Cellular Strategies to Promote Repair in the Damaged CNS Using a Combined Therapeutic Approach

Rebecca Lamond BSc (Hons) M-Res

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College of Medical, Veterinary and Life Sciences
Institute of Infection, Immunity and Inflammation
University of Glasgow

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This thesis is dedicated to my Mum; for teaching me to work hard for everything that I've wanted in life

Abstract

Following disease or injury to the CNS, the formation of a glial scar represents a physical and molecular barrier to repair. Although some therapies have promoted axonal sprouting into the lesion site, these fibres are often tangled and disorientated. To date, there has been little evidence of regenerating fibres successfully exiting the glial scar to reform functional connections. Furthermore, remyelination after disease or injury is limited, often consisting of shorter internodes of myelin and thinner sheaths. Thus, potential therapies aimed at enhancing CNS repair should support the outgrowth of neurites, guide their exit from the glial scar and perhaps aid remyelination. Since multiple factors impede the regeneration of the CNS, a combinatorial approach to therapies including cell-transplantation may be a more promising strategy.

The focus of this thesis was to investigate the interactions of olfactory ensheathing cells (OECs) and Schwann cells (SCs) with CNS glia and neurons in order to determine their effects following transplantation *in vivo*. This was carried out by performing a detailed study *in vitro*, focusing on how these interactions could impact upon endogenous CNS myelination. Based on these observations, this thesis aimed to provide novel evidence that the use of one cell type may be more advantageous than the other for cell-mediated repair of the CNS. Furthermore, the use of a biodegradable scaffold was investigated for its ability to support a complex CNS system and to direct cellular alignment *in vitro*. It was hoped that the data provided from both strategies could aid the design of an optimised cell-seeded scaffold, with the long-term aim of designing an effective combinatorial strategy to enhance repair of the CNS.

Previous studies using well defined myelinating co-cultures have demonstrated that they recapitulate many features of the CNS, allowing the study of neurite density and myelination, with correctly formed nodes of Ranvier. In this thesis I have shown that the addition of olfactory bulb-derived OECs (OB-OECs) to these cultures significantly enhanced oligodendrocyte myelination. Conversely, endogenous CNS myelination was significantly reduced by exogenously added SCs, which did not appear to ensheath axons with peripheral myelin. The addition of conditioned media derived from SCs (SCM) reduced oligodendrocyte myelination,

suggesting that this effect was mediated via SC-secreted factors. A direct biological comparison between purified OB-OECs and SCs demonstrated that SCs expressed significantly higher levels of connective tissue growth factor (CTGF) mRNA and protein. In addition, the antibody-mediated neutralisation of CTGF in SCM restored myelination to the level of untreated controls. Treatment with SCM and exogenous CTGF significantly reduced the differentiation of purified OPCs in culture in the absence of other glial/ neuronal influences. However, pre-treatment of the astrocyte monolayer with CTGF or SCM, prior to its use in the myelinating cultures suggested that CTGF may also induce key changes in astrocytes, causing them to up or down-regulate their expression of vital factors which control the myelinating capability of oligodendrocytes.

I have also demonstrated that biodegradable scaffolds fabricated from poly-ε-caprolactone (PCL) supported the outgrowth of CNS neurons and proliferation of glia; whilst pre-embossed microgrooves promoted cellular alignment. Furthermore, when compared with a range of other biomaterials, excluding glass, PCL supported the highest level of oligodendrocyte myelination in the cultures.

Taken together, data from this thesis suggests that OECs may be more beneficial than SCs in a cell-mediated repair strategy for the injured CNS, due to the latter's negative effects on oligodendrocyte myelination *in vitro*. Since PCL has also been shown to support the differentiation and survival of a complex CNS culture system, this thesis may also provide novel information for optimising an OEC-seeded PCL scaffold for implantation *in vivo* to effectively promote CNS regeneration.

Author's Declaration

I declare that, except where explicit reference is made to the contribution of others, that this dissertation is the result of my own work and has not been submitted for any other degree at the University of Glasgow or any other institution.

Signature _____

Printed name: REBECCA LAMOND

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Table of Contents

Chapter 8 Final Discussion

List of Figures

Results Chapter 4

Results Chapter 5

Results Chapter 6

Results Chapter 7

Chapter 8: Final Discussion

List of Tables

Publication List

Donoghue PS, **Lamond R,** Boomkamp SD, Sun T, Gadegaard N, Riehle MO, Barnett SC (2013) The Development of a Polycaprolactone Scaffold for Central Nervous System Repair. Tissue Eng Part A 19:497-507. (*Thesis Chapter 7)*.

Lamond R, Riehle MO, Barnett SC. Schwann Cells, but not OECs, Negatively Affect Oligodendrocyte Myelination In Vitro via the Expression of CTGF (Manuscript in Preparation). *(Thesis Chapters 4-6)*.

Lamond R, Barnett S.C. Isolation and Characterisation of Olfactory Bulb-Derived Ensheathing Cells. American Journal of Neurorestorology. (Submitted).

List of Abbreviations

2D	two-dimensional
3D	three dimensional
µm	micrometres
µM	micromoles
µl	microlitres
Aß	Amyloid beta protein
ACM	astrocyte conditioned medium
AD	Alzheimer's disease
BBB	blood brain barrier
BDNF	brain derived neurotrophic factor
BMP	bone morphogenetic protein
BrdU	5-bromo-2'-deoxyuridine
Caspr	contactin associated protein
cDNA	cellular deoxyribonucleic acid
CM	conditioned media
CNP	2',3'-cyclic nucleotide 3'-phosphohydrolase
CNS	central nervous system
CNTF	cilliary neurotrophic factor
CO_2	carbon dioxide
CSF	cerebrospinal fluid
CSPGs	chondroitin sulphate proteoglycans
CTGF	connective tissue growth factor
αCTGF	neutralising antibody to CTGF
CXCL10	C-X-C motif chemokine 10
DAPI	4'-6-diamidino-2-phenylindole
D*IV*	days *in vitro*
DMEM	Dulbecco's Modified Eagle Medium
dNTP	Deoxyribonucleotide triphosphate
DRG	dorsal root ganglion
E	embryonic day
EAE	experimental autoimmune encephalitis
ECM	extracellular matrix
EGF	epidermal growth factor
E-NCAM	embryonic (polysialylated) neural cell adhesion molecule
FBS	foetal bovine serum
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
GalC	galactocerebroside
GFAP	glial fibrillary acidic protein
hr/ hrs	hour/ hours
h.OCM	heat-treated olfactory ensheathing cell conditioned media
h.SCM	heat-treated Schwann cell conditioned media
IGF	insulin-like growth factor
IL-	Interleukin
K_v	voltage-gated potassium channels
KO	knockout
L-15	Leibovitz medium
LIF	leukaemia inhibitory factor
LP-OECs	lamina propria-derived olfactory ensheathing cells
LPS	Lipopolysaccharides

MAG	myelin associated glycoprotein
MBP	myelin basic protein
MSCs	mesenchymal stem cells
min	minute(s)
MOG	myelin oligodendrocyte glycoprotein
mRNA	mitochondrial ribonucleic acid
MS	multiple sclerosis
Na_v	voltage-gated sodium channel
N-CAM	neural cell adhesion molecule
Nf155	155kD isoform of neurofascin
Nf186	186kD isoform of neurofascin
Nrg	neuregulin
OB-OECs	olfactory bulb-derived olfactory ensheathing cells
OECs	olfactory ensheathing cells
OCM	olfactory ensheathing cell-conditioned media
OPCs	oligodendrocyte precursor cells
$p75^{NTR}$	low affinity nerve growth factor receptor p75
PBS	phosphate buffered saline
PCL	poly-ε-caprolactone
PDGF	platelet-derived growth factor
PDGFR	platelet-derived growth factor receptor
PLL	poly-L-lysine
PLLA	poly-L-lactic acid
PLP	proteolipid protein
PMMA	poly(methyl)methacrylate
PNS	peripheral nervous system
qRT-PCR	quantitative real time polymerase chain reaction
rpm	revolutions per minute
RT	room temperature
SCs	Schwann cells
SCI	spinal cord injury
SCM	Schwann cell conditioned media
TGF-β	Transforming growth factor beta
THBS	thrombospondin-
TnC	Tenascin-C
TNFα	Tumour necrosis factor-alpha
TnR	Tenascin-R

Chapter 1

Literature Review

1.1 Organisation of the Central Nervous System (CNS)

The Nervous System can be divided into the Central Nervous System (CNS) and the Peripheral Nervous System (PNS), which is then further categorised into the somatic and the autonomic PNS, with its enteric, sympathetic and parasympathetic branches (Figure 1.1). Comprising of the spinal cord and the brain, the role of the CNS is to process sensory information relayed from afferent fibres in the PNS and to co-ordinate motor outputs from the brain to various effectors via efferent fibres by propagating both electrical and chemical impulses. Furthermore, the spinal cord itself contains intricate neuronal circuitry which can mediate the control of various reflexes and central pattern generators. A delicate interplay between CNS and PNS nerves and support cells (glia) regulates all aspects of behaviour, such as the control of voluntary and involuntary movement, whilst also mediating the necessary changes to maintain homeostasis in response to environmental stimuli, such as changes in temperature or the sensation of pain.

1.1.1 Basic Anatomy of the Spinal Cord

The spinal cord itself is a long, thin almost-spherical bundle of nerves, which extends from the medulla oblongata (brain stem) and is protected by 33 spinal vertebrae (Figure 1.2). Although the vertebrae extend to the region of the coccyx where they are fused, the spinal cord terminates between the space of the first and second lumbar vertebrae at the connus medullaris, giving rise to the filum terminale. Thereafter, the continuing bundle of nerves that extends out from the cord without exiting the vertebral column is called the cauda equine, or “horse’s tail”.

As well as the spinal vertebrae, the cord is surrounded by 3 meningeal layers; namely the most superficial dura mater, the arachnoid membrane and the innermost pia mater, which contains many of the blood vessels supplying oxygen and nutrients to the spinal cord. Furthermore, in between the arachnoid and pia mater in the subarachnoid space lies cerebrospinal fluid (CSF), which acts to cushion the spinal cord and to promote homeostasis by removing metabolic waste from the CNS via the blood brain barrier.

For each vertebra, a pair of spinal nerves containing both sensory (dorsal) and motor (ventral) roots emerge from the vertebral column at regular intervals, serving to connect the spinal cord to specific nerves innervating the rest of the body. Thus, there are 8 pairs of cervical spinal nerves, 12 pairs of thoracic, 5 pairs of lumbar, 5 pairs of sacral and 1 pair of coccygeal spinal nerves (Figure 1.2). The neuronal cell bodies of sensory fibres are located within a swelling known as the dorsal root ganglion in the periphery, with their afferent projections extending into the spinal cord via the dorsal roots. Conversely, the cell bodies of motor neurons are located throughout the cord, whilst their efferent fibres exit the spinal cord in the ventral roots (Figure 1.3). Dorsal roots and ventral roots come together and exit through the intervertebral foramina between each vertebrae to become mixed spinal peripheral nerves, which branch out to innervate specific regions in the periphery, with the exception of the C1 nerves which exit between the occipital bone and the atlas.

Internally the spinal cord is composed of both gray matter and white matter, which contains axons that are ensheathed (myelinated) in a compact lipid/ protein layer known as myelin to provide insulation, thus promoting the rapid conduction of axonal electrical impulses. The centre of the spinal cord comprising the grey matter is shaped like a butterfly or the letter “H”, and contains the cell bodies of interneurons and motor neurons, neuroglia, unmyelinated axons and the dendrites of interneurons and motor neurons (Figure 1.3). The projections of the gray matter (the “wings”) are called horns; namely the anterior, posterior and lateral horns. Neuronal cell bodies in the grey matter form functional groups of “nuclei”, such that the anterior grey horns innervate skeletal muscle, the posterior horns contain somatic and autonomic fibres and the lateral horns, present only in the thoracic, upper lumber and sacral regions of the spinal cord, contain autonomic motor nuclei which innervate smooth muscle, glands and cardiac muscle.

White matter surrounds the grey matter, and contains mostly myelinated axons, of sensory, motor and interneurons, although there are some unmyelinated axons in this region. Unlike the grey matter, white matter is segmented into columns as opposed to horns, giving rise to the anterior, posterior (dorsal) and

lateral columns. Fibres within the white matter columns are organised into ascending (sensory) and descending (motor) tracts with a common function/ destination.

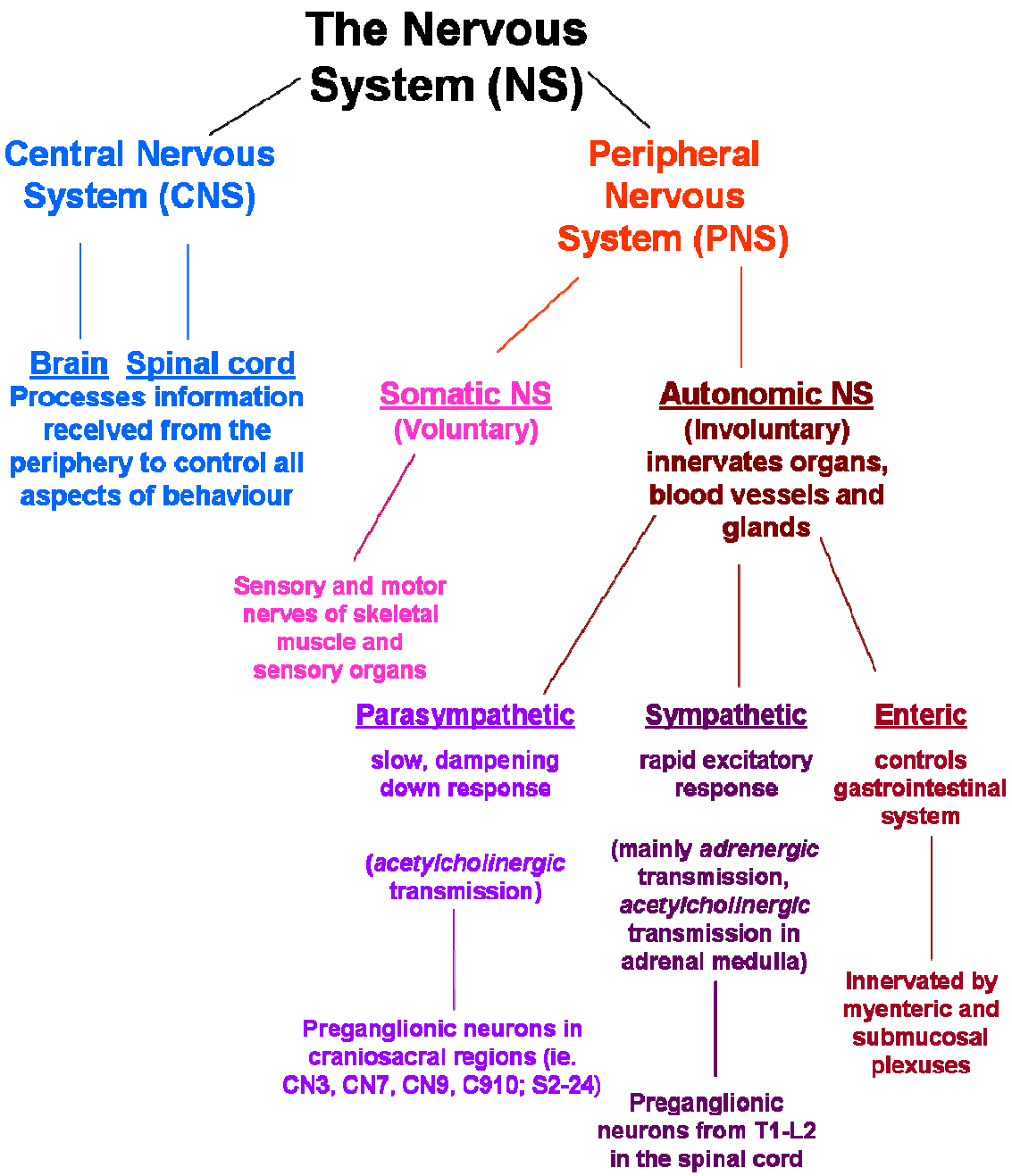


Figure 1.1 – *A schematic of the organisation of the Nervous System*. The nervous system (NS) can be divided into the central nervous system (CNS), comprising of the brain and spinal cord, and the peripheral nervous system (PNS). Further branches of the PNS include the somatic NS, which controls voluntary movement, and the autonomic NS, with its sympathetic, parasympathetic and enteric divisions, which control involuntary movement. The CNS processes sensory information from the periphery to co-ordinate all aspects of behaviour.

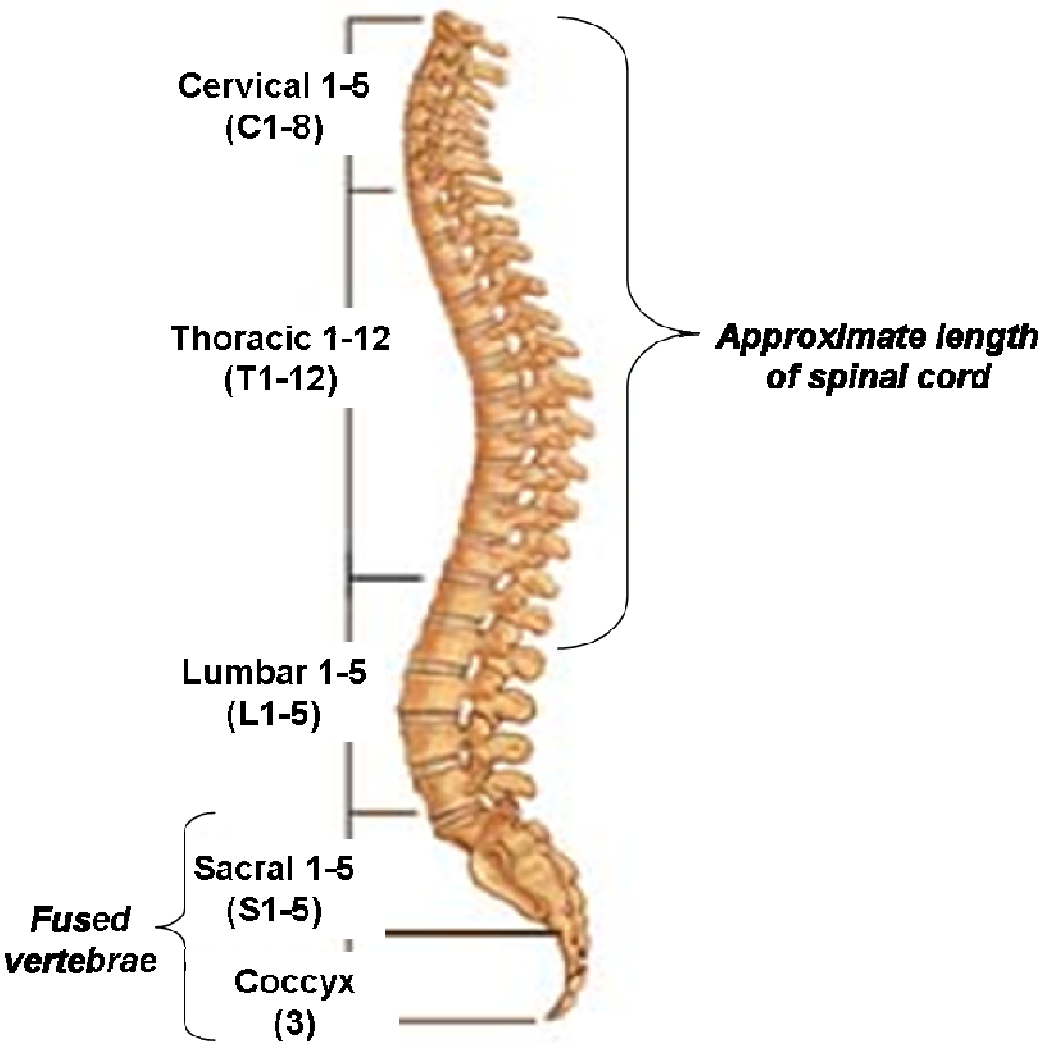


Figure 1.2 – *The 33 vertebrae surrounding the human spinal cord.* The spinal cord is surrounded by 33 bony vertebrae, beginning at the cervical region of the neck and terminating at the coccyx. Each vertebra can be named and numbered in accordance to its anatomical position, though the 3 most-caudal fused vertebrae constitute the coccyx. The spinal cord itself, however, arises from the foramen magnum and ends at the intervertebral space between the first and second lumbar vertebrae (L1, 2). There are 30 pairs of mixed spinal nerves, one arising from each spinal segment from the cervical to the sacral region, and one final pair of nerves exiting the cord at the coccygeal region. Diagram modified from http://www.spineuniverse.com/anatomy/vertebral-column.

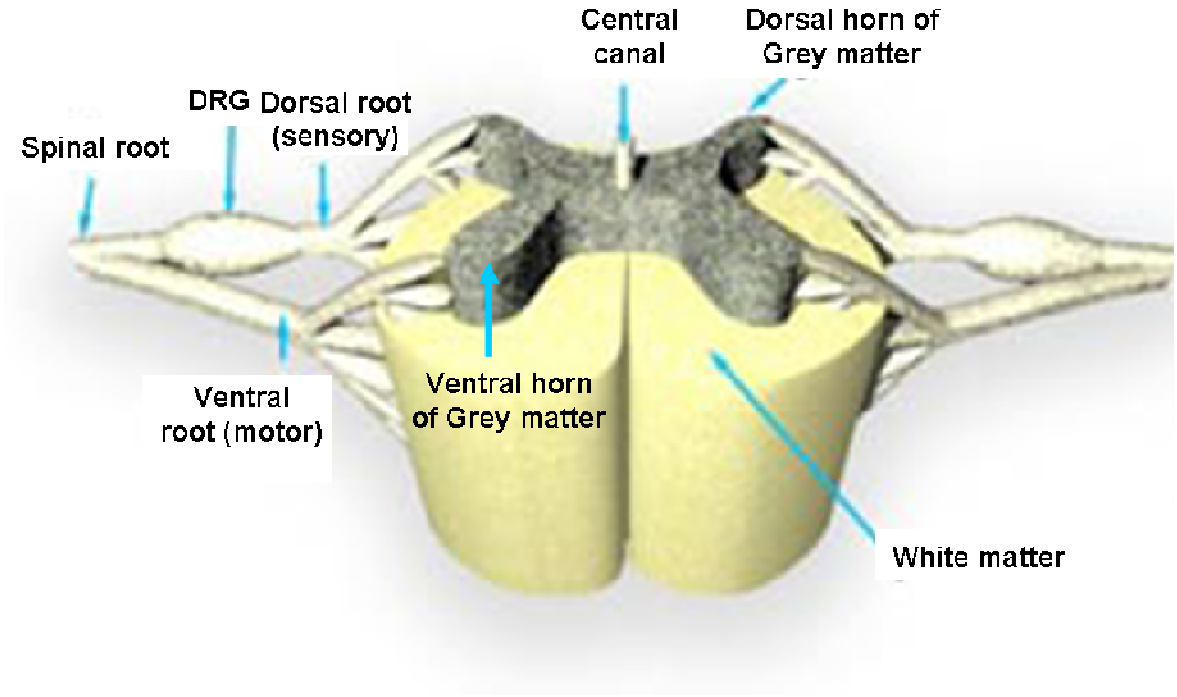


Figure 1.3 – *The basic internal anatomy of the spinal cord*. The spinal cord can be divided into grey matter, containing neuroglia, the cell bodies of motorneurons and interneurons and unmyelinated axons, and the white matter, which contains mostly myelinated axons. The cell bodies of sensory neurons lie in the periphery in a swelling known as the dorsal root ganglia (DRG), whilst sensory fibres project into the spinal cord via the dorsal roots. Both the ventral roots (motor fibres) and dorsal roots (sensory fibres) come together to form pairs of right and left mixed spinal nerves. Through the centre of the spinal cord runs the central canal, which is continuous with the ventricles of the brain and contains cerebrospinal fluid (CSF). Modified from http://www.apparelyzed.com.

1.2 Glial Cells within the Nervous System

Glial cells, or neuroglia, are present in abundance throughout the nervous system and are thought to be imperative for competent neurological functioning, as their Latin name would suggest. Their roles include helping to maintain homeostasis, aiding neurotransmission, forming myelin, providing trophic support and protecting neuronal cells and axons. In the CNS, astrocytes, oligodendrocytes and microglia carry out these various functions; whilst in the PNS, Schwann cells (SCs) and satellite cells constitute the glial population. Although they are not classed as major glial sub-type, Müller cells and Bergmann glia are also specialized radial glial cells related to astrocytes, which function in the retina and cerebellum, respectively (Del Cerro & Swarz., 1976; Mori et al., 1976; Komine et al., 2007; Koirala et al., 2010; Kuang et al., 2012; Lin et al., 2012).

There is a clear segregation of both CNS and PNS glia *in situ* in the absence of disease or pathology. However, olfactory ensheathing cells (OECs) are present both in the peripheral olfactory mucosa *and* in the central olfactory bulbs, making them the only glial cell to span both the CNS and PNS under non-pathogenic conditions. As well as playing vital roles in maintaining the healthy nervous system, glial cells have been widely implicated in disease and injury states; both in contributing to pathology and in modulating the repair of the nervous system.

1.2.1 Microglia

Microglia are the resident macrophages of the CNS and the smallest glial cell type. Originally described by Rio-Hortego (1932), microglia are considered to be of mesodermal origin and it is thought that they migrate into the CNS during early development, where they adopt a highly branched, ramified morphology termed as "resting" in the intact CNS (Kettenmann et al., 2011). Following disease or injury, however, microglia become "activated" and they adopt an amoeboid phenotype with retracted branches (Rio-Hortego & Penfield, 1927; Aliosi, 2001; Xiang et al., 2006).

The brain and spinal cord are considered to be immune-privileged, in that they are segregated from circulating immune cells by the blood brain barrier (BBB), which represents both a physical and immunological barrier, preventing the entry of foreign molecules and pro-inflammatory factors to the CNS (Janzer & Raff, 1987; Bouchaud et al., 1989; Janzer et al., 1993). Therefore, microglia function to maintain homeostasis by scavenging debris and by rapidly responding to insult (Liberto et al., 2004; Dissing-Olesson et al., 2007) by phagocytosing potential pathogens (Frautschy et al., 1992; Bauer et al., 1994; Neumann et al., 2008). In addition, microglia contribute to the pro-inflammatory environment following trauma or disease by secreting cytokines such as IL-1α, IL1-ß and TNF-α (Sawada et al., 1989; Auro et al., 1998; Davies et al., 1999; Clausen et al., 2005) The role of microglia in inflammation following spinal cord injury will be discussed in more detail in section 1.4.3.

In non-pathological situations, microglia have also been implicated in a number of physiological processes, and contrary to previous beliefs, advances in imaging techniques have suggested that "resting" microglia are in fact highly motile i*n vivo* in the healthy brain (Nimmerjahn et al., 2005). Several studies have provided evidence to suggest that microglia may be involved in the "synaptic pruning" that takes place to compensate for the over-population of neurons which occurs during development and to aid synaptic remodelling, particularly in areas such as the hippocampus (Perry et al., 1985; Dalmau et al., 1998; Fiske & Brumes, 2000). Data obtained from time lapse experiments has demonstrated

microglia engulfing retinal ganglion cells (RGCs) during synaptic remodelling of the visual system (Stevens et al., 2007; Huberman et al., 2008). In addition, as well as contributing to inflammation, microglia can also *alleviate* the inflammatory environment by phagocytosing apoptotic cellular debris, thus reducing the abundance of pro-inflammatory cytokines in the micro-environment (Magnus et al., 2001). Finally, microglia are also a source of growth factors, such as basic fibroblast growth factor (bFGF) and transforming growth factor beta (TGF-β) (Araujo & Cotman, 1992).

1.2.2 Astrocytes

Astrocytes, so-called due to their stellate morphology, are multi-process bearing cells present throughout the brain and the spinal cord. They are distinguishable by their expression of intermediate filaments composed of glial fibrillary acidic protein (GFAP) (Eng et al., 1971; Bignami et al., 1972), though they express other markers such as the intermediate filaments vimentin and nestin, as well as the calcium-biding protein, S100-β. Astrocytes fill the spaces between neurons and synapses, where their functions include the support of neurons and the regulation of neurotransmission by absorbing excess neurotransmitters, thus preventing the inappropriate spread of electro-chemical information (Westergaarde et al., 1995; Walz., 1989; Molnar et al., 2011). Furthermore, astrocytic end-feet from the brain and spinal cord parenchymal basal lamina form the glia limitans, or glial limiting membrane, which lies deep to the pia mater (Black & Waxman, 1985; Goto & Hashimoto, 1988; Wolburg & Risau, 1995) and forms part of the blood-brain barrier.

