presented in this research project, microglia are implicated as one potential source, either directly or

indirectly, of the migratory signal(s) driving OEC migration in an EtBr-induced demyelination animal model. Why is it important to identify the source of these migratory signals? Cell migration would be a far more efficient way of delivering myelinating glia to multiple areas of CNS injury, such as the isolated MS plaques, than trying to deliver the cells directly to each injured area.

Although the distances over which migratory signals are working in the present study is only one spinal cord segment, this is still 2-3 times further than either oligodendrocyte progenitors or Schwann cells will migrate in response to a demyelinated lesion. OEC migration occurred along a migratory path containing many reactive astrocytes and microglia and this migration was significantly reduced when the microglial reactivity was dampened using a 4 week minocycline treatment. This indicates that the first 4 week period after cell grafting and EtBr injection is the time period when the microglia contributed to the generation of a migratory signal(s). By varying the duration and when in the first 4 week period the minocycline treatment is started, it will be possible to determine whether it is the early (i.e. 3-7 days) or late (1-4 weeks) microglial response to injury that is likely to be most important in contributing to the generation of a migratory signal(s). Knowing this time frame is important for the design of experiments to identify the molecular mechanisms. One can hypothesize that the microglial reactivity during the first two weeks after EtBr injection is the most critical for generating a migratory signal(s) and for creating a migratory pathway conducive to OEC migration because this would be the time interval during which the microglial cells would be the most reactive. For example, the early treatment with clodronate of young adult female rats that have a lysolecithin-induced demyelinated lesion in their spinal cord resulted in the depletion of microglia and macrophages and in a decreased remyelination by oligodendrocytes, whereas a late clodronate administration had no effect on the repair outcome even though still depleting numbers of activated macrophages (Kotter *et al.*, 2001). In MS the microglial response to a progression of the pathogenesis in each plaque is reflected in the profile of released cytokines (e.g., the type and level of cytokine), excitatory amino acids, oxidative radicals, and nitric oxide. By identifying the time frame of the microglial response to

plaque progression, we can predict whether early (e.g., IL-1b) vs. late (e.g., IL-12) proinflammatory and/or anti-inflammatory (e.g., IL-10) cytokines contribute to the migratory signal(s) inducing OECs to migrate.

Transplantation of myelinating glia will be an essential part of potential therapeutic approaches to neural repair in MS and spinal cord injury. In addition to finding the best cell candidate, the method of cell delivery is also an important issue of any therapeutic approach involving cell transplantation. Although various degrees of success in CNS tissue regeneration and remyelination have been achieved after cell transplantation, several aspects specific to MS remain neglected in these animal models. The demyelinated lesions in MS are disseminated throughout the brain and spinal cord and, thus, it is certainly not possible to transplant cells into each and every lesion. Moreover, some demyelinating lesions can be located within vitally important areas such as the brain stem or located in CNS areas that are surgically inaccessible without causing undue mechanical damage to surrounding tissues (e.g., the optic nerve). Therefore, delivery of cells into the CSF by means of a lumbar puncture has many advantages over direct injection into the neuropil of the CNS. Without inflicting unnecessary additional damage to the CNS tissue, the lumbar puncture route could use the CSF to distribute the grafted cells to those parts of the CNS that were demyelinated. As reported in this thesis, OECs grafted into either normal or demyelinated areas of the CNS neuropil can be induced to migrate towards a focal area(s) of demyelination. When the OECs were grafted into the CSF, some were found attached to the pia mater at some distance from the site of grafting as well as being found within the neuropil of the same brain areas. One can hypothesize, therefore, that once OECs are grafted into the subarachnoid space they will float in the CSF, attach to the pia matter at various levels of the neuraxis, and then migrate across the damaged blood-brain barrier into the demyelinated lesions where they will contribute to neural repair.

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