1.2.2.1 Astrocyte Morphology and Classification

In vivo, astrocytes exist either as long, multi-process bearing fibrous astrocytes found predominantly in the white matter or as protoplasmic astrocytes, which contain fewer and shorter branches and are most prevalent in the grey matter. Whilst the processes of fibrous astrocytes tend to have regular contours and are cylindrical and elongated, those arising from protoplasmic astrocytes are described as being irregular, flat sheet-like processes (Peters et al., 1976; Wilkin

et al., 1990). Astrocyte morphology *in vitro*, however, is classed as being either Type 1 or Type 2 based largely upon the findings of Raff and colleagues (1983 a, b). Using cultures derived from rat optic nerves, they demonstrated the existence of a sub-population of astrocytes, termed Type 1, which had a fibroblast like morphology and did not bind to tetanus toxin or to the monoclonal antibody, A2B5, which recognizes cell surface tetrasialogangliosides (Eisenbarth, 1979). Conversely, Raff et al., (1983 a) described the presence of Type 2 astrocytes, which were said to be neuronal-like in their morphology and able to bind both tetanus toxin and the A2B5 antibody. Whilst epidermal growth factor (EGF) was mitogenic for Type 1 astrocytes, it did not induce proliferation in Type 2 astrocytes. Raff and colleagues also reported that immunolabelling of cultures derived from white matter tissue indicated the presence of both Type 1 and Type 2 astrocytes, whilst grey matter cultures only contained the former astrocyte sub-type.

1.2.2.2 Astrocyte Development

Astrocytes are though to be generated from precursors from the sub-ventricular zone (SVZ), which is derived from the neuroepithilium of the neural tube (Levison & Goldman, 1993, 1997; Luskin et al., 1993; Luskin & McDermott, 1994). Perinatally, these precursors are said to arise in the cerebellum and they then migrate throughout the white matter (Milosevic & Goldman, 2002). It is postulated that the cells that make up the CNS are derived from neural stem cells (NSCs), which are multi-potent non-committed cells. However, evidence suggests that NSCs may in fact initially give rise to intermediate precursor cells with a semi-committed lineage. For example, glial-restricted precursors (GRPs) derived from NSCs can differentiate into astrocytes and oligodendrocytes, neuronal-restricted precursors (NRPs) and possibly astrocyte-restricted precursors (ARPs) (Liu & Rao, 2004; Dietrich et al., 2007). Raff and colleagues (1983) demonstrated the ability of the GRPs to differentiate into both astrocytes and oligodendrocytes *in vitro* depending upon the culture medium, whilst GRPs have also been shown to be present in the developing spinal cord from embryonic day 12 (E12), and are distinguishable from surrounding epithelial cells based upon their immunoreactivity with the A2B5 antibody (Rao et al., 1998).

In cultures derived from optic nerve, radial glial cells in the epithelium have also been shown to produce astrocyte precursor cells (APCs), which initially express Pax2, vimentin and cell-surface gangliosides labelled by the A2B5 antibody. Initially, these APCs are negative for GFAP and S100-β, though GFAP expression is eventually gained upon their differentiation into astrocytes, which is induced by CNTF and LIF in culture (Mi & Barres, 1999).

Raff et al., (1983 b) showed that a common progenitor expressing both NG2 and platelet derived growth factor receptor α (PDGFRα) cultured from the neonatal rat optic nerve (denoted as the O-2A progenitor) could also give rise to either Type 2 astrocytes or oligodendrocytes depending upon the presence of serum. For example, culturing of these cells in foetal calf serum (FCS) resulted in their differentiation into Type 2 astrocytes, whilst an absence of serum in the culture medium induced oligodendrogliogenesis. GRPs are said to be distinct from O-2A progenitors in that they do not express NG2 or PDGFRα and they exhibit differential adhesive properties in response to laminin and fibronectin (Rao & Mayer-Proschel, 1997). Miller & Raff (1984) attempted to correlate these findings *in vivo* by demonstrating that fibrous astrocytes in the optic nerve of frozen tissue sections from the adult rat labelled with the A2B5 antibody, similarly to Type 2 astrocytes *in vitro*, whilst protoplasmic astrocytes from the cerebral cortex were A2B5 negative, like their Type 1 counterparts. Miller et al., (1985) also described the presence of 3 distinct glial populations in the optic nerve *in vivo*: galactocerebroside positive (GalC^{+ve}) oligodendrocytes, A2B5 $^{-ve}$/ GFAP $^{+ve}$ Type 1 astrocyte, which first appear embryonically, and A2B5^{+ve}/ GFAP^{+ve} Type 2 astrocytes, appearing from post-natal day 7 onwards.

However, the existence of Type 1 and Type 2 astrocytes derived from O-2A progenitors *in vivo* is difficult to prove and the previous findings have been contested somewhat by others. Espinosa De Los Monteros et al., (1993) demonstrated that pre-labelled O-2A progenitors injected into the neonatal rat brain differentiated into GalC $^{+ve}$ oligodendrocytes, and not Type 2 astrocytes. However, transplantation of an O-2A cell line, which demonstrated similar bi-potential characteristics to O-2A progenitors *in vitro,* into glial-free areas of the adult rat spinal cord resulted upon their differentiation into both myelinating

oligodendrocytes and Type 2 astrocytes (Barnett et al., 1993). Whilst the work of Espinosa De Los Monteros et al., (1993) and others could indicate that the existence of a common progenitor for both the oligodendrocyte and Type 2 astrocyte lineage is an artefact of *in vitro* culture, it is also worth considering the effects of age and CNS region (ie. spinal cord, cerebellum etc) on the ability of O-2A progenitors to differentiate into either cell type. Even subtle changes in the cellular composition of the micro-environment could greatly influence cellular behaviour by modifying paracrine/ autocrine signalling mechanisms as well as interactions with the extracellular matrix (ECM), thus altering cell-fate. Due to the lack of consistent *in vivo* evidence to prove the existence of Type 2 astrocytes derived from O-2A progenitors, these cells have since been termed as oligodendrocyte precursor cells (OPCs).

Free-floating sphere cultures can also be utilised to study NSC fate *in vitro*. Reynolds & Weiss (1992) initially demonstrated that neurospheres from the mouse striatum, which were responsive to the mitogenic properties of epidermal growth factor (EGF) possessed antigenic similarities to both neurons and astrocytes, such as the expression of Substance P and gamma-aminobutyric acid (GABBA), as well as GFAP (Doetsch et al., 1999; Laywell et al., 2000; Seri et al., 2001). Furthermore, these spheres could be triturated and differentiated into astrocytes under culture conditions which included serum (Thomson et al., 2006, 2008), though others have also demonstrated the ability of neurospheres to differentiate into oligodendrocytes and neurons (Maciaczyk et al., 2009; Darsalia et al., 2010).

1.2.2.3 Astrocyte Differentiation

It has been reported that the majority of astrocytes are generated during gestation and in the first 2 weeks postnatally in rodents (Skoff & Knapp, 1991). Thereafter, astrocyte numbers are said to remain fairly consistent throughout the adult CNS (Hommes & Leblond, 1967, Korr et al., 1973; Paterson, 1983). Cilliary neurotrophic factor (CNTF) secreted by Type 1 astrocytes has been shown to induce the differentiation of OPCs *in vitro* into Type 2 astrocytes (Hughes et al., 1988; Lillien et al., 1988). In addition, factors such as

interleukin-6 (IL-6), oncostatin M (OSM) and cardiotrophin-1 all from the IL-6 family have also been shown to stimulate astrocyte differentiation in embryonic CNS and optic nerve cultures, possibly via the activation of the JAK/ STAT pathways, which induce GFAP expression (Gard et al., 1995; Johe et al., 1996; Bonni et al., 1997; Ochiai et al., 2001). Bone morphogenic proteins 2 and 7 of the TGF-β super-family have also been implicated in regulating aspects of astrocyte differentiation via activation of the Smad transcription factors (Gross et al., 1996). Furthermore, constitutively activating Notch in the E9 mouse brain induced the initial production of radial glial cells, which differentiated into astrocytes in the adult brain (Gaiano et al., 2000). It would appear that the delicate and timely balance of all of these factors, amongst others, is necessary for mediating the differentiation of astrocytes from their precursors.

1.2.3 Oligodendrocytes

The primary function of oligodendrocytes in the CNS is to ensheath axons in myelin by extending multiple processes which wrap around several axons, thus providing electrical insulation. Oligodendrocytes are smaller in size than astrocytes and lack $GFAP^{+ve}$ intermediate filaments; however, they contain large numbers of microtubules in their dynamic processes, unlike the latter glial cell type (Peters et al., 1991; Lunn et al., 1997),

1.2.3.1 Oligodendrocyte Development

Similarly to astrocytes and neurons, oligodendrocytes are thought to be derived from precursor cells arising from neuroepithelial cells in the SVZ (Hardy & Reynolds., 1991; Doetsch et al., 1997; Holz & Schwab, 1997). It has been reported that oligodendrocytes first appear in the optic nerves from birth and that oligodendrogliogenesis can continue for up to 6 weeks postnatally in rodents (Skoff et al., 1976; Barres & Raff, 1993; Baumann & Pham-Dinh., 2001).

In the spinal cord, oligodendrocyte precursors arise in the ventral ventricular zone and then migrate dorsally during development (Warf et al., 1991). Thereafter, the dorsal regions acquire the ability to give rise to oligodendrocytes

during further development. Cultures of the thoraco-lumbar rat spinal cord demonstrated that the capacity for oligodendrogliogenesis was restricted to the ventral region of the spinal cord until approximately E14 (Warf et al., 1991). Studies of transgenic mice lacking a floor-plate have demonstrated its importance in inducing the development of ventrally derived oligodendrocytes, namely via the expression of sonic hedgehog (*Shh*), which induces the necessary transcriptional changes required for oligodendrocyte differentiation (Lu et al., 2000; Zhou et al., 2000); and bone morphogenic proteins (BMPs), which negatively regulate oligodendrogliogenesis by promoting astrogliosis (Orentas & Miller., 1996; Pringle et al., 1996; McMahon et al., 1998; Liem et al., 2000).

As discussed in section 1.2.2.2, oligodendrocytes most commonly differentiate from a pre-cursor, the O-2A progenitor/ OPC, which can also give rise to Type 2 astrocytes *in vitro* (Raff et al., 1983). A subset of these OPCs is also thought to give rise to a population of adult $NG2^{+ve}$ OPCs. These so called NG2 cells are a distinct population of glia, which are antigenically and morphologically similar to OPCs, although they proliferate, migrate and differentiate more slowly, and have been described in abundance throughout the adult CNS (ffrench-Constant & Raff, 1986; Wolswijk & Noble, 1989; Reynolds et al., 1997; Butt et al., 1999; Horner et al., 2000; Dawson et al., 2003). Furthermore, $NG2^{+ve}$ glia are also said to have stem cell-like properties and are capable of prolonged self-renewal *in vitro* (Wren et al., 1992).

Zhu et al., (2008) used transgenic mice engineered to express GFP derived from the NG2 lineage to demonstrate that these cells were able to readily differentiate into oligodendrocytes, and to a sub-set of protoplasmic astrocytes present only in the grey matter of the spinal cord but not in the white matter. Typically, however, $NG2^{+ve}$ glia are thought to preferentially give rise to oligodendrocytes *in situ* (Levine et al., 1988 a,b). Furthermore, Yoo & Wrathall (2007) suggested that there may be a bias towards oligodendrocyte differentiation from NG2 glia following CNS injury by culturing free-floating spheres and single cell suspensions derived from the spinal cord of injured and uninjured rats. They reported that whilst the NG2 cells co-expressed markers such as GFAP or CC1 in culture, suggesting their bi-potential for both astrocytes

and oligodendrocytes, NG2 glia from the injured spinal cord differentiated into oligodendrocytes but rarely astrocytes.

1.2.3.2 Oligodendrocyte Differentiation

The process of oligodendroglial cell maturation involves the progression of OPCs into terminally differentiated oligodendrocytes with myelin-forming capacity, via several intermediate stages. Oligodendrocyte differentiation can be assessed by morphological changes, including the formation of highly complex branching as maturation proceeds, as well as via the expression of several phenotypical antigenic markers, which can be transiently expressed through multiple stages of differentiation (Figure 1.5). It is estimated that the initial overpopulation of oligodendrocytes results in approximately 50% undergoing programmed cell death upon failing to myelinate (Knap et al., 1986; Barres et al., 1992). Trapp and colleagues (1997) reported that in the cerebral cortex of rats, approximately 20% of the pre-myelinating oligodendrocyte population were degenerating between day 7 and day 21 after birth. Upon reaching maturation, oligodendrocytes become mitogenically unresponsive and lose their ability to migrate (Raff et al.,1978; Ranscht et al., 1982; Zhang & Miller, 1996).

Typically, markers such as the A2B5 antibody recognizing gangliosides such as GT3 and its *O*-acetylated derivative, which are both down-regulated with increased differentiation and prior to the onset of myelination, can be used to identify OPCs (Eisenbarth, 1979; Dubois et al., 1986; Farrer et al., 1999). Furthermore, the cell surface proteoglycan, NG2, was also shown to be expressed in approximately 95% of $A2B5^{+ve}$ bipotential glia derived from the optic nerves (Stallcup & Beasley, 1987); whilst the expression of platelet derived growth factor receptor alpha (PDGFRα) is also commonly used as a means of identifying OPCs (Pringle et al., 1992). The expression of GalC and the binding of the oligodendrocyte 4 (O4) antibody, which recognises cell-surface sulfatides, seminolipids and a pro-oligodendrocyte antigen, are used to identify intermediate stages of differentiation, though both can persist throughout the maturation of oligodendroglial cells (Sommer & Schachner, 1981; Uchida et al., 1981; Bansal et al., 1989). Mature oligodendrocytes and myelin sheaths can be

labelled with antibodies which recognise glycoproteins such as myelin basic protein (MBP), myelin oligodendrocyte protein (MOG) and proteolipid protein (PLP), amongst others (Baldwin & Carnegie, 1971; Lennon et al., 1971; Scolding et al., 1989). The antibody to PLP also recognises its splice variant, DM20, which can be detected in oligodendroglial cells before the onset of maturation and myelin formation thus, antibodies to PLP can also label less differentiated oligodendrocytes (Nave et al., 1987).

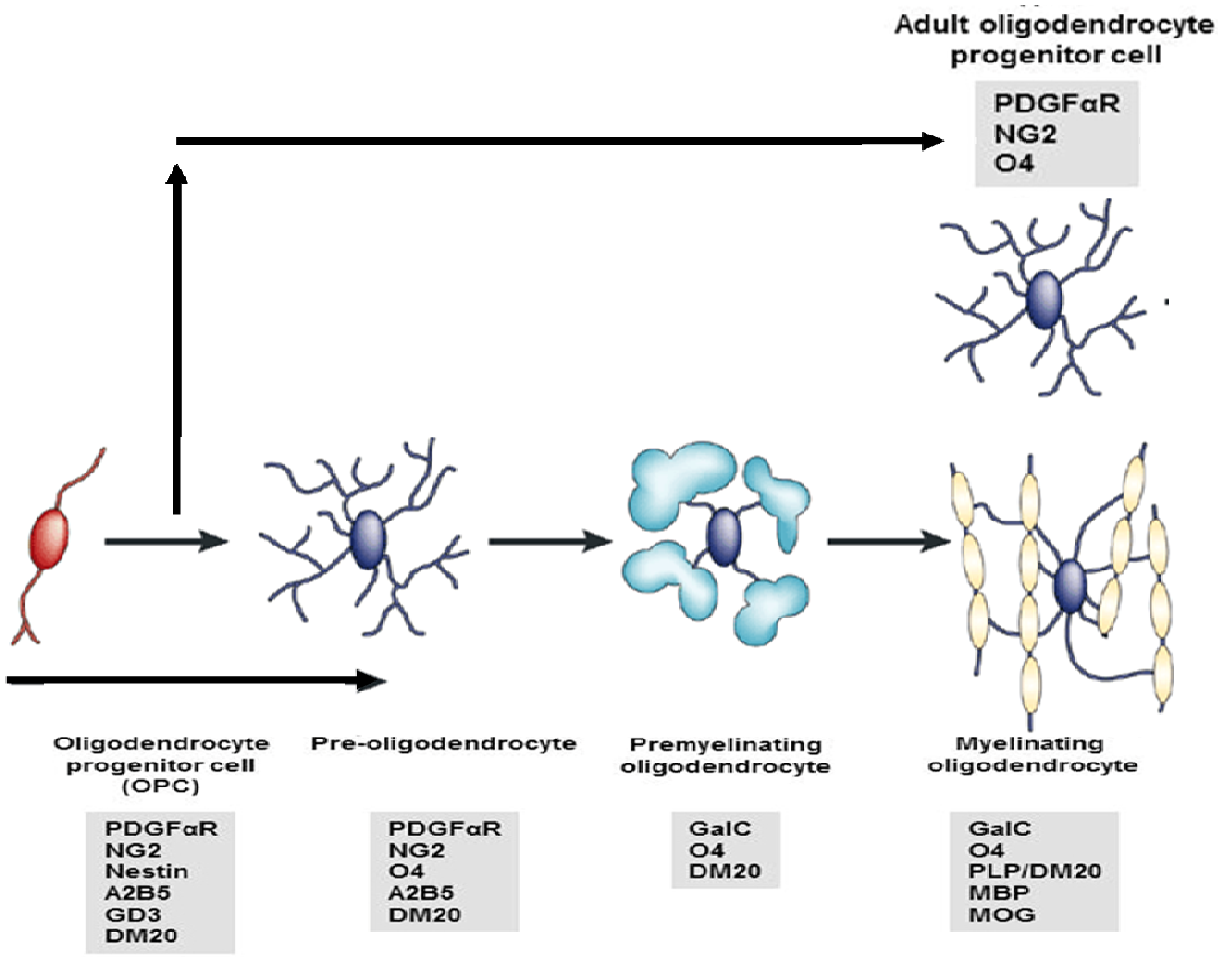


Figure 1.4 – *The stages of oligodendroglial cell differentiation*. Cells of the oligodendroglial lineage can be characterised in terms of their maturation status by their morphology and their expression of several antigenic markers. OPCs typically express NG2 and PDGFRα and label with the A2B5 antibody, which recognises cell-surface gangliosides. Intermediate markers include Gal C and the O4 antibody, which can also remain present in myelinating oligodendrocytes. Mature markers, such as PLP, MBP and MOG typically denote terminally differentiated oligodendrocytes and are also present throughout the myelin sheaths. The antibody to PLP also recognises its less mature isoform, DM20. Adult progenitors, thought to be derived from OPCs, express NG2 and PDGFRα and in some instances the O4 antibody. Although they are more branched than immature OPCs, they lack mature myelin markers, such as MBP, MOG or GalC. Modified from Zhang (2001) *Nature Neuroscience Reviews* **2**: 840 -843 and www.frontiersin.org/NeuroendocrinScience/10.3389/fnins.2012.00010/full.

1.2.3.2.1 Factors Controlling Oligodendrocyte Differentiation

The mechanisms which govern the differentiation of cells of the oligodendroglial lineage from progenitors into mature oligodendrocytes are highly complex and as yet, not fully understood. However, it is thought that an intricate balance of several factors which regulate survival, proliferation and differentiation are imperative for mediating these events. The presence of specific environmental cues can greatly influence the fate of progenitors; for example, the postnatal repertoire of growth factors favours gliogenesis as opposed to neurogenesis, thus most neurons are formed embryonically (Levison et al., 1993; Johe et al. 1996; Calver et al., 1998; Yandava et al., 1999).

Platelet derived growth factor (PDGF), known to be secreted by Type-1 astrocytes (Raff et al., 1988) is a potent mitogen and survival factor for oligodendrocyte precursor cells (OPCs) (Noble et al., 1988), which express the cell surface PDGFRα (of which there are α and ß) (Hart et al., 1989a). Several studies have demonstrated the effects of PDGF on cells of the oligodendroglial lineage, such as the addition of increasing concentrations of PDGF in rat optic nerve cultures, which resulted in a significant decrease in the number of oligodendrocytes that underwent apoptosis (Barres et al., 1992). Furthermore, over-expression of PDGF in mice demonstrated the hyperproliferation of OPCs (Calver et al., 1998), thus emphasising the roles of PDGF on cell survival and expansion. Upon binding of PDGF, the PDGFR dimerises and subsequently activates signal transduction of anti-apoptotic pathways such as the phosphatidyl inositol 3 kinase (PI3-K) pathway. It is said that oligodendroglial cells eventually become mitogenically unresponsive to PDGF, perhaps due to disruption of downstream signalling pathways, thus inducing their differentiation (Hart et al., 1989 b).

Others expanded upon these findings by reporting that the heparin-binding growth factor, FGF, was also a potent mitogen for OPCs (Eccleston & Silberberg, 1985). Unlike with PDGF alone, exposure to a combination of both bFGF and PDGF resulted in the *sustained* proliferation of perinatal OPCs *in vitro* and the

inhibition of their differentiation (Bogler et al., 1990; Noble et al., 1990). Furthermore, Wolswijk & Noble (1992) demonstrated that bFGF was also mitogenic for adult OPCs *in vitro*. McKinnon & colleagues (1991) reported that the synergistic effects of the combination of FGF with PDGF were likely due to the ability of FGF to positively regulate PDGFR expression on OPCs, as well as their sensitivity to PDGF; whilst PDGF was required to make bFGF treated OPCs motile (McKinnon et al., 1993).

The actions of other regulators of oligodendrocyte maturation, such as transforming growth factor-beta (TGF-ß), exert their effects by modulating the actions of PDGF (McKinnon et al., 1993) to cease proliferation and to enhance the differentiation state of cells of the oligodendroglial lineage. In addition, factors including neurotrophin 3 (NT3), neuregulin, glial growth factor-2 and leukaemia inhibitory factor (LIF); (Richardson et al., 1988; Barres et al., 1993; Cohen et al., 1996; Adachi et al., 2005) are also reported to be mitogenic for oligodendroglial cells.

Insulin-like growth factor-1 (IGF-1) and insulin-like growth factor-2 (IGF-1) are also vital for regulating oligodendrocyte behaviour (McMorris et al., 1986; Ye et al., 1995, 2002). Ubiquitously present throughout the body, the effects of IGF-1 and IGF-2 are mediated via the interaction with insulin-like growth factor 1 receptor (type 1 IGF-1R). Binding to the IGF receptor leads to the subsequent activation of the anti-apoptotic pathways PI3-K and Akt, and thereafter, the activation of ERK-1 and ERK-2 which regulate the transcription of several cell survival factors, such as c-fos and c-jun (Feldman et al., 1997). IGFs under normal physiological conditions are usually bound to an insulin growth factor binding protein (IGFBP), of which there are 6 in total with differing structural characteristics depending upon their specialised function, so that their distribution can be targeted throughout the body whilst simultaneously stabilizing their metabolic clearance. In the CNS, it is thought that IGFs are produced by glial and neuronal cells and interestingly, IGF-1 is also reported to be abundant within the olfactory bulbs (Rotwein et al.,1988; Werther et al., 1993; Russo et al., 1994) and olfactory epithelium (Federico et al., 1999).

The importance of IGFs and IGF-1R signalling on oligodendrocyte behaviour has unequivocally been demonstrated through various studies whereby treatment with IGF-1 in particular was shown to protect OPCs from glutamate induced excitotoxic death (Wood et al., 2007). Conditional knock out studies of IGF-1R in OPCs reported a decrease in the volume of the corpus callosum and anterior commissure, as well as a reduction in cell numbers of the $NG2^{+ve}$ and mature oligodendrocyte population and an overall reduction in myelination (Ye et al., 2002; Zeger et al., 2007). Similarly, administration with IGF-1 into the rat spinal cord resulted in an increase in 2', 3'-cyclic nucleotide 3'-phosphohydrolase (CNP), which labels intermediate and mature/myelinating oligodendrocytes and myelin sheaths (Brunner et al., 1989), and an increase in overall myelin sheath formation (Goddard et al., 1999).

As well as secreted factors, interactions with molecules within the extracellular matrix (ECM) can also affect the differentiation state of oligodendrocytes. For example, laminin has been demonstrated to potentiate the response of oligodendrocytes to growth factors such as PDGF (Frost et al., 1999; Colognato et al., 2002, Baron et al., 2003). Furthermore, the laminin-2 receptor α-dystroglycan has been implicated in IGF-1 signalling in that si-RNA knock down studies of dystroglycan resulted in an inhibition of IGF-1-mediated differentiation of oligodendrocytes on a laminin substrate (Galvin et al., 2010), though it is not thought to play a role in cell survival. Conversely, the α6β1 laminin receptor has been implicated more in the activation of the pro-survival pathways, PI3-K/ AkT (Colognato et al., 2002, 2004; Barros et al., 2009), thus suggesting that the effects of laminin on oligodendroglial cell behaviour may differ substantially depending upon receptor activation.

In addition, Tenascin-C and Tenascin-R as substrates for oligodendroglial cells have been shown to inhibit aspects of maturation such as MBP expression (TnC), as well as oligodendrocyte process extension via Rho GTP signalling (TnC and TnR), and myelin formation (Kiernan et al., 1996; Nash et al., 2011). Studies of TnC KO OPCs suggested that whilst they demonstrated an increased rate of maturation, when cultured on PLL coated coverslips their survival was limited compared to wild-type controls, suggesting that the role of TnC in governing

oligodendroglial cell behaviour may be to confer protection against pro-apoptotic signalling (Garwood et al., 2004).

The Notch family is also considered to be important in mediating different aspects of oligodendroglial behaviour. The Notch receptor, present on OPCs, binds to its ligand, jagged 1 (expressed by astrocytes), to induce the activation of the transcription factors hairy and enhancers of split 1 and 5 (Hes1 and Hes5), which are suppressors of several genes controlling cell differentiation. Activation of the notch pathway controls the timely differentiation of oligodendrocytes, as was evidenced in a study of the rat optic nerve, whereby jagged 1 expression on the axons of retinal ganglion cells decreased temporally with the onset of myelination in the optic nerve tracts (Wang et al., 1998). Since the Notch receptor is said to be expressed in adult OPCs (Stidworthy et al., 2004) and in MS lesions (John et al., 2002), it is hypothesised to play a potential role in the remyelinative failure of the CNS during disease progression. However, Stidworthy and colleagues reported that the conditional knock down of Notch 1 in PLP expressing cells did not affect the rate of remyelination in a cuprizone lesion of the trigeminal tracts. Conversely, a study by Zhang et al., (2009) whereby Notch 1 was inactivated in Olig 1 expressing cells in a mouse model of demyelination via administration of lysolethicin into the corpus callosum, remyelination was enhanced, thus suggesting that the inhibition of the Notch pathway mediated repair by suppressing the inhibition of oligodendrocyte differentiation. Whist PLP/DM20 is said to be a marker for both mature and immature oligodendroglial cells, Olig 1 expression is associated more with OPCs. Thus, these conflicting reports could be attributed to the differing maturation stages at which Notch was knocked down. Also, since both studies looked at remyelination in different areas, distinct differences in the surrounding cellular environment could also play a role in the control of oligodendrocyte differentiation and remyelination.

The Wnt group, which consists of wingless and integration 3 (Wnt3), ß catenin and transcription factor 4 (Tcf4), also acts upon oligodendrocytes to prevent their differentiation via the translocation of ß catenin to the nucleus where it activates Tcf4 to suppress differentiation genes. Constitutively activated Wnt signalling in mice resulted in the delayed formation of myelin sheaths and the

appearance of mature oligodendrocytes, without an increase in OPC cell number, suggesting that the role of Wnt in oliogdendrocyte maturation is independent of proliferation (Feigenson et al., 2009).

As mentioned throughout this thesis, cilliary neurotrophic factor (CNTF) is known to mediate aspects of oligodendroglial behaviour, such as cell survival, particularly against the activation of pro-apoptotic pathways via tumour necrosis factor alpha (TNFα) (Barres et al., 1993; Tsukamoto et al., 1995; D'Souza et al., 1996). Proliferation of OPCs from the optic nerve is also said to be enhanced by increased expression of CNTF, whilst mice with ablated CNTF expression exhibit a reduction in OPC proliferation and eventual losses in oligodendrocyte numbers (Barres et al., 1996). CNTF also plays a role in oligodendrocyte maturation, in that an increase in CNTF in cultures of OPCs resulted in increased MBP expression compared with controls. The mechanisms underlying this CNTF-mediated enhancement of differentiation do not appear to be oligodendrocyte specific, however, given that this treatment can also induce the differentiation of OPCs into astrocytes in culture depending upon the extracellular environment. Furthermore, myelination is enhanced by CNTF *in vitro,* probably indirectly via astrocyte secreted factors, (Nash et al., 2011) and *in vivo* when OPCs over-expressing CNTF where transplanted into the contused thoracic spinal cord of rats (Cao et al., 2010).

1.2.3.2.2 Intrinsic Differentiation of Oligodendrocytes

Whilst the importance of environmental cues on oligodendrocyte differentiation has been discussed, it has also been reported that oligodendrocytes may differentiate in response to intrinsic mechanisms. For example, cultures of OPCs derived from the embryonic brain proliferated a set number of times before forming oligodendrocytes around the time when the donor animals would have been born and thus, oligodendrogliogenesis would have begun (Abney et al., 1981). Furthermore, Noble & Murray (1984) demonstrated that OPCs developed into oligodendrocytes in the presence of Type 1 astrocytes, regardless of the age of the astrocyte, which could arguably alter the micro-environment in which the

OPCs were grown. These findings were therefore interpreted as suggesting that intrinsic mechanisms within OPCs cells also controlled their differentiation. Most compellingly, studies of clonally related OPCs reported that these cells displayed a tendency to undergo differentiation after a similar number of divisions (Temple & Raff, 1986). It has been suggested that the cyclin-dependent kinase (Cdk) inhibitor, p27/Kip 1, progressively accumulates in OPCs as they proliferate and is present at high levels in oligodendrocytes, thus suggesting that the accumulation of p27 is part of the intrinsic counting mechanism that ceases precursor cell proliferation to initiate differentiation by arresting the cell cycle (Durand et al., 1997).

1.2.4 Myelination of CNS axons

Myelination, first described by Virchow (1846), occurs by the wrapping of axons in lipid-rich extensions of the plasma membrane of oligodendrocytes in the CNS in order to improve their conduction velocity. A single oligodendrocyte is capable of extending multiple processes to simultaneously wrap several axons in myelin segments, known as internodes. These internodes of myelin are separated by nodes of Ranvier, whereby the axolemma is exposed to the extracellular space (Figure 1.5), thus allowing for action potentials to jump from node to node via Saltatory conduction. It has been reported that both $NG2^{+ve}$ glia and astrocytes contact axons at the nodes of Ranvier (Black & Waxman, 1988; Butt et al., 1999), though the reasons for this are not yet fully understood.

Compact myelin can be characterized by the periodicity of electron dense and light layers; whilst the cytoplasmic loops, which form around the axon at the inner and outer ends of the myelin sheath, known as paranodal loops, represent less compact areas (Rosenbluth et al., 1995). Recent advances in *ex vivo* imaging have provided novel evidence to suggest that following initial axoglial contact, myelination may proceed by the initial spiralling of oligodendrocyte processes around the axon followed by the thickening up and spreading of "cuffs" of myelin which join together to form longer internodes; termed the ofiomosaic (serpent) model (Bauer et al., 2009; Sobottka et al., 2011; Ioannidou et al., 2012).

Myelination proceeds caudorostrally in the brain and rostrocaudally in the spinal cord, with the peak of myelination typically occurring in the first year of life in humans, although it can continue into adolescence in associated brain areas, such as the hippocampus (Yakolev & Lecours, 1966; Baumann & Pham-Dinh, 2001).

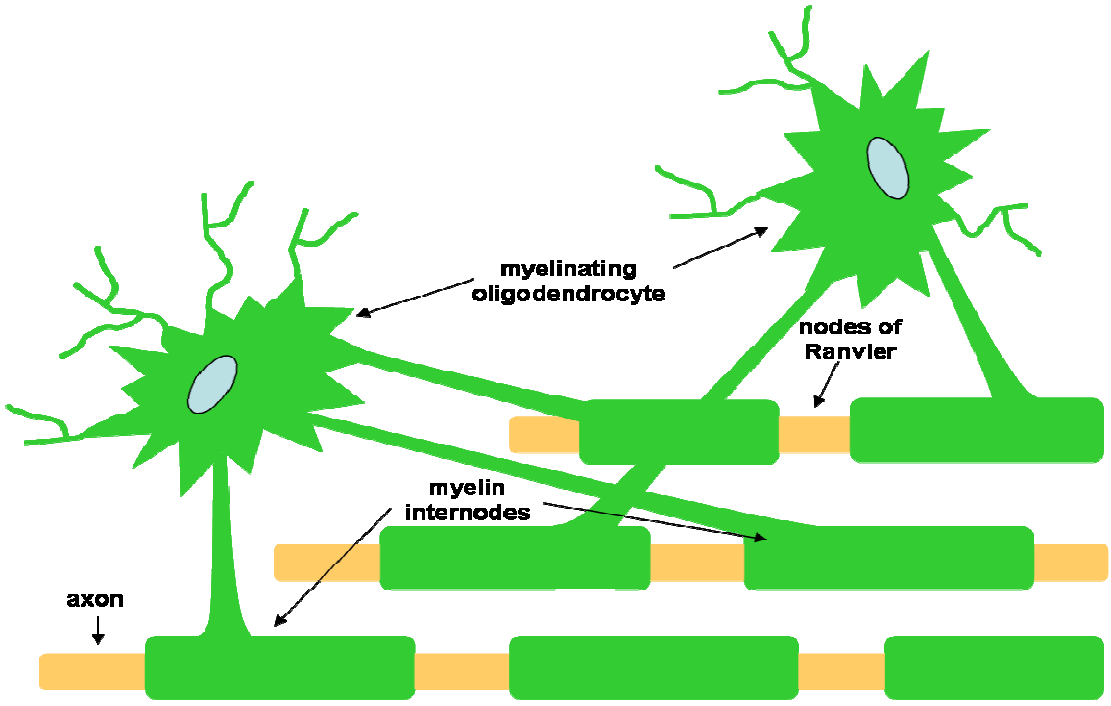


Figure 1.5 – *Oligodendrocyte myelination*. A mature oligodendrocyte can extend its processes to wrap several adjacent axons in internodes of compact myelin, which is composed of several glycolipids and proteins to provide electrical insulation. Rapid transmission is achieved by Saltatory conduction, whereby action potentials propagate along gaps in the myelin internodes, known as nodes of Ranvier, where sodium channels are clustered, thus increasing velocity.

1.2.4.1 Composition of Central Myelin

The dry mass of myelin is comprised of approximately 70-85% lipids and 15-30% proteins. Of the lipid component, galactocerebroside is thought to be the most abundant, along with lecithin and cholesterol; whilst sphingomyelin is less prolific but is thought to strengthen the myelin sheath (Gregson et al., 1974; Jungalwala, 1974; Gould & Dawson, 1976).

Proteolipid protein and myelin basic protein are the major proteins in central myelin (Omlin et al., 1982; Brenner et al., 1989) and are thought to be vital in regulating the compaction of myelin. For example, MBP/ PLP mutants show abnormalities in the major dense lines of their myelin sheaths (Klugmann et al, 1997; Stoffel et al., 1997). The CNS-specific glycoprotein, myelin oligodendrocyte glycoprotein (MOG) is located in the most superficial layers of the myelin sheath (Linington et al., 1988). As such, MOG has been implicated in

the pathology of autoimmune disorders targeting the myelin sheath in that subsets of patients with neuromyelitis optica (NMO) (Kitley et al., 2012) and multiple sclerosis (MS) (Zhou et al., 2006; Elliot et al., 2012) demonstrate the presence of autoantibodies against MOG, which can trigger the activation of complement-induced cell death. Myelin-associated glycoprotein (MAG) is a minor protein component of the myelin sheath (Linington et al., 1984; Trapp et al., 1987). 2',3'-cyclic nucleotide 3'-phosphohydrolase, known as CNP, is not present in compact myelin layers but is abundant in the paranodal loops (Sprinkle et al., 1980; Trapp et al., 1988). The aforementioned markers are only some of those found in the myelin sheaths. For example, labelling with the O4 antibody demonstrates its presence throughout the myelin sheath (Schiff & Rosenbluth, 1995), and as yet there may be some myelin-specific proteins and lipids which remain undiscovered.

1.2.4.2 Organisation of the Myelin Sheath in the CNS

The myelin sheath contains a series of domains, namely the internode, the paranodal region and the juxtaparanode (Salzer et al., 2003), whereby the accumulation of myelin proteins and the correct organisation of specific molecules is thought to be crucial for maintaining axoglial contact and in mediating the function of the myelin sheath (Figure 1.6). For example, the neuronal-specific adhesion molecule neurofascin 186 (Nf186) is located at the nodes of Ranvier (Tait et al., 2000), along with voltage-gated sodium channels (Na_v) (Rasband & Trimmer, 2001), which aid the propagation of action potentials along the axon. Ankaryin G has been reported as a requirement for the clustering of Na_v channels (Zhou et al., 1998); although it has also been suggested that oligodendrocyte contact may induce Na_v channel clustering at the nodes of Ranvier (Kaplan et al., 1997). The adhesion molecules contactin and axonal contactin-associated protein (Caspr) are observed at the paranodal regions of the axon (Einheber et al., 1997; Rios et al., 2000). Furthermore, the oligodendrocyte-specific adhesion molecule Nf155 is located at the paranodal loops where it apposes Caspr and contactin (Tait et al., 2000). The ablation of glia-specific Nf155 resulted in the gradual loss of paranodal axoglial junctions (Pillai et al., 2009), thus highlighting its importance in maintaining the structure of the myelin sheath.

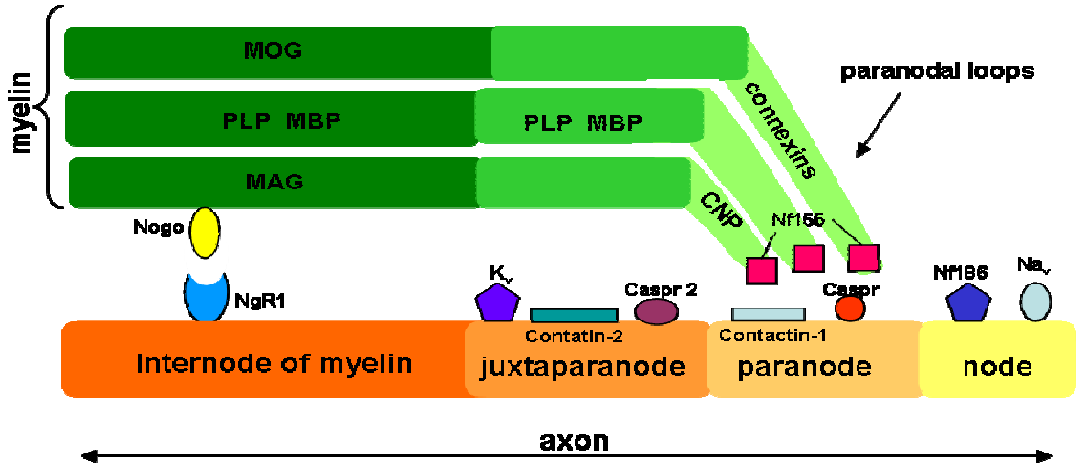


Figure 1.6 – *Organisation of CNS myelin sheaths.* The formation of compact myelin requires the correct assembly of a serious of proteins, which are thought to mediate axonal/ oligo contact, such as neurofascin 155 and 186 (Nf155; Nf186), Caspr and contactin and Nogo along with its receptor, NgR1. Sodium channels (Na_v) cluster at the nodes of Ranvier, whilst potassium channels are located at the juxtaparanodal region of the axolemma to aid the propagation of action potentials. The glycoprotein, myelin oligodendrocyte glycoprotein (MOG), is thought to be located in the most superficial layers of the myelin sheath; whilst myelin associated glycoprotein (MAG) is reported to be concentrated at the innermost layers of the myelin sheath, apposing the axolemma. CNP is most abundant at the paranodal loops. Diagram was drawn based on Mayer et al., (2012) *Journal of Neurological Sciences*. **319** (1-2) p 2-7, with modifications.

1.2.4.3 The Role of Astrocytes in Myelination

There is compelling evidence amongst the literature to suggest that astrocytes play a role in CNS myelination. Using a culture system of RGCs, purified OPCs and optic nerve-derived astrocytes, Watkins et al., (2008) demonstrated that the presence of these astrocytes enhanced the thickness of the myelin sheaths. In addition, Sørenson et al., (2008) reported that an astrocyte monolayer was imperative for the induction of myelination in embryonic spinal cord cultures derived from the rat. Using the same culture system, it was reported that direct modulation of the astrocyte monolayer phenotype using cytokines such as the pro-myelinating factor, CNTF, increased endogenous myelination (Nash et al., 2011). *In vivo*, it has been demonstrated that astrocytes within the rat optic nerve begin to synthesize CNTF at the end of the first post-natal week, which correlates with the onset of myelination (Stockli et al., 1991; Dobrea et al., 1992; Colello et al., 1995). ffrench-Constant and Raff (1986) also suggested that the so-called Type 2 astrocytes had a specialised role to play in regulating

myelination, given that their processes associated with nodes of Ranvier on myelinated axons and they were abundant throughout white matter tracts. Furthermore, GFAP expression is said to increase in the CNS during myelinogenesis (Jacque et al., 1980); whilst a null mutation for GFAP caused abnormal myelination (Liedtke et al., 1996). However, the precise mechanisms by which astrocytes mediate their effects on CNS myelination have yet to be fully elucidated.

1.2.5 Schwann Cells

Schwann cells (SCs) make up the most numerous glial cell population in the PNS, where their role is to myelinate axons and to assist in the regeneration of peripheral nerves following damage, which will be discussed in more detail in section 1.3.1.

1.2.5.1 Schwann Cell Origin

SCs originate from Schwann cell precursors (SCPs), which are derived from neural crest stem cells (NCCs) (Grim et al., 1992; Jessen et al., 1994; Riethmacher et al., 1997). The lateral migration of NCCs from the neural tube is said to drive their differentiation into melanocytes, whilst ventrally migrating NCCs become neurons and glia, suggesting that the fate of NCCs may be pre-determined to some extent (Jessen & Mirsky, 2005). There is also evidence to suggest that transplanted NCCs can differentiate into cells of the oligodendroglial lineage following transplantation into the CNS of a dysmyelinated mouse model (Bindel et al., 2011).

In rats, SCPs appear at approximately embryonic day 14-15 (E14-15), which then differentiate into immature SCs around E15-17 and finally mature myelinating and non-myelinating SCs from E18 onwards (Jessen et al., 1994; Dong et al., 1995). (Figure 1.7). Although SCPs can produce neurons, it is thought that they are biased towards SC differentiation since they appear less responsive to pro-neurogenic factors, such as BMP-2 (White et al., 2001; Kubu et al., 2002; Jessen & Mirsky, 2005). Furthermore, the numbers of SCPs declines as differentiated

SCs appear, whilst SCP-lacking mice mutants do not produce SCs (Britsch et al., 2001), thus suggesting the likelihood that SCPs are responsible for generating SCs. It is postulated that the role of SCPs during development, asides from being a source of SCs, is to aid the survival of neurons. Prior to gliogenesis, SCPs associate closely with developing nerves and in mice lacking SCPs, neuronal survival is poor (Garratt et al., 2000).

Boundary cap cells from the neural crest, which are involved in the formation of the boundaries between the CNS and PNS can also differentiate into subsets of neurons, satellite cells and the SCs in the dorsal and some ventral roots (Murphy et al., 1996; Maro et al., 2004). However, there are no boundary cap-derived SCs in limb nerves, suggesting the existence of 2 populations of SCs with differing origins. Following transplantation into the rodent CNS, boundary cap cells can also become neurons, oligodendrocytes and astrocytes according to fate-mapping studies (Zujovic et al., 2011).

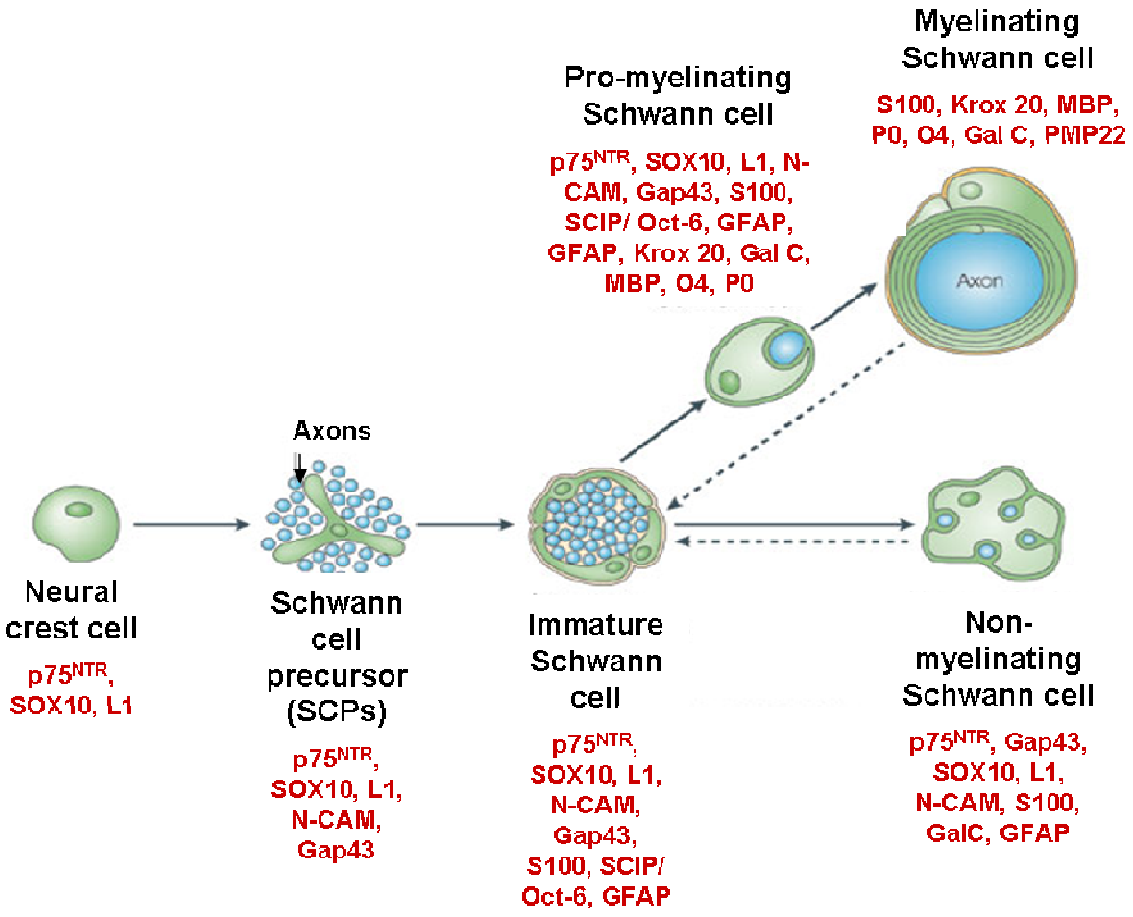


Figure 1.7 – *Schwann cell differentiation from neural crest derived SCPs.* SCPs from neural crest derived stem cells differentiate into immature SCs, which then give rise to mature myelinating and non-myelinating SCs. The low-affinity NGF receptor p75NTR labels cells of the SC lineage throughout differentiation, though its expression is lost in myelinating SCs. Similarly, whilst myelin protein zero (MPZ/P0) gene expression can be detected throughout the SC lineage, P0 is massively up-regulated at the protein level in myelinating SCs, whilst its expression is lacking in non-myelinating cells. Similar increases in myelin proteins such as MBP are also confined to pro-myelinating/ myelinating SCs, as is the expression of the transcription factor Krox-20. Whilst SCPs and immature SCs associate with bundles of axons, the establishment of 1:1 SC/axon relationships precedes the onset of myelination. Adapted from Jessen & Mirsky Nature Reviews Neuroscience (2006) 6 p671-682 and Zorick & Lemke Current Opinion in Cell Biology (1996) 8 p 870-876.

1.2.5.2 The Survival of Cells from the Schwann Cell Lineage

Prior to differentiation, the number of SCs being generated is carefully regulated by counterbalancing pro-survival and apoptotic mechanisms to ensure that the appropriate number of SCs is generated to match axonal numbers (Komiyama et al., 1992). Activation of the $p75^{NTR}$ can trigger pro-death signalling in SCs to reduce cell numbers, as *in vitro* studies have shown that SCs derived from $p75^{NTR}$ deficient mice show enhanced survival in response to serum and growth factor withdrawal (Syroid et al., 2000). Similarly, TGF-ß, which can be expressed by SCs themselves, can also induce apoptosis, though this effect is likely to be under strict developmental control, since mature SCs are less susceptible to TGF-ß-induced cell death (Parkinson et al., 2001).

Conversely, axons are thought to be crucial sources of neuregulins, as NRG1 accumulates in DRGs and axonal tracts during development (Marchionni et al., 1993; Longart et al., 2004), where it is said to aid the survival of SCPs and increase their proliferation (Dong et al., 1995; Morris et al., 1999; Woldeyesus et al., 1999). However, this survival-enhancing effect mediated by axons is reciprocated, as SCP-deficient mice mutants showed limited neuronal survival in limb nerves (Garrat et al., 2000; Britsch et al., 2001). Unlike SCPs, which rely upon factors secreted by neurons, such as NRG1, for their survival (Dong et al., 1995), immature and mature SCs can reduce their vulnerability to programmed cell death via autocrine signalling of factors such as IGFs, NT3, PDGFß and LIF (Chen et al., 1998; Dowsing et al., 1999; Meier et al., 1999; Weiner et al., 1999). IGF-1 has been shown to rescue SCs from apoptosis induced by serum deprivation by inhibiting the JNK pathway, via activation of PI3K (Cheng et al., 2001).

1.2.5.3 Factors Influencing Schwann Cell Differentiation

Each stage of SC differentiation can be characterised by morphological and antigenic changes (Figure 1.7). The low-affinity NGF receptor $p75^{NTR}$ is commonly used as marker of non-myelinating SCs, and is present from the precursor stage and throughout differentiation until it is down-regulated in myelinating SCs (Bonetti et al., 1997). The expression of N-CAM follows a similar pattern throughout SC differentiation. The most striking antigenic difference between (pro) myelinating and non-myelinating SCs is the up-regulation of myelin proteins, including MBP and myelin protein zero (MPZ/P0) in the former cell type, which will be discussed in more detail in section 1.2.6.1 (Zorick & Lemke, 1996; Jessen & Mirsky, 2006). Although it is more commonly associated as being an astrocytic marker, GFAP also labels non-myelinating SCs *in vitro* and *in vivo* (Garrat et al., 2000; Chen et al., 2006). The transcription factor SCIP/Oct 6 (suppressed cAMP-inducible POU), is absent in myelinating SCs (Scherer et al., 1994), whilst Krox-20 is up-regulated only in pro-myelinating and myelinating SCs. (Zorick & Lemke, 1996; Parkinson et al., 2005; Jessen et al., 2006).

The control of SC differentiation is mediated via several factors. Whilst SOX10 is expressed by all NCCs, in SOX10 inactivated mice radial glia and SCPs are missing (Britsch et al., 1998, 2001, Paratore et al., 2001). Neuregulin 1β (NRG1) is also thought to be crucial for aspects of SC behaviour, given that pre-incubation of SCPs with neutralising antibodies to NRG1 reduced SC differentiation (Morrissey et al., 1995 a,b). Furthermore, NRG1 treatment of neural crest cells decreased neurogenesis in culture, suggesting a bias towards gliogenesis (Shah et al., 1994). It has been postulated that the role of SOX10 may be to increase the receptiveness of SCs to NRG1, since SOX10 mutants decrease their expression of the NRG receptor, ErbB3 (Britsch et al., 1998).

Similarly, Notch activation inhibits neurogenesis in cultures of SCs, which correlates with an increase in SC differentiation, thus suggesting that Notch could also be a key mediator in the direct or indirect control of gliogenesis (Morrison et al., 2000; Wakamatsu et al., 2000; Kubu et al., 2002), although its activation inhibits SC myelination (Woodhoo et al., 2009). TGF-β is also a

negative regulator of myelination in DRG cultures (Einheber et al., 1995; Guenard et al., 1995).

Other transcription factors noted to affect myelination in SCs are SCIP/Oct 6 and Krox-20. The down-regulation of SCIP/Oct 6 coinciding with the onset of myelination is said to be necessary for the increased expression of myelin genes, given that SCIP suppresses the P0 and MBP promoters (Scherer et al., 1994). Conversely, Krox-20 is thought to be crucial in regulating myelination by suppressing the JNK pathway, since SCs from Krox-20 KO mice can establish 1:1 relationships with axons but cannot initiate myelination or the necessary up-regulation of myelin genes (Topilko et al., 1994; Zorick & Lemke, 1996; Parkinson et al., 2005; Jessen et al., 2006).

As previously stated SCPs are multipotent but immature SCs appear to be committed solely to differentiating into mature myelinating and non-myelinating SCs, as evidenced by their resistance to pro-neurogenesis factors such as FGF-2 and BMP-2 (Sherman et al., 1993; Morrison et al., 2000). Mature SCs can, however, de-differentiate into immature SCs, often in response to injury (Stewart et al., 1993; Scherer, 1997; Dupin et al., 2003). It is thought that the likelihood of an immature SC to take on a myelinating or non-myelinating phenotype is largely dependent upon axonal contact, with large calibre fibres inducing the induction of the former phenotype. Furthermore, these phenotypes are said to be reversible, in that a non-myelinating SC can transform into a myelinating SC when in contact with the correct axonal signal, and vice versa (Wilkins et al., 1997; Simons & Trotter, 2007).

1.2.5.4 Requirements for Culturing Schwann Cells

In culture, reagents to elevate cAMP levels can be used to influence SC behaviour, thus mimicking the effects of axonal contact. It has been shown *in vivo* that an increase in the level of cAMP enhances the differentiation of pro-myelinating SCs into myelinating SCs (Monuki et al., 1989, 1990; Wegner et al., 2000, 2001). The combination of cAMP elevation and FGF-2 *in vitro* results in increased SC proliferation, whilst in the absence of FGF-2 SCs are encouraged to differentiate (Morgan et al., 1991). NRG 1 also enhances the proliferation of SCs in culture and its effects can be potentiated by the presence of forskolin (Raff et al., 1978; Morrisey et al., 1995).

Typically, protocols for growing SCs include serum in the media, since serum deprivation can induce programmed cell death in SCs (Maurel & Salzer, 2000). A study by Stewart et al., (1991) demonstrated that the presence of serum wasn't required to induce the mitogenic effects of PDGF, FGF or neuregulin. Conversely, without serum, which contains unknown quantities of growth factors, the mitogenic effects of TGF-ß were abolished. Similarly, in serum-free defined media, SCs failed to form a basal lamina or to myelinate DRGs in co-culture (Eldridge et al., 1987). However, these researchers demonstrated that the addition of human placental serum and chick embryo extract was sufficient to induce basal lamina formation and myelination in culture and this effect could also be reproduced when cultures were grown in the presence of serum and ascorbic acid. Thus, the use of ascorbic acid in serum-containing media is now common place in DRG/SC co-cultures where myelination is being studied (Maurel et al.,2000; Melli et al., 2009; Limpert & Carter., 2010).

1.2.5.5 Schwann Cell Purification

Early reports indicated the presence of both fibroblasts and SCs in peripheral nerves (Brockes et al., 1979). However, these researchers showed that in the absence of mitogens (other than those present in serum) fibroblasts divided rapidly in culture, whilst SCs divided more slowly. Furthermore, cultured fibroblasts expressed the Thy-1.1 antigen whereas SCs did not. Brockes & colleagues (1979) exploited these fundamental differences in order to generate purified cultures of SCs. They exposed 2 day old cultures to cytosine arabinoside (AraC) to kill off the rapidly dividing fibroblasts (Aguayo et al., 1975), before placing the remaining cells into medium containing an extract of bovine pituitary extract to support the growth of SCs. However, the resulting cultures were only 80-90% pure. Thus, an antiserum to Thy-1.1 was added to cell suspensions along with rabbit complement to kill of any remaining Thy-1.1^{+ve} cells. Thereafter, the cultures were reported to be approximately <95% pure for SCs. The use of AraC followed by a Thy-1.1 complement-mediated kill is now a commonly used protocol for generating highly purified cultures of SCs (Weinstein & Wu, 1999; Lakatos et al., 2000; Fairless et al., 2005; Honkanen et al., 2007; Higginson et al., 2012).

1.2.6 Peripheral Myelination by Schwann Cells

Myelination in the peripheral nervous system begins with the radial sorting of axons according to calibre and the establishment of 1:1 axon/SC relationships, from approximately E18 (Chen et al., 2003; Xu et al., 2005; Yang et al., 2005). This process requires the initial production of a layer of ECM by SCs, known as a basal lamina, as studies of ß1-integrin deficient mice show defects in radial sorting (Feltri et al., 2002; Li et al., 2005; Benninger et al., 2007; Grove et al., 2007; Nodari et al., 2007). The role of the basal lamina is to induce polarity of the SC/axon complex and to aid the orientation of the SC in relation to its environment and the axo-glial interface. The attachment of SCs to axons is postulated to be mediated via factors including nectin-like 1 (Necl1) in axons and nectin-like 4 (Necl4) in SCs, which accumulates at the periaxonal surface in the region where the myelin internode would develop (Maurel et al., 2007;

Perlin et al., 2007; Park et al., 2008). Gliomedin is also instrumental in facilitating this axo-glial contact, in addition to inducing the clustering of sodium channels to the nodes of Ranvier (Eshed et al., 2005; Feinberg et al., 2010).

Once this 1:1 relationship has been established, the SC extends its cytoplasmic folds several times around the axon to form compact myelin (Figure 1.8). Each layer consists of a double thickness of the plasma membrane, except at the innermost layer where part of the SC cytoplasm gets left behind (Bunge et al., 1989). A SC synthesizes just one myelin internode on a single axon with two half nodes of Ranvier (Garbay et al., 2000). Unlike myelinated fibres in the CNS, peripherally myelinated axons are surrounded by a continual SC basal lamina (Raine, 1984). Non-myelinating SCs ensheathe *groups* of small calibre C fibres in a basement membrane, forming what are known as Remak bundles, whereby individual unmyelinated axons remain segregated by SC processes. It has been suggested that the role of non-myelinating SCs is to enhance neuronal survival, as mutant mice with extensive SC loss show limited survival of unmyelinated C fibres (Chen et al., 2003).

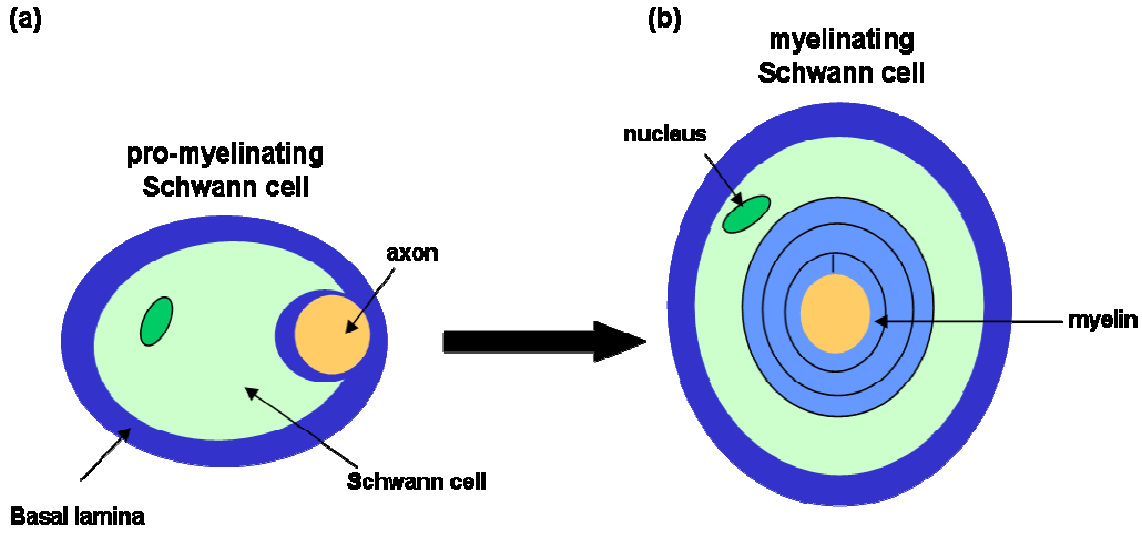


Figure 1.8 – *Myelination of Peripheral Axons*. A 1:1 relationship is established between SCs (pale green) and axons in pro-myelinating cells (a), which coincides with the development of a SC basal lamina surrounding the SC/axon complex (dark blue). Myelination proceeds with the extension of the SC cytoplasm wrapping itself around a single axon several times to form 1 internode of myelin, which has a double thickness of the plasma membrane at each layer (b).

1.2.6.1 Composition of Peripheral Myelin

The gross structure of the peripheral myelin sheath is similar to that of central myelin, in that it also contains myelin internodes, juxtaparanodes, paranodal loops and nodes of Ranvier (Peters et al., 1966; Nasu et al., 1987). Peripheral myelin sheaths also contain Schmidt-Lanterman clefts, which run spirally throughout the myelin sheath to increase space and to ensure access to all aspects of the myelin structure, though these are less common in the CNS (Ghabriel et al., 1980 a.b; Small et al., 1987; Baumann & Pham-Dinh, 2001).

Like CNS myelin, peripheral myelin is also rich in lipids, which account for approximately 70% of its dry weight. However, the concentrations of specific lipids may vary between peripheral and central myelin. For example, sphingomyelin is more enriched in the PNS where it makes up approximately 20-35% of the lipid content, versus 3-8% in the CNS (Norton, 1984). In addition, the ganglioside GM4 is notably absent from peripheral myelin, whilst LM1 is specific to the PNS (Fong et al., 1976; Chou et al., 1985; Fredman et al., 1991).

Similarly, the protein composition of the myelin sheath can vary between the PNS and the CNS (Table 1.2). In the periphery, the major proteins are the glycoproteins P0 and PMP22, which are absent from the CNS, followed by MBP (Kitamura et al., 1976; Roomi et al., 1978; Smith et al., 1979).The importance of P0 has been demonstrated in P0 null mice, which lose the interperiodic lines associated with the compaction of the myelin sheath (Martini et al., 1995 a,b). It is postulated that the role of MBP is also to aid the compaction of the peripheral myelin sheath, as is the case in the CNS (Omlin et al., 1982; Martini et al., 1995 a). Periaxin is another PNS-specific myelin protein, and is located at the periaxonal surface where it makes up 5% of the total myelin protein content in peripheral nerves (Shuman et al., 1983). PLP is present at relatively low levels in peripheral nerves; whilst MAG, present at the periaxonal space and in the paranodal loops and Schmidt-Lanterman incisures constitutes another minor protein in peripheral myelin (Sternberger et al., 1979; Trapp et al., 1989). The central myelin proteins MOG and OMgp are notably absent from the peripheral myelin sheath, though one group reported the presence of MOG mRNA associated

with SCs in rodents and primates, whilst non-myelinating SCs were shown to express cytoplasmic MOG *in vitro (*Pagany et al., 2003*)*.

Myelin Protein	Expression in CNS	Expression in PNS
P2	-	++
PLP	+++	+
MBP	+++	++
MOG	+	-
MAG	+	+
P0	-	+++
PMP22	-	+++
PERIAXIN	-	+
OMgp	+	-
CNP	+	+
O4	++	+
GalC	++	+

Table 1.1 – *Differences in myelin composition between CNS and PNS.* Whilst PLP and MBP are the dominant proteins in CNS, constituting approximately 70% of the total protein in the myelin sheath, in the periphery the PNS-specific glycoproteins P0 and PMP22 constitute the majority of the protein content. Similarly, the contribution of O4 and GalC are slightly greater in the CNS than the PNS. Periaxin and P2 are also proteins specific to PNS myelin, whilst the minor glycoproteins MOG and OMgp are only found in central myelin.

1.2.7 Olfactory Ensheathing Cells (OECs)

Olfactory ensheathing cells (OECs) were originally described as the Schwann cells of the olfactory system due to their antigenic and morphological properties (Gasser, 1956; De Lorenzo, 1957; Frisch, 1967; Doucette, 1990), but it became clear from further research that they are in fact a distinct and specialised population of glial cell, which can reside in both the PNS and the CNS (Barnett et al., 1993).

Olfactory receptor neurons (ORNs) are responsible for detecting odour and transmitting sensory information via action potentials from the peripheral olfactory nerve in the olfactory mucosa (OM) to the centrally located olfactory bulbs (OBs), and then finally to the olfactory cortex via second order neurons, where this information can be processed (Gomez and Celli, 2008) (Figure 1.9). Unlike most adult CNS tissues, ORNs are unique in that they undergo neurogenesis throughout life to allow for normal cell turnover (approximately every 6-8 weeks; Carr & Farbman, 1993) and to replace cells damaged in response to noxious stimuli (Graziadei & Monti-Graziadei, 1978a, 1979; Farbman, 1990, 1992). Furthermore, axons from regenerating ORNs are able to extend and transcend the boundary between the PNS and CNS, which is in contrast to other regenerating PNS axons, which fail to penetrate the dorsal root entry zone to successfully re-enter the CNS (Liuzzi & Lasek, 1987; Stensass et al., 1987; Carlstedt et al., 1989).

The term “olfactory ensheathing cell” was assigned to these cells due to their ability to closely associate with ORNs by ensheathing many unmyelinated axons in a continuous basal lamina to form the olfactory nerves. In addition, OECs are hypothesised to be fundamental in mediating the continual neurogenesis of the olfactory system by directing neurite outgrowth, whilst guiding regenerating ORNs from the periphery to the centrally located olfactory bulbs (Graziadei & Monti-Graziadei, 1978a, 1979; Farbman, 1990, 1992; Lindsay et al., 2010).

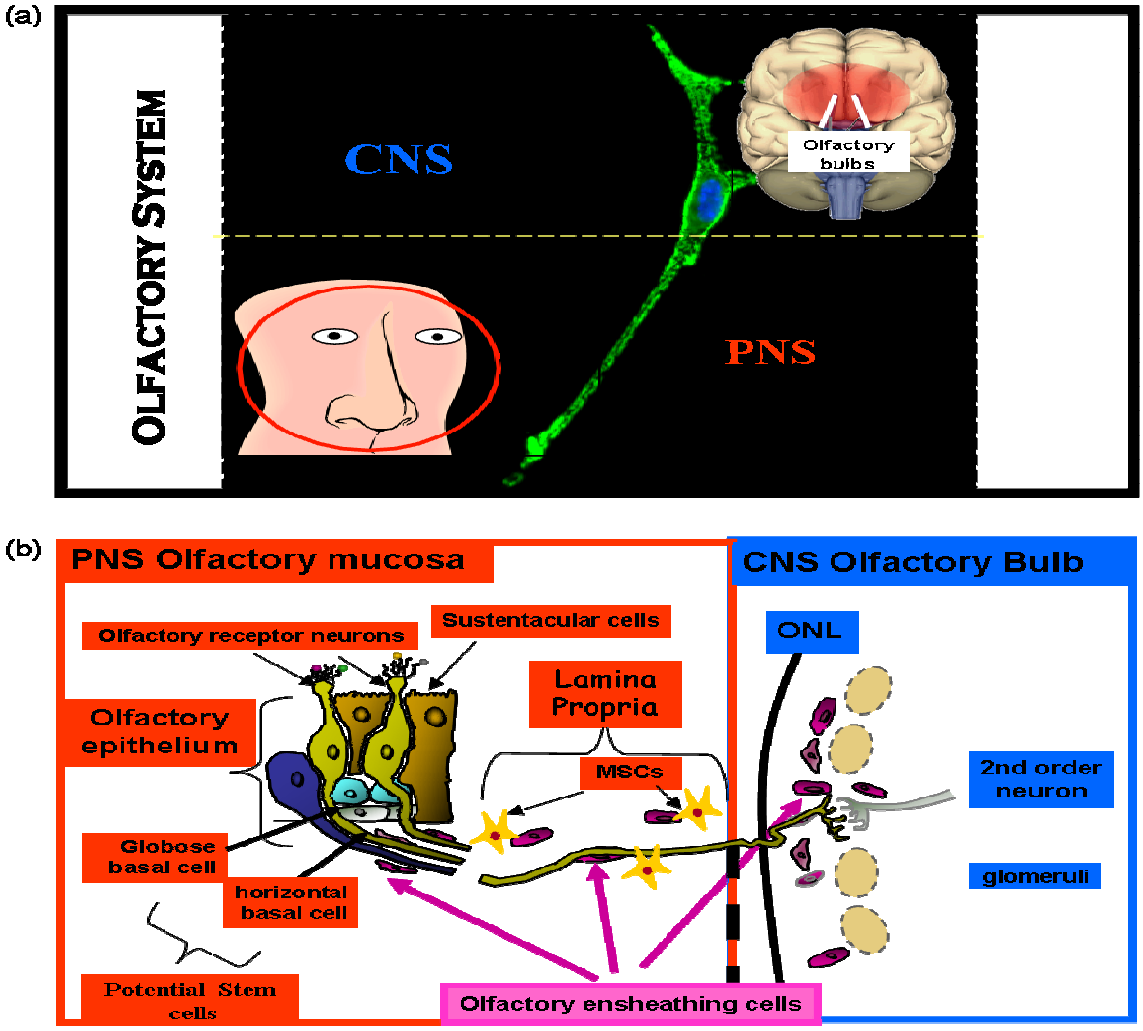


Figure 1.9- *Schematic depicting the central and peripheral regions of the olfactory system*. The olfactory system consists of the peripheral olfactory mucosa located in the dorsal region of the nasal cavity and a central component, comprising of the olfactory bulbs (a). The olfactory mucosa can be further divided into the olfactory epithelium, containing olfactory receptor neurons (ORNs), and the lamina propria, which contains mesenchymal stem cells and OECs. During normal cell turnover or in response to noxious stimuli, globose basal and horizontal basal cells in the olfactory epithelium are stimulated to regenerate the olfactory epithelium, whilst regenerating axons from ORNs extend through the cribriform plate where they re-enter the CNS to synapse with 2nd order neurons in the olfactory nerve fibre layer (ONL) in the olfactory bulbs (b). The regenerative capacity of the olfactory system has been widely attributed to the presence of OECs, which ensheath ORN axons, guiding them to their target destination, as well as the stem cells in the olfactory epithelium.

1.2.7.1 Development of OECs

OECs are classically described as being derived from the olfactory placode (Verwoerd & van Oostrum, 1979; Couly et al., 1985; Chuah et al., 1991). However, a repeat of these original fate-mapping studies has suggested that OECs may be derived from the neural crest, as with SCs (Barraud et al., 2010). These researchers grafted the anterior neural fold, which forms the olfactory placode (Couly et al., 1985) from a GFP^{+ve} donor into a wild-type host to show that the OECs present throughout the olfactory nerve were $p75^{NTR+ve}$ but negative for the expression of GFP, suggesting that they could not have migrated from the olfactory placode. Furthermore, NCCs are thought to form the lamina propria and Barraud et al., (2010) also reported that NCCs from GFP^{+ve} donors labelled p75 ^{NTR} positive OECs in the olfactory nerves. This data could perhaps suggest a dual origin for the development of OECs.

1.2.7.2 The Peripheral Olfactory System: *Olfactory Epithelium*

The olfactory mucosa of the peripheral olfactory system is located in the dorsal region of the nasal cavity and is comprised of an olfactory epithelium and a lamina propria and both regions are compartmentalised by the presence of a basal lamina (Doucette, 1990). Within the epithelium, sustentacular cells provide support for olfactory receptor neurons (ORNs) (Schwob, 2002), whilst putative stem cells, the globular basal cells (GBCs) (Graziadei & Monti-Graziadei, 1979; Caggiano et al, 1994; Huard et al., 1998) and horizontal basal cells (HBCs) (Mackay-Sim and Kittel, 1991; Carter et al., 2004; Leung et al., 2007) are thought to be responsible for replacing ORNs as well as other cellular components of the olfactory mucosa. More specifically, GBCs are thought to give rise to olfactory glia, neurons and the sustentacular cells of the olfactory system following moderate damage or normal cell turnover, whilst HBCs have been shown to give rise to GBCs as well as glia and the aforementioned cell populations. It is thought that the HBC response is more associated with severe damage to the olfactory system (Leung et al., 2007; Lindsay et al., 2010).

1.2.7.3 The Peripheral Olfactory System: *Lamina Propria*

The lamina propria (LP) is the underlying connective tissue containing the extending axons of ORNs, which are organised into bundles as they traverse into the CNS (Graziadei, 1971; 1973). At approximately 0.1–0.4 μm in diameter, these axons fall below the threshold diameter for myelination and therefore, remain unmyelinated within the olfactory system (Field et al., 2003). However, these fibres are ensheathed in basement membrane by the peripheral OECs residing within the lamina propria as they course to the olfactory bulb in the CNS. Recent data has also demonstrated the presence of a STRO-1 positive population of mesenchymal-like stem cells (MSCs) in this tissue in the rat (Tomé et al., 2009) and in human tissue (Delorme et al., 2010; Lindsay et al., 2010). Furthermore, non-myelinating Schwann cells reside in the lamina propria, where they associate with unmyelinated sympathetic axons and blood vessels, though these cells are difficult to distinguish from OECs given their almost-identical antigenic and morphological profile.

1.2.7.4 The Central Olfactory Bulbs

The anatomy of the olfactory bulb is somewhat more complex than that of the mucosa. Positioned ventrally to the orbital surface of the frontal lobe of the brain, the olfactory bulb is comprised of, from the most superficial tissue level inwards: the outer olfactory nerve fibre layer, the glomerular layer, the external plexiform layer, the mitral cell layer, the internal plexiform layer and the granule cell layer (Doucette, 1990). ORNs penetrate the nerve fibre layer before going on to terminate and form specialised odour-specific synaptic configurations, known as glomeruli, within the glomerular layer with mitral/tufted cells, whose axons extend into the olfactory tract. Fine-tuning of odour recognition is mediated via the inhibitory regulation of these mitral cells by interneurons (granule cells) in the granule layer of the olfactory bulb. From the olfactory tract, mitral cell axons project to the olfactory cortex of the brain, which in turn outputs to brain regions such as the hypothalamus, hippocampus and amygdala to process important emotional/behavioural aspects of olfaction. The superficial layers of the olfactory bulb also contain OECs as well as astrocytes and meningeal fibroblasts (Doucette., 1990; Schwob et al., 1992).

1.2.7.5 The Myelinating Capacity of OECs

Although Devon & Doucette (1995) reported the myelination of DRG neurites by OB-OECs *in vitro* in the absence of ascorbic acid, others have been unable to reproduce these results *in vitro*, regardless of the culture conditions (Plant et al., 2002), thus the issues surrounding the myelinating potential of OECs in culture has remained controversial. A recent study, however, has reported that OECs myelinated DRG neurites in culture in the absence of ascorbic acid, but that this process did not get underway until 3wks *in vitro* and 7 days after the induction of SC myelination in DRG/SC cultures containing serum and ascorbic acid (Babiraz et al., 2011). These findings could explain conflicting reports regarding the myelinating capacity of OECs *in vitro*. As previously stated, ORNs are unmyelinated, thus OECs are non-myelinating cells *in situ* under non-pathological circumstances. Whilst this is thought to be due to the fact that axons of ORNs fall below the threshold diameter at <1 μm, it could also be argued that the micro-environment of the olfactory system lacks the necessary cues to induce OECs into a myelinating phenotype.

1.2.7.6 Growth Requirements for OECs

Serum has been shown to aid the expansion of OECs in culture, although in its absence neonatal OECs adopt a more multi-process bearing phenotype (Barber et al., 1987), whilst embryonic cells become spindly under these conditions (Devon & Doucette, 1995). OB OECs, however, vary their morphology from spindly to stellate, regardless of whether or not they are grown in serum (Ramon-Cueto & Avila, 1998), thus demonstrating the plasticity of their phenotype (Franceschini & Barnett, 1996; van den Pol & Sanitarily, 2003; Vincent et al., 2005).

Astrocytes are thought to secrete many factors that promote the growth and differentiation of many glial cell types and have, in particular, been shown to be mitogenic for oligodendrocyte precursor cells (Noble & Murray, 1984). Subsequent studies demonstrated that serum-free conditioned media from type I cortical astrocytes (astrocyte conditioned medium, ACM) was also a potent mitogen for OECs (Franceschini & Barnett, 1996). Further research indicated that ACM contained an isoform of the growth factor neuregulin hence, its mitogenic properties (Pollock et al., 1999; Yan et al., 2001). Though the effects

of ACM were said to be growth-restrictive after approx. 14 days *in vitro* in neonatal rat cells (Alexander et al., 2002), these authors also demonstrated that OEC proliferation could then be restored and maintained for up to 9 weeks by the addition of a mitogen mix (termed olfactory mitogen medium (OMM), containing fibroblast growth factor 2 (FGF2), forskolin and heregulin. Without this cocktail of growth factors, neonatal OECs cultured in serum alone were shown to cease proliferating after 3-4 weeks *in vitro*. Furthermore it was reported that neonatal cells cultured in the absence of mitogens (serum alone) often lost the expression of markers such as O4 and polysialylated (embryonic) neural cell adhesion molecule ((PSA)-E-N-CAM) (Alexander et al., 2002), though this could possibly be attributed to the presence of serum rather than to the lack of growth factors.

Similarly, adult rat OB-derived OECs grown in serum-containing media supplemented with forskolin (2 µm, Sigma) and bovine pituitary extract (20 µg/ml, Biomedical Technologies) (Takawi et al., 2002) were able to divide for up to 5 weeks in culture, whilst removal of these mitogens significantly reduced the ability of these cells to proliferate (Rubio et al., 2008). The culture of mouse OB-derived OECs also seems to require the presence of growth factors, namely forskolin and bovine pituitary extract, as described for rat OECs (Au & Roskams, 2002; Richter et al., 2008), though these cells could not be expanded in rat OMM-ACM (unpublished data from Barnett lab).

1.2.7.7 Characteristics of OECs

Early analysis of glial cells from the olfactory bulb demonstrated their expression of glial fibrillary acidic protein (GFAP), a common marker for astrocytes (Barber & Lindsay, 1982; Denis-Donini & Stenoz, 1988), and $p75^{NTR}$, which is typically used to define non-myelinating Schwann cells (Jessen et al., 1990; Ramon-Cueto & Nieto-Sampedro, 1992; Barnett et al., 1993; Pixley, 1996). However, there is also heterogeneity amongst OECs of the olfactory bulb in that the olfactory nerve layer in the bulb is subdivided into two layers, whereby only the OECs residing in the outer layer express $p75^{NTR}$ (Au et al., 2002).

It has been reported that two morphologically distinct populations of OECs can be observed *in vitro*, termed astrocyte-like and Schwann cell-like depending upon their antigenic profile (Ramon-Cueto et al., 1993; Franceschini & Barnett, 1996). Although both cell types express S100ß (Devon & Doucette, 1992; Franklin et al., 1996; Pixley, 1996; Barnett et al., 2000, Sasaki et al., 2006), Schwann cell-like OECs are spindly in morphology, much like Schwann cells, and widely express p75 ^{NTR}, however they demonstrate weak immunoreactivity to GFAP. Conversely, astrocyte-like OECs are strongly GFAP positive with little expression of p75^{NTR} and are flat and multi-process bearing with a larger cytoplasm than in Schwann cell-like OECs. Ramon-Cueto et al., (1993) reported a tendency towards the multi-process bearing phenotype (i.e. astrocyte-like) when culturing from the adult rat OB, however, Huang and colleagues (2008) described the appearance of both phenotypes in adult cultures. Furthermore, they suggested that these distinct phenotypes could readily transform in culture thus demonstrating the plasticity of OECs.

Neural cell adhesion molecule (NCAM) and its embryonic form (E-NCAM) have also been shown to be expressed by OECs cultured from an adult mouse. An early study by Miragall et al., (1988) demonstrated the expression of NCAM in all glia within the OB, as well as the expression of E-NCAM at the glia limitans within the nerve fibre layer of the OB, which is principally composed of OECs. Further studies in rat species have also highlighted the expression of polysialylated (embryonic) neural cell adhesion molecule ((PSA)-E-NCAM) in astrocyte-like OECs; an effect which is most likely to be observed when the cells are cultured in the absence of serum. Though (PSA)-E-NCAM expression was reduced with time regardless of culture conditions, it could be retained for longer when OECs were grown in serum-free media (Franceschini & Barnett, 1996).

The oligodendrocyte 4 (O4) antibody is commonly used as a marker for oligodendrocytes and oligodendrocyte precursor cells (OPCs), however it can also label OECs, as originally demonstrated by Barnett et al., (1993). They observed O4 staining in the superficial layers of the olfactory bulb of neonatal rats and further labelling with p75 ^{NTR} confirmed that these cells were in fact OECs. Subsequent research has confirmed the expression of O4 antigens in mammalian OECs both *in vitro* (Doucette & Devon., 1994; Smith et al., 2002; Wewetzer et

al., 2005) and *in vivo* (Franceschini & Barnett, 1996, though this expression is lost with time in culture (Franceschini & Barnett ,1996).

In addition, a wide panel of biomarkers, including laminin, vimentin, nestin and nerve growth factor (NGF), have also been described in OECs under particular culture conditions (summarised by Ramon-Cueto & Avila, 1998 and Kawaja et al., 2009). Their expression is more variable, however, and less well documented than the aforementioned markers, perhaps making them less appropriate candidates for the identification of OECs.

1.2.7.8 OECs Isolated from Human Tissue

It is more acceptable to obtain human OECs from mucosal biopsies, since the procedure for isolating this particular niche of cells is relatively non-invasive, in comparison to the isolation of OB-OECs (Fèron et al., 2005; Mackay-Sim et al., 2008; Mackay-Sim & St John 2010). However, Barnett et al., (2000) demonstrated that human OECs can also be cultured from olfactory bulbs obtained during surgery where a bulbectomy is a necessary part of routine surgery.

Whilst both foetal calf serum (FCS) and ACM were shown to be mitogenic for human OB-OECs (hOB-OECs), culture in DMEM-FCS (10%) without additional factors caused the cells to lose their expression of $p75^{NTR}$, making it more difficult to identify these cells as OECs. However, the addition of Hrg ß1 and forskolin to DMEM-FCS (10%) caused the cells to retain their expression of $p75^{NTR}$ whilst also allowing them to proliferate in culture for up to 5 passages. These findings suggest that whilst human OB-OECs have similar growth factor requirements to rats, additional growth factors may be required for their long-term culture so that they can be grown to confluency for transplantation. Olfactory bulbs from human cadavers can also be isolated during surgery to remove other organs for donation up to 3 hours after the onset of circulatory arrest, as an alternative source of hOB-OECs (Miedzybrodzkil et al., 2006).

1.2.7.9 Purification of OECs

In order to truly understand the regenerative potential of OECs, it is necessary to generate relatively pure cultures for transplantation to eliminate contaminating cells, such as meningeal fibroblasts. There are several methodologies which can be employed for this purpose, however positively selecting for a population of cells based upon their antigenic profile can produce highly pure cultures of cells.

1.2.7.9.1 Easy Sep Purification

The magnetic nano-particle system is a comparatively new means of purifying cells and is based upon the principle of using magnetic nano-particles to bind to cells which have been labelled with an antibody against a particular antigen of interest. A tube containing the cell/ antibody suspension is then placed into a specialised magnet forcing the nano-particles, which are bound to the +ve population of cells, to stick to the sides of the tube whilst unlabelled/ unbound cells can be poured out. This washing process is repeated up to 4 times and the remaining cell pellet is then spun down and re-suspended in the appropriate growth medium. A DIY EasySep^{TM} kit (Stem Cell Technologies) containing mouse IgG1 can be purchased for the selection of p75 ^{NTR} positive cells, making this an appropriate tool for the purification of OECs. Using this methodology, cultures of OECs can be produced which are approximately 95-98% p75^{NTR} positive (see Higginson & Barnett, 2010 for more details).

1.2.7.9.2 FACS Sorting

Fluorescence-activated cell sorting (FACS) is another antibody-mediated method for cell purification. Briefly, a heterogeneous mix of cells is labelled with a specific cell type antibody for 1-2 hr, followed by its fluorescent class specific secondary antibody for up to 45 minutes. The labelled cells are then placed in a FACS analyser which forms droplets of cells, which can be separated by magnetic charge and collected in different vials based upon the differential excitation and emission wavelengths of the fluorochrome associated with each secondary

antibody. It is possible to use more than one antibody to positively select for multiple populations within a heterogeneous cell mix.

FACS was first described for the purification of OECs by Barnett et al., (1993), using olfactory bulbs taken from the rat as a source of tissue (Barnett & Roskams, 2008). In this context olfactory bulb cells were resuspended in the O4 hybridoma supernatant, which also contained an antibody to galactocerebroside (GalC) and incubated at 4^{o}C for 30-45 min, before the appropriate class-specific fluorescently labelled secondary antibodies were added. Using this methodology, the group were able to select for a population of cells that were positive for O4 but negative for GalC, distinguishing them from oligodendrocytes. Staining of olfactory bulb sections with the O4 antibody confirmed their location in the ONL of the olfactory bulbs, indicating that these cells were in fact OECs. Though perhaps expensive due to the necessary use of specialised machinery, this methodology can be used to produce highly purified populations of OECs (Fairless et al., 2005; Tomé et al., 2007, Santos-Silva et al., 2007).

1.2.7.9.3 Immunopanning

Immunopanning is a third method that also involves purifying cells based upon their antigenic profile. Briefly, olfactory-derived cells can be plated onto tissue culture plastic dishes, which have been coated with p75^{NTR} IgG as a means of purifying OECs. After several washes to remove unbound cells, attached cells could be removed from the dish as a relatively pure population (Ramón-Cueto & Nieto-Sampedro, 1994).

1.2.7.10 Summary of OECs

In summary, OECs are a highly specialised type of glial cell which are thought to aid the continual regeneration of the olfactory system by ensheathing the axons of unmyelinated olfactory receptor neurons, guiding them from the periphery back into the CNS to form secondary functional connections with the mitral cells in the olfactory bulb. In light of this, OECs are proposed to be a suitable cell candidate for the transplant mediated repair of the CNS. However, differences in culture conditions and purification techniques can alter the antigenic profile of these cells as well as affect their purity, which may alter their efficacy as a treatment to promote repair. Thus, to fully maximise their repair potential it is necessary to have a full and comprehensive understanding of the complex biology surrounding the culture of OECs.

1.2.8 Antigenic Similarities Between OECs and SCs

Given their antigenic and morphological similarities, Boyd et al., (2006) suggested that calponin may be a useful marker for distinguishing OECs from SCs, since they reported its expression in foetal OB-OECs but not in adult SCs. However, these findings were contested by Ibanez and colleagues (2007), who failed to detect calponin in cultured adult LP-OECs or in the peripheral olfactory system *in vivo*. These anomalies in findings could be attributed to the fact that LP-OECs and OB-OECs are thought to behave differently after transplantation (Richter et al., 2005), which may be due to differences in their antigenic and secretory profiles. It's also worth noting the distinctions in donor age used in each study. Indeed, Tomé et al., (2007) reported that calponin was expressed by OB-OECs and SCs both of neonatal origin, perhaps suggesting that the expression of calponin could be associated with a more plastic phenotype found only in infancy.

Global gene analysis of OEC and SC transcriptomes using gene-ontology overrepresentation analysis reported that 257 genes were up-regulated in OECs compared with SCs. Based upon their function, these changes could be grouped into genes associated with blood vessel development, migration and wound healing (Franssen et el., 2008). A microarray study by Vincent et al., (2005) was more specific in its findings, describing the up-regulation of specific genes, such as *Chl1*, Ccl2/*MCP1* and *Gro* in OECs, which are said to modulate various aspects of immunological responses. Robinson & Franic (2001) reported that the action of Gro1 could be to control the proliferation of oligodendrocytes in cooperation with PDGF. Subsequent analysis by Vincent et al., (2005) using immunohistochemistry, however, revealed little or no expression of these proteins by either cell type. Furthermore, Chl1, a homolog to the L1 adhesion molecule, has been shown to be present in invading SCs following SCI (Wu et al., 2011).

Taken together, these findings highlight that there are currently no known markers which can definitively define OECs from SCs *in vitro* or *in vivo,* though the identification of such a marker would be of great benefit, particularly for transplantation studies where it is extremely difficult to distinguish grafted OECs from endogenous infiltrating SCs.

1.3 Damage to the PNS

After damage to the PNS, neurons may undergo necrosis or apoptosis leading to cellular death. Alternatively, the neuronal cell body may remain intact after becoming transected from its axon, which, along with its myelin sheath, is then phagocytosed by macrophages (Stoll et al., 1989) (Figure 1.10). This process, which occurs over several days post-injury, is known as Wallerian Degeneration. However, the PNS is unique in that unlike the CNS, neurons residing in the periphery are able to regenerate remyelinated axons following trauma (Kraft, 1972; Hentz, 1989; Bonnaud-Toulze et al., 1980; Gupta et al., 2004).

In the first week or so post-injury, regenerating axons appear with highly motile projections, known as growth cones, at their leading edge. The initiation of the Rho/ ROCK pathway and its downstream pathways, including JAK/ STAT, JNK and MAPK, are thereafter thought to enable process extension by regulating cytoskeleton dynamics (Schwaiger et al., 2000; Kury et al., 2001; Boyd & Gordon, 2003; Sun et al., 2012). Once the regenerated axons have reached the distal ends, they begin to grow along the clefts in between non-myelinating SCs. Remyelination typically occurs over a period of approximately 3 weeks in rodents, though the newly formed internodes of myelin are shorter than those formed developmentally (Cragg & Thomas, 1964; Ghabriel & Allt, 1977; Minwegen & Friede, 1985). This may explain why in severe cases of peripheral nerve injury, nerve function is often only partially restored (Minwegen & Friede, 1985).

Although there is some SC loss following injury, this is minimised to some extent by the autocrine secretion of pro-survival factors, as discussed in section 1.2.5.2. Following injury to peripheral nerves, SCs can also de-differentiate, which may assist in the repair of peripheral nerves (Stewart et al., 1993; Scherer et al., 2001; Dupin et al., 2003; Chen et al., 2007; Vargas & Barres, 2007; Gordon et al., 2009).

It is said that the rate of regeneration occurs at approximately 1.5 mm per day according to a mathematical model, which includes regenerative failures, after an initial delay of approximately 3 days (Holmquist et al., 1993). In some instances, whereby the distance between the proximal and distal stumps is too great as occurs in more severe injuries, surgical intervention to bring both ends of the transected axon into close apposition is required, along with other possible therapeutic interventions to be discussed in section 1.5.2.2 (Aird, 1946; Dreissen, 1975; Nawabi et al., 2006; Sun et al., 2009).

1.3.1 The Role of SCs in Peripheral Nerve Repair

Arguably what confers the regenerative capacity of the PNS is the presence of bands of Bungner at the distal stumps of the transected axon, formed by proliferating SCs and the SC-produced basal lamina, which surrounds the axon prior to injury (Spencer & Schaumburg, 1977; Oldfors & Persson, 1982; Ohara & Ikuta, 1988). Bands of Bungner represent spared and viable tissue in which regenerating axons are contained within the milieu of SCs and excluded to some extent from the microenvironment induced by injury. In their absence, as occurs when the proximal and distal stumps are experimentally disjoined, the regeneration of peripheral nerves is significantly reduced (Weinberg et al., 1978; Roytta et al., 1988; Meeker et al., 1993; Watson et al., 1993).

Whilst macrophages are said to phagocytose myelin debris (Perry et al., 1987; Lawson et al., 1994; Goodrum et al., 2004), SCs are also capable of this function following injury (Fernandez-Valle et al., 1995; Liu et al., 1995; Wang et al., 2004). McQuarrie et al., (1985) reported through EM and immunohistochemistry studies of damaged peripheral nerve tissue that myelin fragments could be detected in the cytoplasm of SCs prior to the infiltration of macrophages. Post-injury, SCs in fact mediate the infiltration of macrophages via the secretion of leukaemia inhibitory factor (LIF) and monocyte chemoattractant protein-1 (MCP-1) (Toews et al., 1998; Tofaris et al., 2002; Karanth et al., 2006).

SCs have also been demonstrated to be a vital source of growth factors following peripheral nerve injury. For example, levels of NGF are reportedly low in intact nerves but increase significantly in SCs after injury, as do levels of FGF 1 and 2 and IGF 1 and 2 (Sobue et al., 1988; Hammarberg et al., 1998; Rogister et al., 1999). Each of the aforementioned factors has been well-documented for their ability to promote neurite outgrowth (Lipton et al., 1988; Walicke et al., 1988; Sjöberg & Kanje, 1989; Yasuda et al., 1990; Inagaki et al., 1995; Jiang et al., 1995). In addition, SCs express laminin and fibronectin, which also enhance neurite outgrowth, thus contributing to a growth-permissive ECM (Baron-Van Evercooren et al., 1982; Edgar et al., 1984; Lander et al., 1985; Millaruelo et al., 1988; Culley et al., 2001).

Whilst neurogenesis and neuronal targeting during development is not said to entail SCs (Grim et al., 1992; Riethmacher et al., 1997), their required presence for the regeneration of peripheral nerves would suggest that the mechanisms underpinning these two events are distinct.

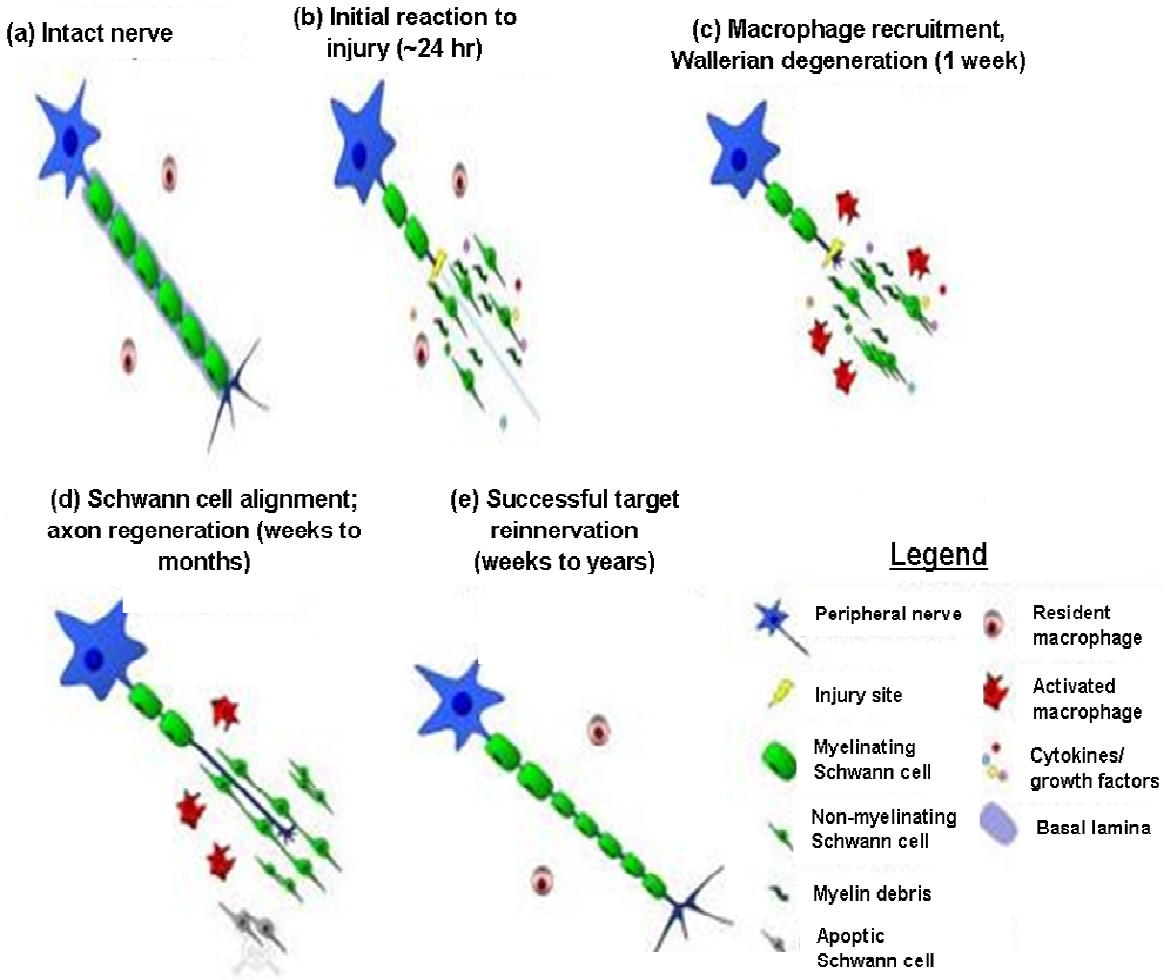


Figure 1.10 – *Peripheral nerve regeneration*. In the initial stages of nerve damage, non-myelinating Schwann cells surround the injury site to phagocytose myelin debris (b) and to contribute to the Wallerian degeneration of the nerve. Thereafter, activated macrophages are recruited to the injury site to facilitate this process (c). Furthermore, non-myelinating SCs secrete axonal growth-promoting neurotrophins at the site of the injury. In the coming weeks to months, regenerating fibres begin to appear, facilitated by the bands of Bungner formed at the distal stumps from the pre-existing Schwann cell basal lamina (d). The process of regeneration can take anything from weeks to years depending upon the severity of initial nerve damage (e).Modified from http://neurowiki2012.wikispaces.com/Secondary+Degeneration+Mechanisms.

1.4 Damage to the CNS

Following disease or trauma to the central nervous system (CNS), injured neurons fail to regenerate competently, resulting in severe losses of function in damaged areas (Ramon y Cajal, 1928). Although there is some evidence of continual neurogenesis in areas of the adult CNS, such as the hippocampus (Eriksson et al., 1998; Nilsson et al., 1999; Yagita et al., 2001; Kamada et al., 2004), by in large neurogenesis is limited, thus any damage to the CNS is permanent and irreversible. It is postulated that this lack of repair is due to a non-growth permissive environment post-injury/disease, as opposed to an intrinsic inability to regenerate.

In the case of spinal cord injury (SCI), the effects can be catastrophic due to the disruption of vital tracts. In particular, damage to the descending motor pathways, such as the reticulospinal, corticospinal and tectospinal tracts of the lateral pathways, can result in significant losses of motor function and muscle tone; whilst damage to the ascending pathways, such as the spinocerebellar and spinothalamic tracts, causes debilitating sensory defects for the patient. The extent of functional loss relates to the area of the injury, in that damage to the cervical region of the cord typically results in deficits of all 4 limbs, whilst thoracic injuries often retain some upper limb function (Figure 1.7). Using the American Spinal Injury Association's (ASIA) motor scale to assess the neurological function in ten groups of muscles and dermatomes, the severity of the injury can be described as either complete, where no motor or sensory function is preserved below the lesion, or incomplete, whereby varying degrees of function can be preserved. Incomplete injuries can also be classified as syndromes, such as Anterior Cord Syndrome, where damage to the anterior portion of the spine results in a loss of motor and sensory function although proprioception is retained; and Central Cord Syndrome, which typically results in a loss of function in the upper limbs but spared lower limb function (Waters et al., 1994 a,b; McKinley et al., 2007; El Masri et al., 2011).

Spinal cord region of damage	Outcome following injury
C1-2 (cervical)	Often fatal, likely to cause respiratory defects resulting in the use of a ventilator
C3-C4	Damage to the innervation of the diaphragm via phrenic nerves, thus impacting upon breathing; loss of function in biceps and shoulders as well as hands
C5-C6	Limited use of upper arms and likely complete loss of function in hands and wrists
C7-8	Some upper arm movement but limited dexterity, limited function in wrists and hands
T1-8 (thoracic)	Complete loss of control of abdominal muscles and trunk stability
T9-12	A degree of loss of abdominal muscle control
Lumbosacral (L1-5; S1-5)	Decreased control of hips and legs, plantar flexion of foot, flexion of toes; loss of bowel and bladder control, loss of sexual function at lower sacral regions

Table1.2– *Typical Functional deficits following damage to the spinal cord*. The severity of damage following spinal trauma relates largely to the area of the injury. For example, cervical injures often result in either complete or partial loss of function in all 4 limbs, whilst lower thoracic injuries tend to affect the lower limbs and trunk stability. Injuries can be assessed as either complete or incomplete using the ASIA scale, which scores neurological function in ten areas of muscle and skin. Whilst complete injuries show a total loss of motor and sensory function corresponding to the site of the injury, incomplete injuries may result in a patient retaining varying degrees of sensory or motor function.

1.4.1 Pathology of a Spinal Cord Injury (SCI)

The primary injury occurs as a result of direct trauma, causing localised axotomy, cell death and haemorrhaging, particularly in the grey matter (Bunge et al., 1994; Beattie et al., 2002; Norenberg et al., 2004) either as a result of contusion, transection or laceration of the spinal cord. During this time many oligodendrocytes are lost, even at sites distal to the initial injury (Emery et al., 1998). It has been suggested that myelinated axons are particularly susceptible to the blunt trauma induced by contusion injuries, since the mechanical stretching forces exerted upon the axon are concentrated on the nodes of Ranvier under these circumstances (Maxwell et al., 1996).

Thereafter, the secondary injury progresses and after approximately 6-8 hrs a penumbra of ischemic tissue surrounds the lesion core and oedema occurs (Guth et al., 1999). Usually within the first 24 hrs, Wallerian degeneration begins and the transected axons begin to degenerate distally to the site of the injury and demyelination persists. After the axon cytoskeleton is dismantled via mechanisms which are dependent upon ubiquitin and calpain (Glass et al., 2002; Zhai et al., 2003; Touma et al., 2007), the myelin sheath begins to degrade and macrophages infiltrate the lesion. The process of Wallerian Degeneration in the CNS, however, is said to occur far more slowly than in the PNS, with activated microglia occupying the site of the injury for several years after injury (Avellino et al., 1995; Lazar et al., 1999; Jander et al., 2001). Furthermore, George & Griffin (1994) reported that myelin and axonal debris were cleared after 30 days *in vivo* in the dorsal roots, whilst in the central dorsal columns, they persisted for up to 90 days.

Haemorrhaging ceases after approximately 7-10 days, when the lesion site becomes filled with cellular debris (Beattie et al., 2002). A large degree of secondary damage can be attributed to the increased concentration of extracellular glutamate, which over-stimulates its AMPA, NMDA and kainite receptors to mediate Ca^{2+} dependent apoptosis of neurons and oligodendrocytes in particular (Mattson et al., 1991; Doble, 1999; Matute et al., 2001). However, the up-regulation of inflammatory cytokines may also be responsible for inducing

necrotic and apoptotic cell death after injury (Bartholdi et al., 1998; Carlson et al., 1998; Popovich et al., 1997).

1.4.1.1 Formation of an Inhibitory Glial Scar

Trauma to the CNS caused by injury or pathology induces varying degrees of astrogliosis as part of the secondary injury, whereby resident astrocytes undergo changes such as increased expression of GFAP, nestin, vimentin and chondroitin sulphate proteoglycans (CSPGS), in addition to becoming hypertrophic (Bignami & Dahl, 1976; Eng & Ghirnikar, 1994; Sofroniew, 2009; Sofroniew & Vinters, 2010). A glial scar begins to form at the site of damage approximately 48 hrs post-injury, composed of these reactive astrocytes, fibroblasts and infiltrating microglia, which are thought to cause cavity and cyst formation, leading to enlargement of the initial wound (Balentine, 1978; Fitch & Silver, 1997; Zhang et al., 1997; Fitch Et al., 1999) (Figure 1.11). In addition, $NG2^{+ve}$ glia infiltrate the scar where they also undergo reactive gliosis, causing them to up-regulate their expression of the inhibitory CSPG, NG2, at their cell surface (Levine et al., 2001; Zhang et al., 2001; Jones et al., 2002). At the more chronic stages of glial scar formation (approx 3-4 wks), SCs have also been shown to infiltrate the lesion into areas which are less astrocyte-dense when the BBB has been breached (Buss et al., 2007; 2008).

Although it was initially thought of as no more than a marker for damaged tissue and thereafter, purely a mechanical barrier to repair due to the altered ECM and increased collagen deposition (Windle & Chambers, 1950; Windle, 1954; Reir, 1986), it is now widely accepted that the glial scar may play a vital role in impeding competent regeneration of the CNS by altering the molecular composition at the injury site (Fitch & Silver, 1997; McGraw et al., 2001; Silver & Miller, 2004). Whilst the glial scar is largely viewed as a negative consequence of CNS damage, in the acute stages of injury it functions to seal off the area from inflammatory infiltrates, thus reducing further secondary damage (Faulkner et al., 2004; Myer et al., 2006; Rolls et al., 2009). In mouse models of cortical stab wounds whereby astrocytes surrounding the injury site were ablated, a

prolonged infiltration of inflammatory mediators was reported, along with increased neuronal degeneration (Bush et al., 1999; Faulkner et al., 2004).

1.4.1.1.1 Growth-Inhibitory Molecules Up-regulated within the Glial Scar

Reactive astrocytes associated with the glial scar have been shown to up-regulate growth-inhibitory molecules such as the path-finding slit proteins and ephrin-B2 (Bundesen et al., 2003; Hagino et al., 2003) as well as CSPGs, including versican, neurocan, brevican, aggrecan and NG2 (Friedlander et al.,1994; Milev et al., 1994; McKeown et al., 1995; Yamada et al., 1997; Asher et al., 2000; Jones et al., 2003; Rhodes & Fawcett, 2004; Sandvig et al., 2004; Fitch & Silver, 2008). Thus, extending neurites are limited in their outgrowth within the glial scar region without intervention. In particular, CSPGs have been demonstrated both *in vitro* (Canning et al., 1993, Inatani et al., 2001; Tom et al., 2004) and *in vivo* (Bradbury et al., 2002; Steinmetz et al., 2005; Houle et al., 2006) to be inhibitory to neurite outgrowth. Furthermore, meningeal fibroblasts have also been reported to up-regulate axonal growth-inhibitory semaphorins within the glial scar (Pasterkamp et al., 1998, 2001).

The mechanisms involved in the inhibition of neurite outgrowth are postulated to involve the activation of the Rho-associated coiled coil kinase (ROCK) pathway, which negatively regulates actin dynamics at the leading edge of the axon (Gallo et al., 2004; Kalli et al., 2005). Thereafter, the collapse or retraction of the growth cone is induced, thus inhibiting neurite outgrowth (Dergham et al., 2002; Fournier et al., 2003; Monnier et al., 2003; Mimura et al., 2006). In addition, ephrins have been reported to simultaneously cause the down-regulation of Rac and Cdc42 of the Rho GTPase family, which are responsible for the actin polymerisation and filopodia formation required for neurite extension (Wahl et al., 2000; Da Silva et al., 2003; LaPlante et al., 2004; Mulder et al., 2004). Semaphorin 3A has also been shown to reduce branching in cortical astrocytes *in vitro*, which may have detrimental effects on the path-finding abilities of these neurons (Dent et al., 2004).

1.4.1.1.2 Myelin Debris within the Glial Scar

In addition to those factors which are up-regulated after injury, components of myelin debris have also been shown to be inhibitory to neurite outgrowth, including MAG (Ng et al., 1996; Tang et al., 1997; Niederőst et al., 2002), OMgp (Wang et al., 2002; Vourc'h et al., 2003) and Nogo-66 (Nogo A) (Chen et al., 2000; Fournier et al., 2000; Oertle et al., 2003).

MAG, Nogo-66 and OMgp bind to the Nogo receptor, NgR, on the axolemma (Mikol & Stefansson, 1988; Mikol et al., 1990; Wang et al., 2002). In addition, $p75^{NTR}$ acts as a co-receptor for signalling (Wong et al., 2002) for each of these ligands. Knock-out studies of $p75^{NTR}$ reported that neurons were less susceptible to the inhibitory activity of myelin debris than in wild type controls (Wang et al., 2002). MAG in particular has a high affinity for the gangliosides GDIa and GTIb, which facilitate its binding to NgR (Yamashita et al., 2002). Each ligand can associate with $p75^{NTR}$ either independently or together to form a complex, which transmits inhibitory signals to modulate the activity of RhoA. (Vinson et al., 2001; Vyas et al., 2002; Wong et al., 2002; Yamashita et al., 2002); and to induce the inactivation of Rac1/Cdc42 (Niederost et al., 2002; Sandvig et al., 2004).

The phenomenon that myelin debris does not appear to be detrimental to neurite outgrowth in the PNS may be explained by the rapid response of Schwann cells and macrophages, which promptly clear myelin debris from the periphery, whilst this response lags in the CNS, as previously described (Stoll et al., 1989; Fernandez-Valle et al., 1995; Shen et al., 2000). Furthermore, MAG is present at concentrations ten fold less in the PNS compared to the CNS, constituting just approximately 0.1% of the total protein content of the peripheral myelin sheath (Quarles et al.,1973; Figlewicz et al., 1981; Baumann & Pham-Dinh, 2001). Neurotrophins have also been shown to overcome the myelin debris-mediated inhibition of neurite outgrowth by elevating cAMP and by activating the pro-survival ERK signalling pathways (Cai et al., 1999; Gao et al., 2003). In peripheral nerve injury models whereby brain-derived neurotrophic factor (BDNF) was knocked out in SCs, regeneration was significantly reduced

(Wilhelm et al., 2012). This data could suggest that the expression of growth factors by SCs is important in mediating competent regeneration, perhaps due in part to their ability to overcome inhibitors of neurite outgrowth, such as myelin debris.

In non-pathological conditions, it has been hypothesised that the roles of MAG and OMgp could be to prevent inappropriate sprouting of neuronal processes by inhibiting neurite outgrowth, particularly at the nodes of Ranvier (Huang et al., 2005). Chang and colleagues (2010), however, dispute this hypothesis by stating that the antibodies previously used to label OMgp at the nodes were non-specific. Furthermore, they reported no nodal abnormalities in OMgp null mice.

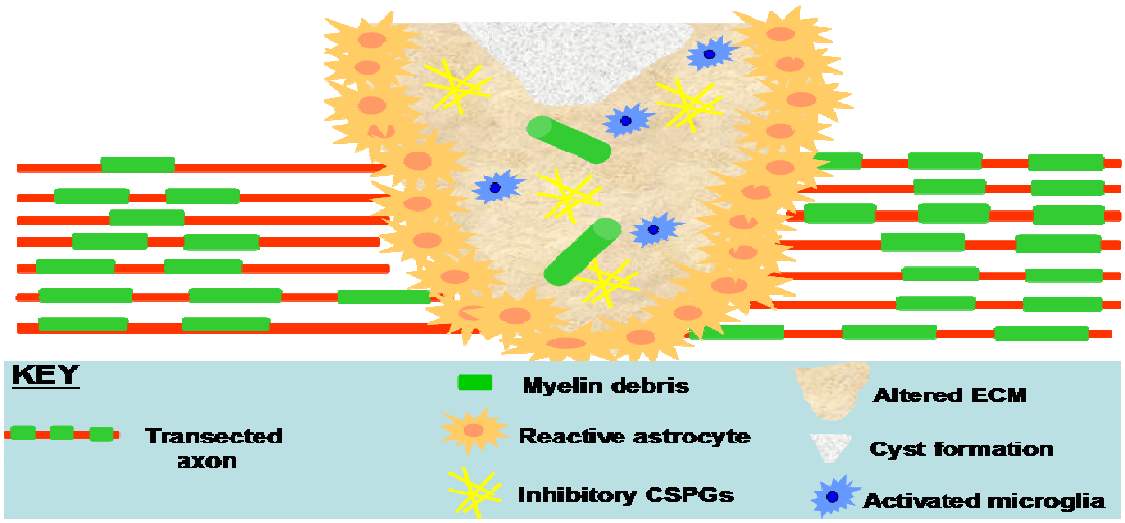


Figure 1.11 – *Schematic of the Glial Scar*. Following disease or trauma to the CNS, a glial scar forms at the site of injury comprised of reactive astrocytes, meningeal fibroblasts and activated microglia, encapsulating a fluid filled cyst. Acutely, the role of the glial scar is to seal of the injury site to reduce the level of secondary inflammatory damage. In the chronic stages of glial scar formation, however, it represents a physical barrier to regeneration due to the altered ECM and a molecular barrier to repair via the presence of growth inhibitory molecules, such as CSPGs and myelin debris. Although some fibres can re-enter the glial scar, there is little or no evidence to suggest that they can successfully exit to reform functional connections.

1.4.1.2 Immune response to SCI

Typically upon breaching of the BBB, classic inflammatory and wound healing mechanisms are triggered, including the activation of astrocytes, microglia and leukocytes, which release localised cytokines, such as TGF-ß, IL-1 ß and IL-6, contributing to the neurotoxic environment (Koyanagi et al., 1989; Armao et al., 1997; Velardo et al., 2004; Arabi et al., 2006). TGF-ß in particular has been shown to be up-regulated within CNS lesions and to amplify glial scarring (Smith et al., 2005; Okada et al., 2006; Wang et al., 2007). Thrombin, which is required for blood clotting at the injury site can also be neurotoxic by potentiating NMDA signalling, thus exacerbating Ca^{2+} dependent mechanisms of apoptosis (Gingrich et al., 2000). Furthermore, circulating antibodies from the periphery can activate complement proteins and microglia/ macrophages, causing them to secrete yet more neurotoxic cytokines, mediating cell death (Stahel et al., 1998; Kadota et al., 2000; Anderson et al., 2004; Ankany et al., 2006).

In post mortem studies of spinal cord tissue taken from patients who survived for up to 1 year after injury, results demonstrated the presence of neutrophils, activated microglia and few macrophages as little as 24 hrs after the initial injury. Whilst neutrophils were present for only approximately 10 days after injury, microglia and macrophages persisted for several months (Fleming et al., 2006). Furthermore, these researchers demonstrated oxidative activity in neutrophils and activated microglia, thus suggesting their ability to contribute to oxidative stress. Both cell types have been shown to be sources of the superoxide anion and nitric oxide post-injury, which combine to form the potent oxidant, peroxynitrite (Colton et al., 1987; MacMicking et al., 1992; Liu et al., 2002).

What this data suggests is that there may be a relatively short therapeutic window for intervention strategies to attenuate the inflammatory response following SCI, perhaps within the first 8-12 hrs. However, there is also evidence to suggest that aspects of the immune response may be beneficial to the repair of the CNS. For example, macrophages can take up glutamate, thus reducing excitotoxic cell death and exerting neuroprotective effects (Rimaniolac et al.,

2000; Van Landeghem et al., 2001). In addition, the release of cytokines such as IL-1ß has been shown to stimulate the production of nerve growth factor (NGF) (DeKosky et al., 1996; Fagan and Gage, 1990) and to induce neurite sprouting, thus facilitating CNS regeneration (McIntosh et al., 1996; Norenberg et al., 2004). Therefore, a more measured therapeutic approach whereby only certain detrimental features of the immune response were ablated could be more beneficial for the treatment of CNS pathologies.

1.4.2 Spontaneous Remyelination in the Damaged CNS

As previously discussed, demyelination of damaged axons occurs as a consequence of spinal cord trauma (Waxman, 1992). However, whilst regenerative repair of axons is limited following disease or injury, there is evidence to suggest that a degree of spontaneous remyelination can occur in response to CNS damage, as was initially demonstrated in chemically-induced demyelinating lesions in adult cats (Bunge et al., 1960; 1961). Remyelination of demyelinated axons is said to be neuroprotective, since axonal degeneration is significantly increased in X-irradiated mice following a chemically induced demyelinating lesion (Irvine & Blakemore, 2008). Restoration of the remyelinating capacity of the animal, however, reduced axonal degeneration in this model.

Recent research has also demonstrated that demyelination occurs to some extent in the human CNS following SCI and can persist for up to a decade in some patients (Guest et al., 2005; Cohen-Adad et al., 2011). However, the extent to which demyelination and remyelination occurs in humans following SCI is still unclear (Bunge et al., 1993; Emery et al., 1998; Abe et al., 1999). In rodent models of SCI, demyelination can be observed within the first 24 hrs after injury. Furthermore, Siegenthaler et al., (2007) reported that the extent of demyelination can vary depending upon the injury model; in that contusion injuries in rodents resulted in demyelination both at the lesion epicentre and at distances from the injury site. Conversely, hemisection injuries demonstrated a more focal pattern of demyelination, with little demyelination at distances from the lesion. Evidence of extensive remyelination can be witnessed by

approximately 12 weeks in spared fibres, however, there is evidence to suggest that demyelination may persist in small subsets of dystrophic fibres, though this occurrence is far less common (Lasienne et al., 2008).

It has been reported that remyelinated fibres possess shorter and thinner internodes of myelin, which some have reported to result in sub-optimal restoration of axonal conduction velocity (Griffiths et al., 1983; Talbot et al., 2005; Lasienne et al., 2008). However, others have reported that axonal conduction is fully restored in remyelinating fibres and functional deficits caused by demyelination are reduced (Smith et al., 1979; Utzschneider et al., 1994; Jeffrey et al., 1979). These anomalies in findings could be attributed to variations in axonal calibre and the extent of remyelination, in that larger axons may repair less efficiently than their smaller counterparts.

Keirstead and Blakemore (1997) provided the first evidence to suggest that remyelination in the adult CNS is not carried out by pre-existing oligodendrocytes, but rather via the recruitment of $NG2^{+ve}$ adult progenitors, which initially up-regulate their expression of NG2 and increase their rate of proliferation (Carroll et al., 1994; Redwine et al., 1998; Levine et al., 1999; McTigue et al., 2001). Thereafter, they differentiate into myelinating oligodendrocytes in response to injury (Gensert et al., 1997; Nishiyama et al., 1997; Levine et al., 1999; Dawson et al., 2003; Fancy et al., 2004). $NG2^{+ve}$ glia have also been reported to increase their expression of the transcription factors Olig2 and NKx2.2, which is thought to aid their differentiation (Sun et al., 2001; Zhou et al., 2001). It is hypothesised that similar signals which promote developmental oligodendrocyte differentiation (ie. FGF, PDGF and IGF) also contribute to the maturation of $NG2^{+ve}$ glia into myelinating cells in an injury scenario (Patel & Klein, 2001). However, factors such as IL-1 and TNF-α may also be crucial, given that remyelination was impaired in rodent models whereby both of these factors were experimentally reduced (Arnett et al., 2011; Mason et al., 2001). A recent study by Huang et al., (2010) also highlighted the importance of the retinoid X receptor γ (RXR γ) in mediating spontaneous remyelination. Data from this study reported that not only was RXR γ expressed in remyelinating oligodendroglial cells in rodents, but that experimental knock

down of RXR γ inhibited oligodendrocyte differentiation in culture and inhibited remyelination by NG2^{+ve} glia *in vivo*.

In addition to NG2^{+ve} glia, infiltrating SCs have also been reported to play a role in spontaneously remyelinating CNS fibres, preferentially in areas devoid of astrocytes (Blakemore, 1975; Snyder et al., 1975; Itoyama et al., 1983; Dusart et al., 1992; Felts et al., 2005). Remyelinating SCs are thought to migrate into the CNS via the cranial roots and blood vessels when the BBB is compromised; therefore SC-remyelination in the CNS is most abundant around the spinal and cranial nerves and around blood vessels (Baron-Van Evercooren et al., 1993; Duncan & Hoffman, 1997; Sim et al., 2002; Zawadzka et al., 2010). However, Zawadzka and colleagues (2010) also demonstrated using fate mapping studies of transgenic mice that NG2^{+ve} glia could also produce myelinating SCs, as determined by their expression of SCIP/OCT6, the peripheral myelin protein, periaxin, and their lack of Olig2.

In demyelinating diseases such as MS, remyelination can also be evidenced in some patients, particularly in the acute stages of the disease, with complete or partial repair of lesions, thereafter referred to as shadow plaques (Lassman et al., 1997; Patrikos et al., 2006; Chari, 2007). It is hypothesised that the ability of CNS axons to remyelinate may explain to some extent the "relapse and remission" pattern observed in some MS patients. However, with disease progression remyelination fails for reasons which are as yet not fully understood, although it is likely that the complex microenvironment of the diseased tissue may be responsible for this abortive repair strategy (Franklin, 2002). For example, it has been shown that CSPGs, present in abundance throughout glial scar tissue, can reduce remyelination by negatively affecting oligodendrocyte attachment and differentiation (Siebert & Olsterhout, 2011; Lau et al., 2012).

A greater understanding as to the mechanisms which induce and impair endogenous remyelination could be vitally important for the treatment of CNS pathologies (Franklin & Kotter, 2008).

1.5 Strategies to Promote Repair of the Spinal Cord

As discussed in the previous sections, spinal cord injuries are complex and multi-faceted, thus there is currently a wealth of literature describing multiple repair strategies targeting specific aspects of regenerative failure, with reports of varying degrees of success.

1.5.1 Pharmalogical Intervention

An enzyme which removes the glycosaminoglycan (GAG) side chains from CSPGs, chondroitinase ABC (ChABC), has been used in the treatment of SCI models *in vivo* with conflicting results. Whilst several groups have reported increased axonal sprouting and, in some cases, functional recovery following treatment with ChABC (Bradbury et al., 2002; Huang et al., 2006; Barritt et al., 2008; Shields et al., 2008; Alilain et al., 2011; Starkey et al., 2012), others have reported contradictory findings. Kadakia et al., (2009) reported that whilst they did observe some increased collateral sprouting depending upon the site of the injection of ChABC and its position rostral or caudal to the injury, they did not observe any functional recovery as a result of treatment. However, technical differences may account for these discrepancies in that certain behavioural tests are more sensitive than others; whilst the severity of the lesion and time-course for treatment may also greatly affect the efficacy of ChABC.

Inhibitors of the Rho pathway, such as C3 ADP-ribosyltransferase, have also been shown to promote neurite outgrowth in *in vivo* models of SCI (Ichikawa et al., 2008; Boata et al., 2010). In addition, the inhibition of ROCK, the downstream effector involved in Rho signalling, using Y27632 also attenuates the inhibition of neurite outgrowth *in vivo* (Ling et al., 2007; Sagawa et al., 2007). However, an *in vitro* model of SCI recapitulating several key features of glial scar formation was used to demonstrate that C3 promoted neurite outgrowth into the cut or lesioned area; whilst Y27632 did not (Boomkamp et al., 2012). Nonetheless, this study also reported that Y27632 was able to enhance myelination and neurite density in the areas surrounding the lesion, suggesting that it may play a role in

neuroprotection or in enhancing plasticity of spared fibres. A combination of both of these compounds proved to be synergistic, with dose-dependent effects.

The role of myelin debris and its signalling via NgR in impeding axonal regeneration in the CNS has been discussed in section 1.4.1.1.2. NEP1-40, an antagonist to NgR, has been shown to promote sprouting of seritonergic and corticospinal axons in a rodent model of thoracic SCI, in addition to increasing locomotor recovery (GrandPré et al., 2002; Li & Strittmatter, 2002; Cao et al., 2008).

Methylprednisolone, a corticosteroid drug used to suppress inflammation, has also been administered to patients with SCI within the first 8-10 hrs immediately following the injury (Bracken et al., 1990). In one study, patients receiving this treatment for 48 hrs (167 patients) showed significant clinical improvements of at least one neurological point after 6 mths compared to those who were only treated with methylprednisolone for 24 hrs (166 patients). However, those patients in the former group also showed a significantly increased occurrence of pneumonia compared with the latter (Bracken et al., 1997; 1998). Further clinical studies have shown no evidence of methylprednisolone improving neurological function in SCI patients (Ito et al., 2009) Thus, the use of methylprednisolone to treat SCI is controversial and clinical practice varies considerably between hospitals (Frampton & Eynon, 2006).

What all of the aforementioned studies have in common is the fact that whilst they may report varying degrees of functional recovery and increased neurite outgrowth, they demonstrate very little anatomical evidence of extensive regeneration *in vivo*, whereby fibres within the lesion are able to successfully exit the graft to reform functional connections.

1.5.2 Cell Transplantation

The transplantation of cells into the CNS is postulated to hold great promise in the treatment of CNS injury by replacing lost or damaged tissue or by enabling a transplanted population of cells to take on the vital functions lost as a consequence of injury or disease progression.

Neural stem cells (NSCs) have been shown to promote a degree of functional recovery following their autologous transplantation into rodent models of SCI (Bambikidis et al., 2004; Karimi-Abdolrezaee et al., 2006; Parr et al., 2007). Karimi-Abdolrezaee and colleagues (2006) observed that of those NSCs transplanted into a contusion injury model, approximately 50% differentiated into OPCs or myelinating oligodendrocytes, correlating with improvements in behavioural scores.

More promisingly, human-derived NSCs have also been xenografted into the rodent CNS, based upon the original work of Gumpel et al., (1987), who used CNS fragments from aborted embryonic tissue to demonstrate this phenomenon (Buchet & Baron-Van Evercooren; 2009; Buchet et al., 2011; Uchida et al., 2013). Since then, researchers have been able to amplify the NSCs population in culture either as an adherent monolayer (Buc-Caron et al., 1995; Carpenter et al., 1999; 2001) or as free-floating spheres (Murray et al., 1997; Flax et al., 1998; Vescovi et al., 1999; Uchida et al., 2000). Further research showed that the transplantation of human embryonic NSCs (hENSCs) into the intact brain and spinal cord of rodents resulted in their preferential differentiation into astrocytes and neurons, as opposed to oligodendrocytes (Fricker et al., 1999; Buchet et al., 2002; Englund et al., 2002). However, Cummings et al., (2006) reported that the transplantation of hENSCs into the contused rat spinal cord resulted in their extensive differentiation into myelinating oligodendrocytes. This data therefore appears to suggest that the host environment greatly influences stem cell fate post-transplantation. Similarly, whilst Jin and colleagues (2012) reported the differentiation of hENSCs into astrocytes, neurons and oligodendrocytes within the corpus callosum and white matter of the rodent spinal cord, they observed a lack of migratory ability when these cells were

injected into the corpus callosum. Nonetheless, transplanting OPCs pre-differentiated from hENSCs into a cervical contusion model of SCI demonstrated improvements in forelimb function, compared to non-transplanted animals, and an increase in the number of spared fibres surrounding the lesion (Sharp et al., 2010). Furthermore, human induced pluripotent stem cells (HiPSC) commonly derived from human fibroblasts can also be differentiated into OPCs, capable of extensive remyelination in a dysmyelinated mouse with no evidence of adverse complications (Wang et al., 2013).

Mesenchymal stem cells (MSCs) have also been reported to induce some functional improvement, as assed by behavioural testing, as well as decreases in cavity formation following their transplantation into animal models of SCI (Ankeney et al., 2004; Osaka et al., 2010). Quertainmont et al., (2012) carried out a cytokine array of lesioned spinal cord tissue with or without MSC grafts and reported increased NGF expression, which correlated with enhanced neuroprotection and vascularisation following MSC transplantation. The source of the MSCs may greatly influence these properties, however, since Lindsay et al., (2012) demonstrated that human MSCs derived from the olfactory mucosa enhanced endogenous CNS myelination *in vitro,* whereas those derived from bone marrow did not. A study from Korea has already described the effects of transplanting autologous bone marrow-derived MSCs into patients with SCI (Park et al., 2012). This report claimed that of the 10 patients being studied, 3 were shown to have improvements in upper limb function, which correlated with improvements in electrophysiological testing. Furthermore, they described all of the individuals tested as having no adverse reactions as a result of cell transplantation. However, 3 out of 10 patients is an extremely small number to draw any substantial conclusions from. Furthermore, a more detailed follow-up of these patients over the course of several years may produce more accurate results.

Cell transplantation is also considered as an important potential therapy for the treatment of demyelinating diseases (Blakemore et al., 1995; Duncan et al., 1995; Archer et al., 1997). Windrem et al., (2004) reported that the transplantation of human foetal OPCs into the forebrains of the dysmyelinated

shiverer mouse resulted in extensive remyelination. Similar findings have been reported in organotypic slice cultures from these mice mutants (Bin et al., 2012). OPC cell lines, namely the CG4 line, have also been shown to repopulate a glial-free X-irradiated demyelinated lesion with astrocytes and oligodendrocytes (Franklin et al., 1995), as did a clonal OPC line generated by c-myc transduction (Barnett et al., 1993), suggesting their potential therapeutic use in the treatment of demyelinating diseases. More promisingly, a recent Phase 1 clinical trial demonstrated the safety of transplanting allogenic HNSCs into patients suffering from leukodystrophy, with reports of modest myelination by the grafted cells (Gupta et al., 2013).

The transplantation of non-stem cells, such as glial cells, has also generated much attention. It could be argued that transplanting cells with a pre-determined fate may be advantageous in that it allows the researcher to exert more control over the experimental design, perhaps by limiting the likelihood of the graft differentiating into an undesired cell type. The use of SCs and OECs for cell-transplantation studies following CNS trauma is of particular interest, given their interesting and unique properties *in situ*.

1.5.2.1 OEC Transplant-Mediated Repair of the CNS

The regenerative capacity of the olfactory system has been widely attributed to the specialised supportive functions of OECs, thus making them an attractive candidate for cell-mediated repair of the CNS (Doucette, 1995; Franklin & Barnett, 1997; Ramon-Cueto & Avila, 1998). The expression of neural cell adhesion molecules (N-CAMs) in the plasma membrane of OECs has been hypothesised to provide an appropriate growth substrate on which olfactory axons can grow. Furthermore, OECs are said to be a source of nexin, nerve growth factor (NGF) and extracellular laminin (Miragall et al., 1988; Doucette, 1990; Franceschini & Barnett, 1996), all of which are capable of promoting neurite extension.

One of the earliest studies describing the role of OECs in promoting axonal regeneration out-with the olfactory system reported that following rhizotomy, transplanted adult OB-OECs from the rat enhanced the regeneration of DRGs

throughout the spinal cord. Furthermore, the authors reported that there was no inappropriate innervation of spinal segments by regenerating axons (Ramon-Cueto & Nieto-Sampedro., 1994). Under normal conditions, the axons of peripheral DRGs are able to regenerate, however, they fail to transcend the glia limitans to re-enter the CNS (Carlstedt et al., 1989; Siegal et al., 1990). The injury model utilised by Ramon-Cueto and colleagues to challenge this theory involved transecting the dorsal roots and anastamosing them to the spinal cord at their point of entry. A repeat of this study, however, reported that there was little or no significant anatomical evidence of regenerating fibres following the transplantation of OECs, compared to animals whereby no cells had been transplanted (Riddell et al., 2004). Furthermore, these authors reported that there was no substantial electrophysiological data to suggest that functional connections had been re-formed following injury. However, it is worth noting that subtle differences in experimental techniques between these two research groups may at least partly explain anomalies in their findings. For example, whilst Ramon-Cueto et al., (1994) used adult OB-OECs, Riddell and colleagues transplanted neonatal OB-OECs. Donor age may be an important consideration for transplantation, since Coutts et al., (2012) recently reported functional differences in the ability of 3 donor ages of OB-OECs to remyelinate a demyelinated lesion in the adult rat spinal cord. In this study it was reported that embryonically-derived OECs supported the greatest level of remyelination. Furthermore, Ramon-Cueto and colleagues purified their OEC cultures by immunopanning with an antibody to $p75^{NTR}$, whereas Riddell et al., used FACS as a method for purification based upon the expression of the O4 antibody and a lack of GalC in OECs. Novikova et al., (2010) pointed out that different methodologies for the purification of OECs could also greatly alter their properties. They reported that OECs purified by differential attachment promoted the regeneration of rubrospinal and raphaespinal fibres following cervical hemisection, as well as attenuating retrograde degeneration. However, OECs that had been purified using immunomagnetic beads failed to promote significant regeneration, though they did confer neuroprotection to spared fibres.

Nonetheless, the work of Ramon-Cueto and Nieto-Sampedro has been expanded upon, with various degrees of success following the transplantation of OECs alone, or in combination with other therapies for a multi-faceted approach to

repair (Cao et al., 2004; Bunge et al., 2008; Ma et al., 2010). For example, some groups have demonstrated increased outgrowth of corticospinal fibres (Li et al., 1998; Ramon-Cueto et al., 2000; Nash et al., 2002; Fouladi et al., 2003) and raphaespinal fibres (Ramon-Cueto et al., 1998, 2000; Lu et al., 2001; Ramer et al, 2004; Richter et al., 2005; Andrews & Stelzner, 2007). It has also been reported that the delayed transplantation of OECs is more efficacious at promoting repair than acute transplantation (Ramon-Cueto et al., 1998; Keyvan-Fouladi et al., 2003; Lopez-Vales et al., 2007; Wu et al., 2011).

Some improvements in behavioural testing have also been observed following the transplantation of OECs into models of SCI (Ramon-Cueto et al., 2000; Lu et al., 2002; Li et al., 2003), suggesting a restoration of some function. Furthermore, Toft et al., (2007) demonstrated improvements in the electrophysiological data obtained from sensory neurons following transplantation of OECs into a wire knife hemisection. However, in spite of these improvements, this group saw no substantial evidence of significant axonal regeneration beyond the lesion. Similarly, others have reported that there is little evidence of long-distance axonal regeneration using OEC grafts (Li et al., 1998; Lu et al., 2002; Lopez-Vales et al., 2006). Yamamoto et al., (2009) observed the restoration of forepaw function using OEC grafts, despite failing to observe significant amounts of regenerating fibres crossing the lesion. This data may suggest that the role of OECs in promoting repair following SCI could be in maximising neuronal plasticity and sprouting from spared fibres in order to aid functional recovery.

Recent research has also focused on a direct comparison of the differential growth-promoting properties of OB versus LP-OECs, suggesting that LP-OECs are more migratory *in vitro* and more efficacious at promoting axonal regeneration and reducing scar formation *in vivo* than OB-OECs in a rat model of SCI (Richter et al., 2005). However, these findings cannot detract from the body of literature which reports the efficacy of OB-OECs in transplant scenarios (Ramon-Cueto & Nieto-Sampedro., 1994; Navarro et al., 1999; Huang et al., 2003; Guest et al., 2006).

To date, there have been a few examples of clinical trials of patients with SCI being transplanted with OECs. Following autologous transplantation of LP-OECs from 6 mths after the initial injury, researchers reported that whilst there were

no adverse affects associated with OEC treatment after 3 years, there were also no obvious signs of axonal regeneration or changes to the injury site, as assessed by MRI (Fèron et al., 2005; Mackay-Sim et al., 2008). Lima et al., (2006) demonstrated slightly more promising results in that 2 out of 7 of their patients showed improvements in function after autologous OEC-grafts, as determined by their scoring on the ASIA scale. However, one patient reported an increased level of neuropathic pain post-transplantation, whilst another patient showed deficits in function after OEC treatment. A follow up study by the same group, however, described that a combination of OEC transplantation with pre and post-operative rehabilitation resulted in 11 out of 20 patients showing functional improvements (Lima et al., 2010). However, functional recovery relies upon subjective analysis and may be subject to reporter bias, though Fèron et al., (2005) may have controlled for this to some extent by including a non-transplanted group and "blinding" the assessors. That said, none of these studies demonstrated significant anatomical evidence of neuroregeneration following OEC transplantation. A study carried out in China using homologous transplants of foetal olfactory tissue reported that in addition to there being no functional gains in patients after treatment, 5 out of 7 patients showed an increased instance of pneumonia and meningitis with OEC grafts.

In light of this evidence, autologous transplantation may be less detrimental for the patient. Whilst Barnett et al., (2000) described that human-derived OB-OECs became mitogenically unresponsive giving them a short life-span in culture, others have shown that human OECs take about 4-6 weeks to reach sufficient numbers for transplantation, using NT-3 to aid their expansion (Bianco et al., 2004; Fèron et al., 2005; Mackay-Sim et al., 2008). However, these results may be significant when considering the acute transplantation of OECs, whereby large numbers of cells would be required in the relative aftermath of the injury.

1.5.2.1.1 Remyelination of CNS Axons by OECs

As previously described in this thesis, Devon & Doucette gave the first indication of the myelinating potential of OECs *in vitro* using purified DRG cultures (1995). Until a recent report by Babiraz et al., (2011) confirmed these early findings, the notion that OECs could myelinate *in vitro* was strongly contested. OECs have also been reported to remyelinate axons *in vivo* following their transplantation into the host injury site. They produce a typically peripheral pattern of myelin by wrapping cells in a 1:1 ratio, known as signet ring formation and they express P0, as would be traditionally observed in myelinating SCs (Franklin et al., 1996; Barnett et al., 2000; Sasaki et al. 2004; Lankford et al., 2008). However, others have suggested that this remyelination is actually carried out by infiltrating SCs (Takami et al., 2002; Boyd et al., 2004). To investigate this further, Lac-Z labelled OECs were transplanted into a mild contusion injury model in rodents. Results from this study showed that Lac-Z OECs did not associate with remyelinated fibres, but SC-like cells did, though these may also have been unlabelled OECs (Boyd et al., 2004). Similar labelling studies using GFP to identify transplanted OECs have contradicted these findings, reporting GFP-OEC mediated remyelination in ethidium bromide demyelinating lesions (Sasaki et al., 2006). The differences in these two reports could be attributed to donor age of the transplant cells, in that Boyd and colleagues used embryonic bulb OECs, whilst Sasaki et al., transplanted OB-OECs from a neonatal source. However, as previously discussed, Coutts et al., (2012) reported that although adult, neonatal and embryonically-derived OB-OECs were all capable of remyelination in a toxin-induced model of demyelination, OECs from an embryonic source were most effective in this context. Thus, the injury milieu may be vastly different in a demyelinating lesion versus SCI, which could significantly affect the properties of transplanted cells.

In summary, what this research highlights is that whilst anatomical sourcing and donor age may be important factors to consider when isolating and culturing OECs, purification techniques, growth factor supplementation and the length of time the cells spend in passage must also be taken into consideration (Richter et al., 2005; Kawaja et al., 2009; Higginson & Barnett, 2011; Tetzlaff et al., 2010). Variations in protocols from lab to lab may result in subtle/not so subtle

differences in the morphology and antigenic profile of OECs, which could in turn alter their capacity to promote neuronal repair.

1.5.2.2 Schwann Cell Transplants to Repair the CNS

Nerve grafts have been used extensively to mediate the repair of severely damaged peripheral nerves, whereby the gap between the proximal and distal nerve stumps is too great to consider surgical apposition without placing undue tension on the regenerating axon. In these circumstances, a segment of peripheral nerve can be taken from a lower functioning nerve from either the recipient (autograft) or from a donor of the same (allograft) or different species (xenograft). This segment of nerve is then anastomosed to either end of the transected axon in order to bridge the lesion. Results from these studies indicate that their success rate is high, with regenerating fibres being able to transcend the graft to reinnervate the distal nerve stumps (Osawa et al., 1987, 1990; Ide et al., 1990; Tajima et al., 1991; Best et al., 1999). A recent study has also demonstrated that peripheral nerve autografts were able to bridge a lesion of 7cm in sheep, with reports of robust action potentials in regenerating fibres present throughout the graft (Forden et al., 2011).The effectiveness of this strategy to repair peripheral nerves may be at least partly attributable to the ECM-rich basal lamina in the graft, since studies of muscle grafts, which can form similar basal lamina guidance structures, have also reported their efficacy in promoting peripheral nerve regeneration (Fawcett & Keynes, 1986; Sehrbundt et al., 1991).

Similarly, peripheral nerve grafts have been used as a means of bridging CNS lesions, with reports of extensive innervation of the grafts by CNS axons, though these fibres fail to exit the lesion site (Aguayo et al., 1978; Weinberg & Raine, 1980; Matsuyama et al., 1995). For example, Richardson et al., (1980) transected the thoracic spinal cord in rats and then grafted autogolous sciatic nerve into the lesion in an attempt to enhance repair. They reported that the peripheral grafts became innervated by CNS axons and DRGs but despite this apparent regeneration, there was little or no functional recovery in these animals. However, autologous grafts of peroneal nerve, which were implanted

into the medulla oblongata induced the greatest degree of neurite outgrowth when the grafts were placed in close proximity to the cell bodies of CNS neurons, which may be an important consideration for the success of nerve grafts (Lammari-Barreault et al., 1991). Unlike the repair of peripheral nerves, the ability of nerve grafts to enhance the regeneration of CNS axons may be dependent upon the presence of SCs, as apposed to just their basal lamina. Studies of the lesioned diencephalon whereby nerve grafts with viable SCs were compared to those where the SCs had been ablated reported the presence of myelinated and unmyelinated fibres in the former graft only (Smith et al., 1988).

Purified cell suspensions of mature SCs have also been transplanted into a variety of CNS lesions to mediate repair (Kuhlengel et al., 1990 a,b; Raisman et al., 1993; Brook et al., 1993, 2001; Cheng et al., 1996; Bachelin et al., 2005), with varying degrees of success. Pearse et al., (2004) reported increased sprouting of sensory fibres along with enhanced neurite outgrowth in seritonergic axons following SC transplantation in a rodent contusion model, although regeneration of the corticospinal tract was poor. Similarly, Keyvan-Fouladi et al., (2005) demonstrated that the delayed transplantation of SCs 8 wks after the initial injury resulted in some gains in fore paw function, though these only reached around 5-10% of control values. However, in combination with FGF and IN-1 antibody, which alleviates inhibitory myelin debris, human SC grafts induced some regeneration of the corticospinal tract, along with reduced die-back out-with the lesion site in a complete transection injury (Guest et al., 1998). These differences in observations could be attributable to limited regeneration due to the presence of a more severe injury to begin with. Furthermore, the differing responses of sensory and motor axons following SC transplantation could also suggest that the requirements for achieving regeneration are distinct for each class of neuron.

SCs can also be modified to enhance their repair potential following transplantation into a CNS lesion. For example, Girard et al., (2005) reported that the over-expression of BDNF and NT-3 in SCs that were transplanted into the lesioned spinal cord resulted in enhanced SC differentiation and reduced astrogliosis, correlating with a more robust functional recovery. Similarly SCs

engineered to over-express PSA-N-CAM showed enhanced migratory abilities compared with non-engineered cells following their transplantation into the CNS (Lavdas et al., 2006)

Infiltrating endogenous SCs, which are thought to migrate in through the meninges and blood vessels, rather than via white matter tracts (Baron-Van Evercooren et al., 1993), have also been postulated to assist in the limited repair of the CNS, in that Beattie et al., (1997) reported that in contusion models, SCs were observed in close proximity to the limited numbers of regenerating fibres within the lesion site.

Promisingly, the earlier findings of Morrissey and colleagues (1991), whereby cultures of human SCs required approximately 6 wks to reach sufficient numbers for transplantation, have since been improved upon. Casella et al., (1996) reported that growing human SCs on laminin in combination with the growth factors heregulin and forskolin resulted in the propagation of significantly larger volumes of human SCs in approximately half the time, which could be of great benefit for autologous transplant therapies. Furthermore, the FDA has recently given funding for Phase I clinical trials of SC transplants into patients currently living with SCI, to be conducted by the Miami Project to Cure Paralysis under the direction of Dr Mary Bartlett-Bunge and Dr Damian Pearse.

Immature SCs may also hold therapeutic promise in the treatment of CNS pathologies, in that skin-derived precursors (SKPs) transplanted into the contused rat spinal cord were not only shown to promote a degree of locomotor recovery and remyelination following their differentiation into SCs, they also demonstrated enhanced integration and reduced astrogliosis compared with transplants of differentiated SCs (Biernaskie et al., 20007). Similarly, boundary cap cells have also been reported to differentiate into SCs with enhanced migratory abilities following their transplantation into the demyelinated rodent spinal cord (Zujovic et al., 2010).

1.5.2.2.1 Schwann Cell-Mediated Remyelination in the CNS

There is sufficient evidence to suggest that both endogenous and exogenous SCs are capable of remyelinating CNS axons (Gilmore 1971; Blakemore, 1975; Pearse Baron-Van Evercooren et al., 1997; Pearse et al., 2005), although in the X-irradiated spinal cord, SC remyelination is said to occur in astrocyte free regions when the glial limitans has been breached (Blakemore & Patterson, 1975; Sims & Gilmore, 1983; Gilmore & Sims, 1993). It has also been reported that peripherally remyelinated CNS axons exhibit normal clustering of sodium and potassium channels at the nodes of Ranvier and that conductivity can be restored, though in chronic injuries, some deficits in velocity can persist (Felts & Smith, 1992; Honmou et al., 1996; Black et al., 2006).

1.5.3 Fundamental Differences between OECs and SCs After Transplantation

The previous sections highlight the view that there is sufficient evidence to support the use of either OECs or SCs in a transplant-mediated repair strategy. Each of these cell types is almost antigenically and morphologically comparable, making their definitive detection *in vivo* difficult (Barnett et al., 1993; Ramon-Cueto., 1998; Wewetzer et al., 2002; Barnett, 2004). However, differences in key aspects of their behaviour, such as their ability to interact with resident glial cells in the CNS, may be vitally important when considering their transplantation *in vivo*.

Whilst Schwann cells form boundaries and induce astrogliosis in co-cultures with astrocytes, OECs mingle readily in culture and do not cause reactive gliosis (Figure 1.12). This effect is thought to be mediated by differences in the secretory profile of Schwann cells versus OECs, since it has been demonstrated that conditioned media taken from Schwann cells causes OECs to form boundaries with astrocytes (Lakatos et al., 2000, 2003; Santos-Silva et al., 2007). A recent detailed analysis of conditioned media from both OECs and SCs has suggested that highly sulphated heparin sulphates secreted by SCs may be responsible for this boundary formation, along with the expression of FGF 1 and 9 (Higginson et al., 2012). The differential expression of N-cadherin has also been implicated to play a role in this phenomenon (Fairless et al., 2005); as has the response of SCs to astrocyte-secreted ephrins and aggrecan (Afshari et al., 2010a, 2010b). Given the astrocytic composition of the glial scar, these findings could have significant consequences following transplantation of either cell type.

Similar findings have been reported *in vivo* following the transplantation of OECs or SCs into the lesioned spinal cord. Lakatos et al., (2003) reported that SC grafts exacerbated the glial scar and increased the expression of CSPGs, compared with OECs; whilst transplanted LP-OECs were said to reduce scar formation (Ramer et al., 2004). Furthermore, a report by Plant et al., (2001) demonstrated an increase in CSPGs within the lesion following SC transplantation, particularly localised to the SC-graft interface.

Su & colleagues (2009) suggested that the injury environment may in fact favour OEC migration. They showed that TNFα, expressed by reactive astrocytes, increased the migration of OECs on a Boyden chamber assay. Furthermore, blockade of the TNFα receptor, TNFR1, on GFP-OECs reduced their ability to migrate into the lesion. Whilst this may be the case, Pearse et al., (2007) also highlighted that OEC survival after transplantation is significantly increased when OECs are not transplanted directly into the lesion, but at sites rostral or caudal to it. These findings appear to be echoed by Barakat et al., (2005) who reported that following transplantation into the epicentre of a contusion lesion, very few OECs survived compared with SCs. However, they do not report on any differences in the level of astrogliosis induced by each cell graft, and their results appear to show a self-contained SC graft with little evidence of migration or integration with the scar tissue.

Furthermore, in chemically-induced demyelinating lesions, transplanted SCs do not appear to migrate significantly or to integrate in astrocyte-rich areas (Iwashita et al., 2000; Shields et al., 2000). In addition, infiltrating endogenous SCs in these lesions also favour areas devoid of astrocytes (Blakemore et al., 1989). However, a recent study by Zujovic et al., (2012) reported that in a MOG-induced rodent model of EAE, transplanted SCs were able to myelinate demyelinated axons in close proximity with host astrocytes. This data could suggest that the injury milieu may differ significantly between toxin-induced lesions and inflammatory models of demyelinating diseases, which could be significant for enhancing the integration of transplanted cells within the host tissue.

In summary, although they are almost phenotypically indistinguishable from one another, OECs and SCs differ greatly in important aspects of their behaviour, such as their ability to interact with astrocytes. These findings could suggest that OECs may be a preferential candidate for use in cell-mediated repair of the CNS (Kocsis et al., 2009). However, further evidence that OECs exhibit more favourable behaviour than SCs following transplantation into the lesioned spinal cord could prove invaluable in developing cell-transplant therapies for eventual clinical use.

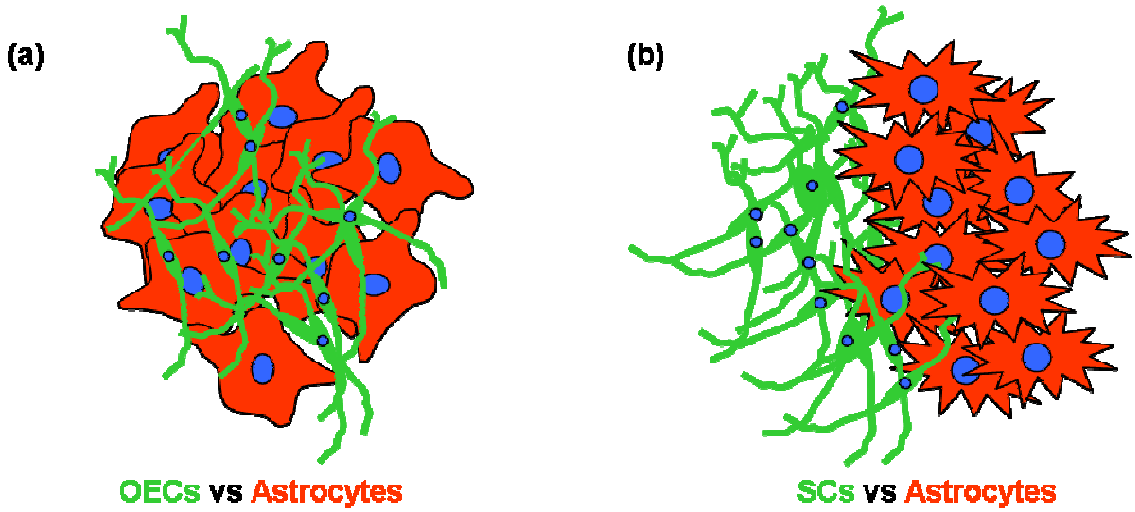


Figure 1.12 – *OECs and SCs differ in their interactions with astrocytes.* Whilst OECs mingle readily with astrocytes *in vitro* (a), SCs form boundaries whereby very few cells cross into the astrocyte territory (b); a phenomenon which is largely mirrored *in vivo*. In addition, SCs induce reactive astrogliosis, causing astrocytes to up-regulate their expression of GFAP and become hypertrophic.

1.5.3 Bioengineering Strategies to Promote CNS Repair

The use of bioengineering strategies is quickly emerging as a potential therapy in the treatment of CNS trauma, by aiming to provide a more suitable growth substrate than the one present at the site of injury and to bridge the lesion (Verreck et al., 2005; Wen et al., 2006; Haile et al., 2007; Amado et al., 2008). Furthermore, biomaterials can be modified so as to enhance their properties, thus maximising their repair potential. For example, poly (ß-hydroxybutyrate) can be impregnated with ECM to enhance cell adhesion and proliferation (Novikova et al., 2009); whilst chitosan can be formed into injectable hydrogels with variable mechanical strengths to best suit its application (Crompton et al., 2007). Thus, a relatively soft hydrogel can mimic the microenvironment of CNS tissue, providing an optimised substrate for the growth of neural and glial cells (Dillon et al., 1998; Balgude et al., 2001). Tabesh et al., (2009) detail a list of suitability criteria for implantable biomaterials; such as high porosity to allow for the diffusion of waste and nutrients and high surface area to volume ratio to maximise cellular attachment.

Furthermore, topographical cues such as grooves, ridges and pillars can also be incorporated into a range of biomaterials to influence cell behaviours, including adhesion, migration, proliferation and differentiation (Yim et al., 2005; Boland et al., 2008). Studies by Dalby et al., (2007, 2012) reported on the ability of nano-scale topography to induce osteogenesis in human MSCs, thus highlighting the potential use of bioengineered therapies in orthopaedic medicine. Similarly, these findings could be optimised to maximise the differentiation of NSCs to facilitate the repair of the damaged CNS.

Previous studies have highlighted the use of bioengineering therapies in the repair of severe peripheral nerve injuries. Ribeiro-Resende et al., (2009) described how poly- ε -caprolactone (PCL) fibres with longitudinal microgrooves could be used to encourage the alignment of SCs *in vitro* and to encourage the formation of bands of Bungner, which are fundamentally important in the regeneration of peripheral nerves. In addition, microspheres of polylactide-glycolic acid (PLGA) infused with glial derived neurotrophic factor (GDNF)

enhanced peripheral regeneration in rodent models of sciatic nerve injury (Wood et al., 2012). Biodegradable scaffolds can also be used as a way to bridge large lesions in peripheral nerve injury, thus facilitating repair (Dai et al., 2000; Yu et al., 2004; Hausner et al., 2007; Ding et al., 2011).

In models of SCI, biodegradable scaffolds may be particularly useful in directing neurite outgrowth, with the aim of promoting the successful exit of re-orientated regenerating fibres from the graft (Chew et al., 2007; Chen et al., 2009). An *in vitro* study using PCL scaffolds embossed with a micro-pattern of grooves and ridges demonstrated their ability to promote neurite alignment and to allow myelination in cultures modelling the intact CNS (Sørenson et al., 2007). However, after 3 weeks this alignment was decreased as cell density increased, indicating the need to investigate the effects of increased groove depth on long-term alignment. Patist et al., (2004) implanted poly (D,L-lactic acid) macroporous guidance scaffolds with or without impregnated BDNF. Their results showed that glial and neuronal cells more rapidly invaded BDNF scaffolds than those without. Furthermore, angiogenesis was almost doubled 8 weeks after implantation of BDNF scaffolds. However, whilst these scaffolds were well tolerated, there was little or no evidence of significant neurite outgrowth within the graft (Patist et al., 2004). These results highlight that the use of a biodegradable scaffold alone may not be sufficient in overcoming the multiple impediments to CNS repair.

1.5.3.1 Cell-Seeded Biomaterials for the Treatment of SCI

Cell-loaded biodegradable scaffolds are also currently attracting attention for the possibility that this kind of combined therapeutic approach may be more effective in the treatment of complex injuries such as SCI (Xu et al., 1995, 1997; Moore et al., 2006; Kubinova et al., 2012).

By in large, the body of research utilising cell-seeded scaffolds in models of SCI have mainly utilised SCs as a cell candidate, perhaps in a bid to recapitulate the guidance channels created by the SC bands of Bungner during the successful repair of peripheral nerves. Olson et al., (2009) reported that following thoracic transection of the spinal cord in rodents leaving a 2mm gap, surgically implanted biodegradable poly-lactic glycolic acid (PLGA) scaffolds seeded with NSCs or SCs resulted in significant increases in neurite outgrowth within the lesion. However, they failed to report the exit of regenerating fibres from the graft, or to observe any significant improvements in functional recovery with treatment. Similar findings were reported by Xu et al., (1997), who described enhanced axonal re-growth within the lesion site but little or no exit of regenerating fibres. Furthermore, Hurtado et al., (2006) observed poor survival of exogenous SCs within the lesion site following the transplantation of pre-seeded poly(D.L lactic acid) porous scaffolds, corresponding with minimal neurite outgrowth.

What these studies highlight is the importance of optimising repair strategies by selecting the most suitable biomaterial and scaffold design coupled with the most effective cell-candidate in order to overcome all aspects of the regenerative inhibition induced by SCI, thus maximising repair.

1.6 Closing Summary

In summary, this literature review has highlighted that whilst peripheral nerves are capable of at least partial regeneration, largely mediated by the actions of SCs, the CNS is incapable of achieving such repair. The reasons for this include the inflammatory response, a lack of trophic support at the injury site and the formation of a physical and molecular barrier to regeneration induced by an astrocytic scar. Current singular strategies to enhance repair of the CNS, including inhibition of negative molecules associated with the glial scar and the implantation of biodegradable scaffolds, have reported some improvements in neurite outgrowth in animal models of SCI, though very little evidence of substantial long-distance regeneration.

Much debate has also surrounded the issue of cell transplantation as a therapy, in terms of which is the most appropriate cell candidate for maximising repair, given that there is sufficient evidence to suggest that either OECs or SCs may be useful in mediating the repair of the damaged CNS. However, previous studies have shown that SCs illicit a negative astrocytic response and exacerbate scar formation following transplantation into SCI models, suggesting that OECs may be a more favourable candidate for repair. The discovery of novel evidence relating to other ways in which these two cell types differ in terms of their behaviour within a CNS environment could prove to be significant in the development of clinically relevant cellular strategies for treating SCI.

By initially selecting the most appropriate cell-candidate, combinatorial approaches to SCI treatment, such as cell-seeded biodegradable scaffolds, could have more promising outcomes for neuroregeneration.

1.7 Thesis Aims

The overall aim of this project was to provide novel evidence to support the use of either OECs or SCs as a preferential candidate for cell-mediated repair of the CNS by demonstrating key differences in their interactions with endogenous glia. Since little was known about the effects of either cell type on endogenous myelination in the CNS, the main focus of this thesis was to assess the ways in which OECs and SCs influenced the myelinating capacity of oligodendrocytes *in vitro*, and to establish, where possible, mechanisms for the resulting effects on myelination.

Furthermore, as the use of a combined therapeutic approach is likely to be more efficacious in the treatment of the damaged CNS, this thesis also aimed to develop a biodegradable micro-grooved scaffold. The initial aims were to demonstrate the most appropriate biomaterial for supporting the survival and differentiation of a complex culture system of glia and neurons. Additional aims included investigating the effects of increasing groove depth on cellular alignment, with the aim of maximising long-term alignment.

Once these aims had been addressed separately, it was hoped that these findings could provide novel data for the development of the optimum paradigm for promoting repair in an *in vivo* model of spinal cord injury using a 3D cell-seeded scaffold. Therefore, the focus of this thesis was divided between two themes:

(i) investigating the effects of exogenous glial cells on oligodendrocyte myelination within an *in vitro* system recapitulating the intact CNS

and

(ii) developing the optimum scaffold design *in vitro* to maximise cell survival, proliferation and differentiation of a complex CNS system.

Chapter 2

Materials & Methods

2.1 Primary Cell Culture

Cultures of astrocytes, oligodendrocyte precursor cells (OPCs) OECs, SCs and dissociated embryonic spinal cord cultures will be described in detail throughout this chapter. Firstly, in order to create a more permissive substrate for optimal cell attachment and growth, a positive charge was created on the surface of tissue culture flasks and glass coverslips by coating in poly-L-lysine (PLL; Sigma, Dorset, UK). Briefly, PLL solution was made up in distilled water (ddH_20) to give a concentration of 13.3 µg/ml and added to a petri dish of autoclaved 13 mm glass coverslips (VWR International, Leicestershire, UK) or to Greiner flasks (Greiner Bio-One Ltd, Gloucestershire, UK), which were then incubated at 37 ºC for 30 min. After this period, the PLL solution was aspirated off and coverslips or flasks were washed with ddH_20 and left to air dry in a sterile tissue culture hood, ready for use.

Non-coated T75 cm^2 Greiner flasks were used to culture neurospheres, whilst PLL coated flasks (both T75 cm^2 and T25 cm^2) were used to maintain all other cell populations. Cells were plated down onto PLL coated coverslips in 24 well plates (Corning Life Sciences, The Netherlands) prior to immunofluorescent labelling, and dissociated embryonic spinal cord cultures were cultured on coverslips and housed 2 per 35 mm petri dish (also Corning Life Sciences).

Cultures were maintained in a humidified incubator, which was kept at a constant temperature of 37 ^oC with 7% carbon dioxide (CO_2), and fed every other day, unless otherwise stated, by removing half the media and replacing with fresh. Media was prepared on a weekly basis and filtered sterile using a 0.22 µm filter (Sartorius Stadium, UK). Gibco Life Sciences (Paisley, UK) were the suppliers for all of the basic media used and each new bottle was supplemented with 25 µg/ml gentamycin (Gibco Life Sciences) upon opening, before being stored in the fridge.

2.1.1 Animal Use and Ethical Considerations

Only Sprague Dawley (SD) rats, which were provided by Harlan Laboratories, UK, were used for work carried out during this thesis. Animals were housed in a secure animal facility and treated in accordance with Home Office Regulations under the Animal (Scientific Procedures) Act of 1986. A trained technician was responsible for the culling of the animals using a Schedule 1 method (namely $C0_2$ exposure or lethal overdose of anaesthetic). Food and water were available ad libitum

2.1.2. Olfactory Ensheathing Cell Preparation

OECs were isolated from the olfactory bulbs of 7-day-old SD rat pups, as summarised in Figure 2.1. Briefly, animals were euthanized by lethal injection of pentobarbital and then decapitated to allow for the skin and the skull to be removed, thus exposing the brain and the olfactory bulbs (OBs). Using curved forceps, the bulbs could then be detached from the brain at the cribriform plate and scooped out before being placed into Leibowitz (L-15) medium to support cell survival in the absence of $C0_2$ equilibration (Gibco Life Sciences, Paisley, UK). Using a scalpel blade, the OBs were chopped finely to mince the tissue, and then incubated for 15-20 min at 37 ^oC in a bijou with 500 µl of collagenase (1.33 %) and 500 µl of L-15 per 5-6 animals. The reaction was stopped by the addition of 1 ml of a mixture of soybean trypsin inhibitor (0.52 mg/ml Sigma Aldrich, Dorset, UK), bovine serum albumin (3.0 mg/ml Sigma Aldrich) and DNase to prevent cell clumping (0.04 mg/ml, Sigma Aldrich); collectively denoted as **SD**. A single cell suspension was prepared by passing the tissue through a series of needles, ranging from gauge size 19G down to 23G and the cells were then pelleted by centrifugation at a speed of approximately 1200 rpm for 4 min at room temperature before being resuspended in their optimum growth media in a PLL coated T25 cm^2 flask.

Though exact protocols for generating the most appropriate growth medium for OECs may vary from lab to lab and may also depend upon the age and source of the tissue, the use of a basic medium such as DMEM supplemented with 5-10 % serum to aid expansion is a commonly used base, which can then be further supplemented with growth factors.

As previously discussed in Chapter 1, Noble and Murray (1984) demonstrated the mitogenic effects of astrocyte-secreted factors (**ACM**) on oligodendrocytes, and this effect was subsequently observed in OECs in combination with fibroblast growth factor 2 (FGF2), forskolin and heregulin (Denoted as olfactory mitogen mix, **OMM**) (Franceschini & Barnett, 1996). Based upon these findings, our protocol for generating a growth-supportive medium for neonatal rat olfactory bulb-derived OECs (OB-OECs), termed **OMM/ACM 10 %**, was as follows: DMEM low glucose (Gibco Life Sciences, Paisley, UK) with 5 % (v/v) foetal bovine serum (Invitrogen, Paisley, UK), further supplemented with fibroblast growth factor 2 (FGF2) (25 ng/ml; Peprotech, London, UK), forskolin (5×10^{-7} M, Sigma Aldrich, Dorset, UK), heregulin (Hrg ß1, 50 ng/Ml; R&D Systems, Oxon, UK), and finally ACM (1:5), which was collected from a confluent T75 cm^2 flask of astrocytes after 48 hr in serum free media (Alexander et al., 2002; Santos-Silva et al., 2007). Cells were fed 3 times a week and grown to confluency in PLL-coated flasks.

2.1.2.1 Olfactory Ensheathing Cell Purification

OECs were purified by the use of a DIY EasySep^{TM} kit (Stem Cell Technologies, Grenoble, France) approximately 5-7 days after the initial dissection (also described in review by Higginson & Barnett, 2011). Briefly, magnetic nano-particles were used to bind to cells which had been labelled with an antibody against a particular antigen of interest (p75 ^{NTR}, nerve growth factor receptor, in this instance). A tube containing the cell/ antibody suspension (anti-p75 ^{NTR}, polyclonal rabbit; Abcam, Cambridge, UK) was then placed into a specialised magnet forcing the nano-particles, which were bound to the antigen +ve population of cells, to stick to the sides of the tube whilst unlabelled/ unbound cells could be poured out. This washing process was repeated 4 times using PBS (phosphate buffered saline, pH 7.4) containing 2% FBS, and the remaining cell pellet was spun down and re-suspended in 100 µl of OMM/ACM 10% to form a strip of cells, which was allowed to adhere to the flask for 15 min at 37 ^{o}C before being flooded with media. Allowing the cells to "sit-down" in this manner maximised their growth potential by encouraging the initial formation of dense, tightly packed colonies.

Immunostaining using p75 ^{NTR} as a marker for OECs revealed that whilst very few cells were positive for p75 ^{NTR} within one hour of being plated down immediately following dissection (Figure 2.2 A), approximately 40% of the heterogeneous population appeared to express this marker 5 days later, prior to positive antigen selection using the EasySep^{TM} kit (Figure 2.2 B). After purification using the aforementioned technique, approximately 98% of the cell population expressed p75 ^{NTR}, suggesting the presence of a highly purified culture of OECs (Figure 2.2 C). Whilst the above highlights that the expression of p75 ^{NTR} may be transient in early OEC cultures, it also demonstrates the usefulness of the EasySep^{TM} kit as a tool for purifying primary cells. Once the cells had begun to reach confluency, they were passaged by light trypsinisation (0.1% in PBS) and split into several flasks and/or seeded onto PLL-coated coverslips in 24-well plates (after approx. 4-7 days) and maintained for no longer than 5 passages.

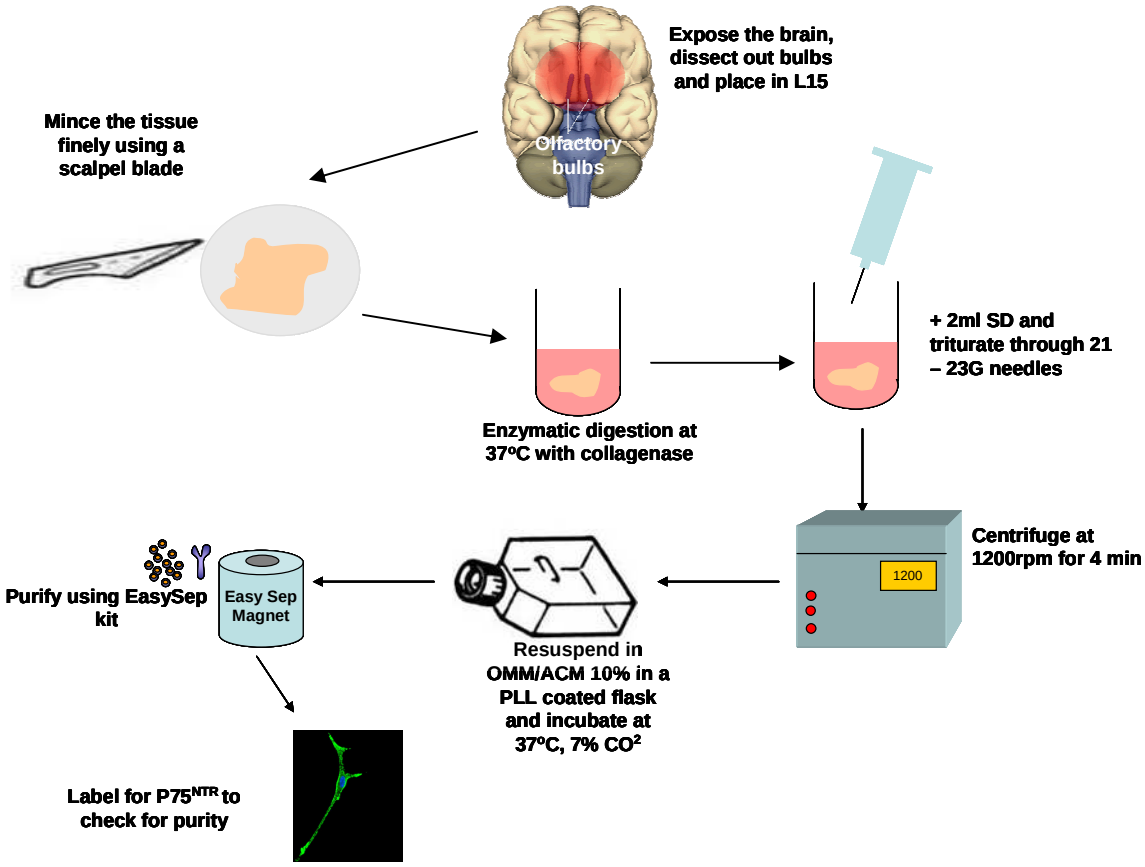


Figure 2.1 – *Primary Culture of OECs*. Olfactory bulbs were dissected out by removing the outer layers of the scalp to expose the brain. The tissue was chopped finely using a scalpel blade, placed into 500 µl of L-15 media and then enzymatically digested by collagenase (1.33%) at 37 °C for 15 min. Soya bean trypsin inhibitor (SD) was added after this period to stop the reaction and the cell suspension was then triturated through a range of needles (21-23G). Cells were pelleted by centrifugation at 1200 rpm for 4 min and resuspended in OMM/ACM 10%, which was replaced (50:50) every 2 days. After 5-7 days, the heterogeneous cell mix was purified using an EasySep™ kit to produce a highly pure population of OECs, which could be confirmed by labelling with an antibody to detect p75 ^{NTR} expression.

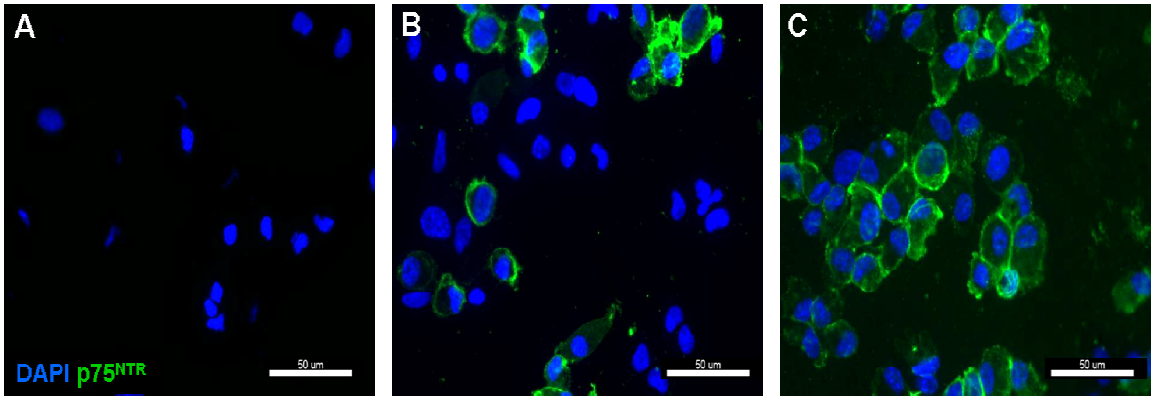


Figure 2.2- *Immunostaining of olfactory bulb tissue for p75^{NTR} pre and post purification using an EasySep™ kit.* After enzymatic digestion of the olfactory bulbs, a heterogeneous mix of cells was seeded down onto PLL coated coverslips and allowed to adhere for 30 min. Immunolabelling with a primary antibody against p75^{NTR} and it's corresponding fluorescently-conjugated secondary antibody revealed that very few of these cells expressed this marker at that time point (A). These same cells were then enriched in OMM/ACM 10% (optimum growth media for OECs) for 5 days and then stained immediately *prior* to purification. Approximately 40% of the population were then positive for p75^{NTR} (B). One hour after purification using the EasySep™ kit to select for a p75^{NTR} positive population of cells, approximately 98% of cells expressed this marker, suggesting that this methodology is effective at producing highly purified cultures of OECs. Cells remained rounded up for 1-2 hr until adequate focal adhesions had been made. Images were taken using an Olympus BX51 fluorescent microscope and Image Pro software using the x 40 objective. Scale bars = 50 µm.

2.1.3 Schwann cell Preparation

The sciatic nerves of P7 SD rats were dissected out and enzymatically digested to produce cultures of SCs (see Figure 2.3). Briefly, the pups were pinned out on a board, anterior surface down, and then 2 small incisions were made on either side of the sacro-lumbar region of the spinal cord. The sciatic nerves were exposed by continuing the incisions down each hind limb, cutting away the skin and then removing part of the piriformis muscle. Using forceps, the sciatic nerves were then gently pushed up to ensure that they were properly detached from the muscle and then a portion of approximately 5-6 mm of each nerve was removed, down to the area where the nerve bifurcates to give rise to the tibial and common fibular nerves. Tissue culture then proceeded exactly as described for OEC preparation, with the addition of 100 µl trypsin (0.25%, Invitrogen) as well as collagenase for digestion of the tissue. Cells were initially resuspended in 10% FBS without any growth factors.

2.1.3.1 Purification of Schwann Cells

Cells were maintained in 10% FBS for 48 hr without any additional factors which were specifically mitogenic for SCs in order to slow down their rate of proliferation. Purification was then performed by the addition of cytosine arabinoside (AraC, 10^{-5} M, Sigma, Dorset, UK) to the media for a further 48 hr in order to kill of rapidly dividing contaminating fibroblasts (modified from Brockes et al., 1979). Further purification was then carried out by trypsinising the cells and resuspending them in a small volume of serum free media with anti-Thy1.1 antibody at room temperature for 15 min (1:50 supernatant, Sigma, Dorset, UK), followed by the addition of rabbit complement (1:4, Harlan Laboratories Ltd., UK) for 45 min at 37 ºC (Lakatos et al., 2000).The purified cell suspension was then pelleted by centrifugation and resuspended in 100 µl of 10% FBS plus heregulin (Hrg ß1, 20 ng/ml; R&D Systems, Europe, Oxon, UK) and forskolin (10^{-6} M), to form a small strip of cells in a T25 cm ^{2} flask, which were allowed to adhere at 37 ºC for 15 min before being flooded with media. As before, cells were passaged by light trypsinisation (0.1% in PBS) and split into several flasks and/or seeded onto PLL-coated coverslips in 24-well plates once they had reached confluency (approx. 4-7 days) and kept for approximately 5 passages.

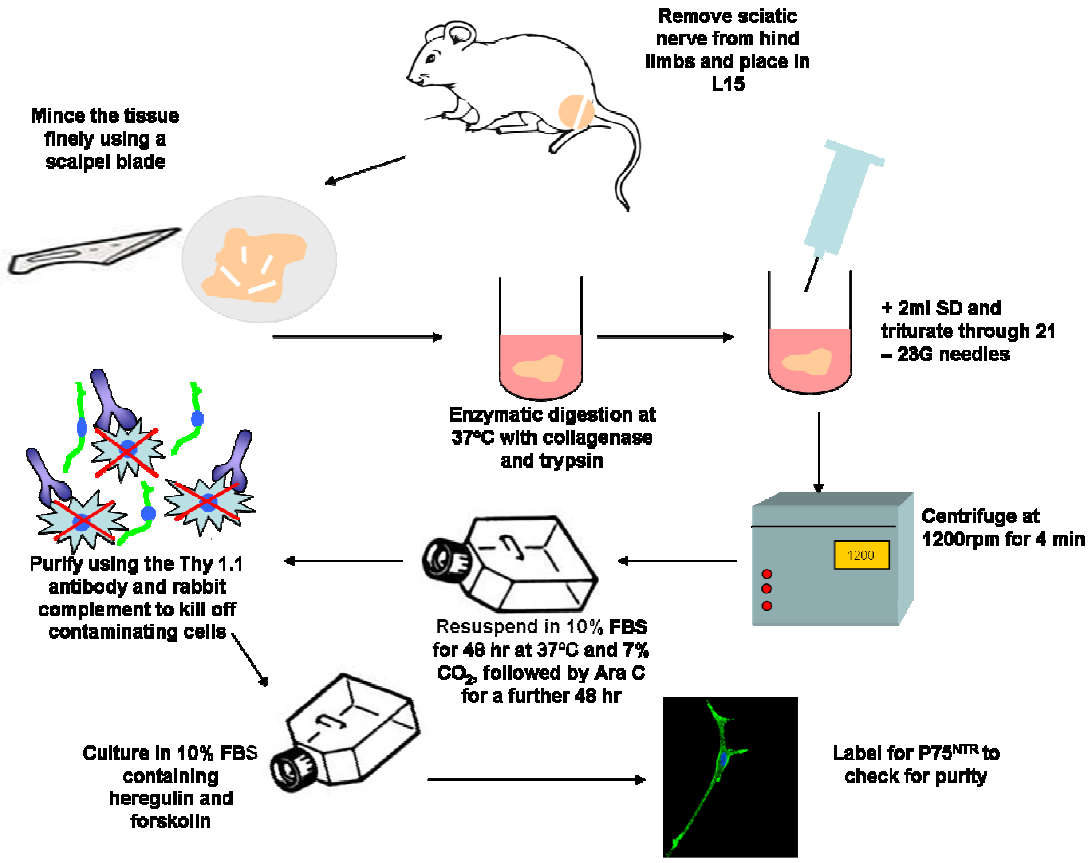


Figure 2.3 – *Primary Schwann cell culture*. SC cultures were obtained by dissecting the sciatic nerves out of the hind limbs of P7 rats. The tissue was chopped finely using a scalpel blade, placed into 500 µl of L-15 media and then enzymatically digested by collagenase (1.33%) and trypsin (0.25%) at 37 °C for 15 min. Soya bean trypsin inhibitor (SD) was added after this period to stop the reaction and a single cell suspension was generated by triturating the tissue through a range of needles (21-23G). Cells were pelleted by centrifugation at 1200 rpm for 4 min and resuspended in 10% FBS for 48 hr, after which cytosine arabinoside (AraC, 10^{-5} M) was added to the media for an additional 48 hr. Following this time period, cells were further purified by incubating with the anti-Thy1.1 antibody (1:50) in serum free media, followed by rabbit complement (1:4) to kill off contaminating fibroblasts. Cells were then spun down again and resuspended in 10% FBS containing heregulin (20 ng/ml) and forskolin (10^{-6} M, Sigma Aldrich, Dorset, UK). Using this method, a highly pure population of Schwann cells could be generated, as assessed by their expression of $p75^{NTR}$ (>95% expression).

2.1.4 Collecting Conditioned Media from Purified Cells

To assess the effects of secreted factors derived from OECs and SCs on endogenous CNS cells, conditioned media (CM) was collected from purified cultures of each cell type by firstly growing cells to confluency in a T25 cm^2 flask (approx 1.5 million cells/flask). Fresh media was then added to each flask (3 ml) and collected after 72 hr, before being filtered sterile (using a 0.22 µm filter, Sartorius) and stored at -20 ^oC until required. The cells were then re-fed and allowed to recover for a further 72 hr before the aforementioned procedure was repeated. A maximum of 2 collections per flask was performed and after this, the cells were not used for any further experimentation. When collecting CM, the media used for collection matched that which was required to maintain each of the cell types/cultures we were investigating, as opposed to the optimum growth media for the cells being collected from. Optimum media was, however, fed to SCs or OECs in between each collection (10% with heregulin and forskolin or OMM/ACM 10%, respectively). When treating cultures with CM, a 1:4 dilution was used with fresh media (1 part CM, 3 parts fresh media) unless otherwise stated. CM was always collected from cells which were approximately passage 3 to allow for accurate comparisons to be made between conditions.

2.1.5 Oligodendrocyte Precursor Cell (OPC) Cultures

Cultures of OPCs were generated by firstly culturing cortical-derived astrocytes from P1 rats. Briefly, the outer layers of the cortex were dissected out (3 pups per flask), making sure to include the corpus callosum, whilst removing the meninges where possible. The tissue was then placed in L-15, minced using a scalpel blade and then collagenase (1.33%) digested for 15-20 min at 37 ºC. The reaction was stopped, as previously described, by the addition of SD and then the cell suspension was triturated through a 21G needle and spun down at 1200 rpm for 4 min. The pellet was resuspended in 10% FBS and the cells were cultured for 10-14 days in a PLL-coated T75 cm^2. After this period, a monolayer of OPCs had formed on top of a confluent sub-layer of astrocytes. To remove the OPCs, the flask was placed on a shaker for 2-3 hr and then the supernatant was removed and placed in a 90 mm petri dish for approx 20 min, whilst fresh 10%

FBS was added to the flask of purified astrocytes, which could then be used for further experiments. The shaken-off supernatant was removed from the petri dish (leaving behind the preferentially attached microglia) and spun down at 1200 rpm for 4 min. OPCs were then resuspended in Sato media (adapted from Bottenstein & Sato, 1979) (defined serum free media: DMEM containing Sato mix, 0.5 mg/ml insulin in 10 mM HCl (Sigma, UK), human transferrin (Sigma, UK), glutamine (100 mM; Sigma, UK) and gentamycin (100 mg/ml; Sigma, UK)), supplemented with the growth factors FGF (10 µg/ml) and platelet derived growth factor (PDGF 2 µg/ml; Peprotech, UK), and plated onto PLL-coated coverslips. Both of these growth factors are known to enhance the survival of OPCs, whilst allowing them to retain a progenitor-like phenotype. The cells were maintained in Sato plus growth factors for 5-7 days, replacing half the media with fresh media every other day. After this time period, FGF and PDGF could be removed and replaced with Sato alone (to promote OPC differentiation) or treated with Sato + conditioned media/experimental growth factors for the duration of the experiment (Raff et al., 1983).

2.1.6 Myelinating Culture System

Previously described by Thomson et al., (2006), this culture system involves generating a confluent monolayer of astrocytes and plating dissociated E embryonic day 15 (E15.5) spinal cord, containing neurons, spinal astrocytes, oligodendrocyte precursor cells and microglia, directly on top (Figure 2.4). After a period of 22-28 days, neurite density and myelination could be assessed using immunocytochemistry and fluorescence microscopy. The nature of this system enables the user to mimic the intact CNS, thereby allowing for the investigation of the complex interactions which occur between endogenous CNS cells and exogenously added glial cells, as well as the effects that a given factor may have upon the culture system. Throughout this thesis, the term “myelinating culture” will be used to denote the aforementioned system.

2.1.6.1 Generation of Astrocyte Monolayers

Astrocyte monolayers were generated by harvesting neurospheres from the striatum of P1 SD rat pups (Figure 2.4). Briefly, the brains were cut mid-sagitally and the outer layers of the cortex pushed back to expose the striatum, which was then dissected out from each hemisphere and placed into L-15 media. For each preparation, approximately 3 heads per flask were used. The tissue was dissociated by trituration through a 21G needle and maintained in a non-coated T75 cm^2 flask in media composed of DMEM/F12 (1:1), supplemented with 0.105% $NaHCO_3$, 2 mM glutamine, 10% pen/strep, 0.6% glucose, 5 mM glucose, insulin (25 mg/ml, Sigma), apotransferrin (100 mg/ml, Sigma), putrescine (60 mM Sigma), progesterone (20 nM Sigma) and sodium selenite (30 nM, Sigma); collectively termed as **neurosphere media (NSM)**. The NSM was further supplemented with 20 ng/ml of mouse submaxillary gland epidermal growth factor (EGF, R&D systems, Abingdon, UK). After the spheres had become confluent (approx. 5-6 days) they were spun down at 800 rpm for 5 min and triturated again using a 21G needle in 1 ml of NSM before being placed in a new flask containing equal measures of fresh media and supernatant, giving a total volume of 20 ml, which was again supplemented with EGF (20 ng/ml). This process was carried out by way of proof of concept to ensure that the cells in suspension where in fact neurospheres and therefore capable of reforming spheres. Once the newly reformed spheres had reached a critical size (approx. 4-5 days), they were once again spun at 800 rpm for 5 min and triturated using a 21G needle. They were then resuspended in DMEM low glucose (Invitrogen, Paisley, Scotland) supplemented with 10% foetal bovine serum and 2 mM L-glutamine (**10% FBS**) to encourage their differentiation into astrocytes, before being plated down onto PLL coated coverslips in a 24-well plate. The monolayer was maintained by removing half the media and replacing it with fresh 10% FBS 3 times a week. A confluent monolayer could usually be observed 7-10 days after spheres were plated down.

2.1.6.2 Dissociated Embryonic Spinal Cord

A pregnant SD rat was euthanized in a $C0_2$ chamber and the embryos were removed by sterilising the abdomen using 70% ethanol and making one large vertical and 2 lateral incisions through the muscle layers to expose the uterus at embryonic day 15 (E15.5). Each embryo was removed from its individual amniotic sac and decapitated, ensuring that the cervical plexus was left intact (located at cervical 1 region, containing fibres which innervate the face, scalp and neck). The upper 5-6 mm of the cord from each embryo was removed and stripped of all traces of meninges to avoid peripheral nerve contamination. By ensuring that the cervical plexus was present, the meninges could be removed more efficiently, thus minimising peripheral contamination. The tissue was then dissociated using trypsin (100 µl of 2.5%) and collagenase (1.33%) for 15 min at 37 ^{o}C and then treated with 2 ml of SD. The cells were triturated through a 21G needle and centrifuged at 800 rpm for 5 min. The resulting cell suspension was resuspended in 2 ml of plating medium containing 50% low glucose DMEM, 25% horse serum, 25% Hanks balanced solution (HBSS) without Ca^{+} and Mg^{+}, (Gibco Life Sciences, Paisley, UK) and 2 mM L-glutamine (Invitrogen) and then further diluted if necessary to give the desired cell density. The mixed culture could then either be plated onto an astrocyte monolayer on glass coverslips, or onto scaffolds depending upon the experimental conditions, at a cell density of 150,000 cells/100µl (unless otherwise stated). After an incubation period of 2 hr to allow the cells to sit down, cultures were maintained in high glucose DMEM supplemented with 0.5 mg/ml insulin, 50 nM hydrocortisone, 10 ng/ml biotin, and a cocktail of hormones including 1 mg/ml apotransferrin, selenium, 20 mM putrescine, 4 µM progesterone (**differentiation media, DM^{+}**). After 12 days in culture, the insulin was removed from the media (**DM^{-}**) to encourage the differentiation of oligodendrocyte precursor cells. Feeding took place 3 times a week by removing half the media and replacing with fresh.

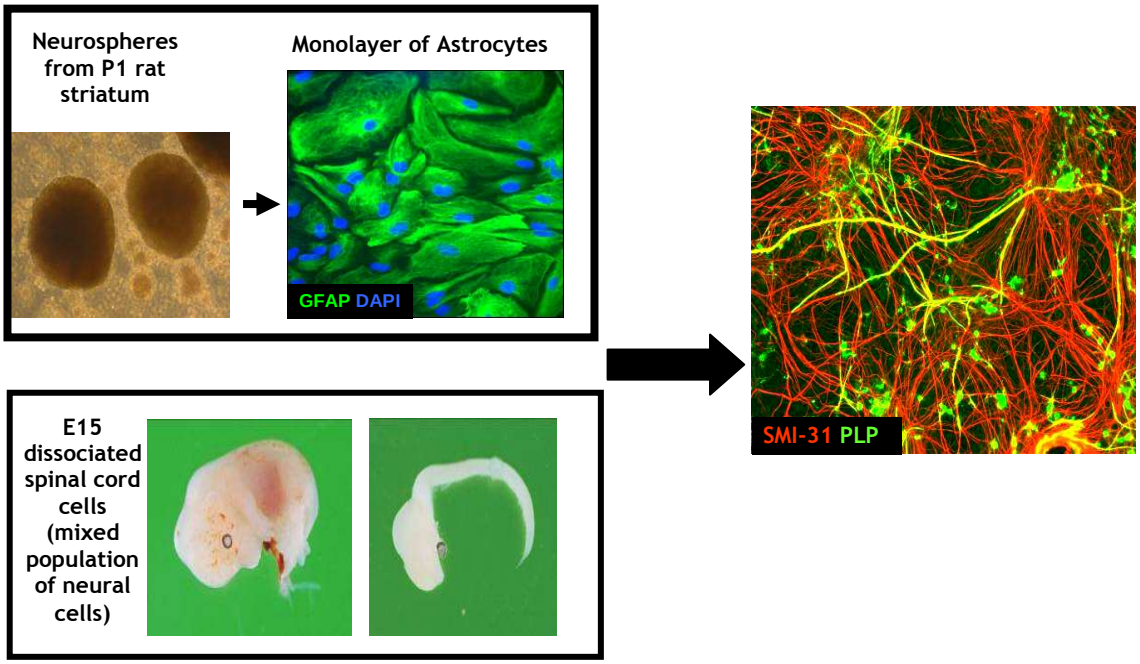


Figure 2.4 – *Schematic describing the methods for producing myelinating cultures.* A confluent monolayer of astrocytes (labelled with glial fibrillary acidic protein, GFAP) was generated by differentiating neurospheres derived from the striatum of post-natal day 1 (P1) rats. A mixed population of cells containing astrocytes, neurons, oligodendrocyte precursor cells and microglia was produced by dissociating embryonic spinal cord tissue and then these cells were plated on top of the monolayer of astrocytes. After 26 days, neurites (labelled with SMI-31 to detect phosphorylated neurofilaments) and oligodendrocytes and myelin sheaths (labelled with an antibody to proteolipid protein, PLP) could be visualised with immunofluorescence, whilst DAPI was used to label nuclei.

2.1.7 Treatment of Myelinating Cultures

In order to assess the biological effects of the interactions between SCs or OECs with endogenous CNS cells, each of these was added exogenously to a myelinating culture or allowed to "condition" the media which was used to maintain the cultures, when focusing solely on factors which may be secreted by each cell type. Conditioned media (CM) was also taken directly from OECs and SCs to treat cultures (as described in 2.1.4). When treating the cultures with CM or indeed, growth factors or antibodies, the standard protocol was to allow the cultures to become established for 12 days before beginning the treatments. Feeding was as previously described: every other day by removing half the media and replacing with fresh DM- containing CM or treatment at the appropriate concentration. Media containing the growth factors/antibodies/CM was freshly prepared every 3-4 days and stored at 4 ^{o}C between each use. Control cultures refer to those which did not have any exogenously added cells, nor were they exposed to any additional treatments during the culture period.

2.1.7.1 Exogenous Addition of OECs or Schwann Cells to Myelinating Cultures

The embryonic spinal cord cells were prepared as described in **2.1.6.2** by enzymatic digestion of the tissue with collagenase and trypsin. During this incubation time, a flask of OECs and a flask of SCs were trypsinised (0.25%), centrifuged at 1200 rpm for 4 min and resuspended in plating media (PM) ready to be counted. The volume used for resuspension was kept as small as possible (usually around 200 µl, depending upon the confluency of each flask) so as to not to greatly alter the concentration of the spinal cord mix (150,000 cells/100µl). Calculations were then performed to work out the correct volume of each cell type to be added to the spinal cord mix in order to produce one cell suspension containing 5,000 or 10,000 OECs or SCs, as well as the spinal cord cells. For further experiments, cell suspensions containing spinal cord cells with both OECs *and* SCs together could also be prepared as outlined above. As previously described, the cultures were allowed to sit down for 2 hr before being fed the usual mix of DM^{+} and PM. Feeding was carried out exactly as normal, by replacing

half the media with fresh DM every other day and by removing insulin after D12 (DM^-).

An alternative method was initially tested whereby the cultures were allowed to become established for 12 days before being removed from their culture dishes and placed into fresh dishes containing no media. OECs or SCs were then plated directly on top of each coverslip in a meniscus at the concentrations previously described, and incubated at 37 ^oC for 1 hr to aid their attachment. However, this method resulted in poor survival of the cultures (including controls, which were treated in the same manner), perhaps due to the mechanical stress exerted on each by the use of forceps and pipetting directly on top of the coverslips at a time when the cultures may be considered to be vulnerable. Similarly, higher concentrations of OECs or SCs were also tested but these again resulted in poor survival of the cultures, which may be attributed to an over-loading of cells onto each coverslip.

2.1.7.2 Conditioning of Myelinating Cultures by OECs or Schwann Cells

Myelinating cultures were allowed to establish for 12 days before treatment began. For each myelinating culture coverslip, 2 confluent coverslips of either OECs or Schwann cells were placed in the same dish. In this manner, cells were exposed to paracrine signalling from factors which may be being secreted by endogenous cells within the culture system (see Figure 2.5). Coverslips containing OECs or Schwann cells were replaced every feeding day as previously described.

Conversely, CM was also collected *directly* from confluent flasks of OECs (OCM) or Schwann cells (SCM) in DM^-. In this paradigm, the CM was collected in an environment where neither cell type was exposed to factors which were being secreted by endogenous cells within the myelinating culture. CM was filter sterilised before use and added to cultures at a 1:4 or 1:8 dilution with fresh DM. To assess the importance of the proteinaceous nature of CM, it was also heated for 1 hr at 55 ^oC (h.SCM) and then treated exactly as above. Each experimental

"n" number relates to a different batch of CM as well as a biological replicate of the myelinating cultures.

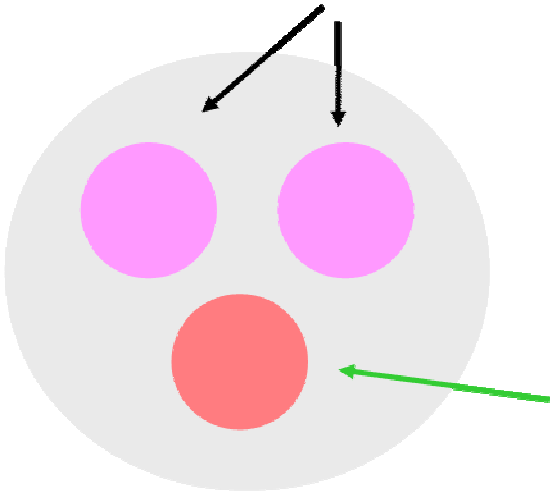


Figure 2.5 – *Methodology of conditioning myelinating cultures.* Two confluent coverslips of Schwann cells (SCs) or OECs were placed in a 35 mm petri dish and allowed to condition a myelinating culture from day 12 onwards. SC/OEC coverslips were replaced every feeding day. In this way, the OECs and SCs were also exposed to factors being secreted by endogenous cells within the myelinating culture, which may have influenced their secretory profile. Black arrows denote SC or OEC coverslips, whilst the green arrow highlights the myelinating culture.

2.1.7.3 Treatment of Myelinating Cultures with Connective Tissue Growth Factor (CTGF)

After 12 days *in vitro,* cultures were treated with CTGF (38.3kD human recombinant protein GWB-932DB7; GenWay Biotech Inc, San Diego) every feeding day by removing half the media and replacing with fresh media containing the growth factor. In order to produce a dose response curve, a range of concentrations was used (500 pg/ml, 750 pg/ml, 10 ng/ml and 100 ng/ml). Each concentration was made up in fresh DM^- , as opposed to being added directly to each dish, and the calculations were done to ensure that the **final** concentration in each dish closely matched those listed above after half the media had been removed and replaced with fresh. A shorter 11kD human recombinant CTGF (Peprotech, UK) was also tested exactly as above at a

concentration of 10 ng/ml. Both growth factors were said to have cross reactivity in rats.

2.1.7.4 Treatment of Myelinating Cultures with a Neutralising Antibody to CTGF

A neutralising antibody to CTGF (rabbit polyclonal; LS Bio, UK) was added to SCM at a concentration of 10 ng/ml and the SCM was then added to cultures as before (1:4 with fresh media every other day). Controls for this experiment included SCM alone, antibody in DM^- and no treatment. The data sheet recommended that a concentration of around 2 µl/ml of antibody should expect to neutralise 200 ng/ml of CTGF therefore, a concentration of 10 ng/ml should effectively neutralise 1 ng/ml.

2.2 Immunocytochemistry

Immunolabelling was assessed by use of a primary antibody against the antigen of interest followed by incubation with an isotype specific fluorescently-conjugated secondary antibody (AlexaFluors; Molecular Probes, UK), which could then be visualised using a fluorescence microscope. The lists of primary and secondary antibodies used for work carried out during this thesis are given in Tables 2.1 and 2.2, respectively. Protocols for staining varied depending upon the location of the antigen (ie. internal or cell surface), however, all coverslips containing cells were initially fished out of 24-well plates or 35 mm dishes using forceps, blotted gently on clean tissue paper to remove excess media and housed in a black lidded box during the procedure (to protect photo-sensitive fluorochromes from bleaching whilst staining). In between each step of the protocol, coverslips were gently washed at least 3 times in PBS for no more than a few seconds. After the final step, an additional wash was carried out using ddH_2O and coverslips were mounted cell-side down on frosted glass slides using Vectashield mounting media (Vector Laboratories LTD, Peterborough, UK). To label the nuclei, Vectashield containing DAPI (*4',6-diamidino-2-phenylindole*, a blue fluorescent stain that binds DNA) was used, however, this stain had to be excluded when a secondary antibody bound to a blue fluorochrome was used during triple labelling of cells (eg. AlexaFluor 350; absorption 346 nm, emission

442 nm). Slides were stored in closed boxes (again, to avoid bleaching) and stored at 4 ^{o}C. Microscopy was carried out within 7-10 days of staining to ensure that the slides had not faded.

2.2.1 Using Cell Surface Markers for Immunocytochemistry

When probing for an external or cell surface antigen, the staining was carried out live (pre-fixation) and primary antibodies were diluted in the cells own media and added to each coverslip at a volume of 50 µl for 20 min at room temperature. Following the washing steps, secondary antibodies also diluted in media were added for a further 20 min under the same conditions. Fixation was then performed using 4% paraformaldehyde and coverslips were washed and then mounted as previously described.

2.2.2 Using Internal Markers for Immunocytochemistry

For internal antigens, cells were initially fixed using 4% paraformaldehyde for 15 min at room temperature and then permeabilized with Triton X (0.1% Triton-X-100 in PBS) for a further 15 min. It was also possible to fix and permeabilize in one step by adding ice cold methanol to the cells for 20 min at -20^{o}C, however, since methanol can damage the epitopes of some antigens, this method of fixation/permeabilization was only used when labelling for GFAP. Cells were then blocked with blocking buffer (0.2% gelatine in PBS) for 15 min at room temperature to minimise non-specific binding of the antibody, before the addition of primary antibody diluted in blocking buffer for 1 hr at room temperature. Following the wash steps, the isotype-specific secondary antibody, also diluted in blocking buffer, was added for 45 min at room temperature. Cells were then washed and mounted as previously described.

2.2.3 Using Cell Surface and Internal Markers

When simultaneously labelling cells using cell surface *and* internal markers (eg. p75^{NTR}, external; SMI-31 and PLP, internal), external markers were labelled first, as described in section 2.2.1. The external primary antibody was added in media

for 20 min at room temperature, followed by the addition of the corresponding secondary antibody for 20 min immediately after washing. Fixation, permeabilization and blocking steps were carried out as described in section 2.2.2 and then staining for internal markers proceeded as previously described. Coverslips were mounted in the usual way, with or without DAPI depending upon the presence of a blue fluorescently conjugated secondary antibody.

Antibody name	Antigen	Cellular Location	Isotype	Dilution	Source
AA3	Proteolipid protein (PLP) and it's isoform DM20	intracellular	Rat IgG	1:100	hybridoma
SMI-31	Phosphorylated neurofilament	Intracellular	Mouse IgG1	1:1500	Abcam
Anti-GFAP	Intermediate filament, glial fibrillary acidic protein	Intracellular	Rabbit polyclonal	1:500	Dako
Anti-$p75^{NTR}$	Low affinity neurotrophin receptor for nerve growth factor	Cell surface	Mouse IgG1 and rabbit polyclonal	1:1, 1:100	Hybridoma, Abcam
Anti-BrdU	Bromodeoxyuridine; binds DNA during S-phase of cell division labelling proliferating cells	Intracellular	Mouse IgG1	1:20	DAKO
Anti-NG2	NG2, extracellular matrix molecule belonging to the CSPG family	Cell surface	Rabbit polyclonal	1:100	Millipore
O4	Oligodendrocyte 4,cell-surface sulfatides and seminolipids	Cell surface	Mouse IgM	1:1	Hybridoma
Anti-MBP	Myelin basic protein (MBP)	intracellular	Mouse IgG2A	1:200	Gift from Prof. Chris Linington

Table 2.1 – *Primary antibody list*. Table 2.1 illustrates the list of primary antibodies which were used for immunofluorescent labelling of primary cells for work carried out during this thesis, along with details of the antigen that they detect, their isotype and working dilutions.

Isotype	Conjugated fluorochrome	Dilution	Source
Anti-mouse IgG1	555 (red) or 350 (blue)	1:600	Molecular Probes (AlexaFluor)
Anti-rat IgG	488 (green)	1:600	Molecular Probes (AlexaFluor)
Anti-rabbit Ig	488 (green) or 555 (red)	1:600	Molecular Probes (AlexaFluor)
Anti-mouse IgM	488 (green) or (555) red	1:600	Molecular Probes (AlexaFluor)
Anti-mouse IgG2A	Fluorescein isothyocyanate (FITC, green) or Tetramethyl rhodamine Isothyocyanate (TRITC, red)	1:100	Southern Biotechnologies

Table 2.2 – *Secondary antibody list*. Table 2.2 lists all of the fluorescently-conjugated secondary antibodies used for immunolabelling studies carried out for this thesis, as well as their working dilutions.

2.3 Quantification of Fluorescent Images

Cells were imaged using an Olympus BX51 epifluorescent microscope and Image-Pro software. For each condition, 2 coverslips were used and 10 images from each coverslip were taken by merging each colour channel to form a composite image, which could be saved as a TIFF file. When imaging myelinating cultures, the DAPI channel was not included so as not to interfere with the myelin quantification (section 2.3.2) Images could then be reopened in Image J (NIH systems, version 1.45) or Adobe Photoshop Elements 7.0 for further analysis to determine neurite density, the % of myelinated axons or to calculate cell numbers and their expression of markers of interest.

2.3.1 Calculating Neurite Density

For myelinating cultures, the SMI-31 positive neurites were generally fluorescently labelled with TRITC-conjugated (red) secondary antibodies, whilst the PLP-positive myelin sheaths and oligodendrocytes were labelled with FITC-conjugated (green) secondary antibodies. In the instances where axons had to be labelled with a blue secondary antibody, each channel was captured on the microscope and then tinted using Image Pro software to ensure that neurites were red and PLP+ cells/sheaths were green. For quantitative analysis of neurite density, images were taken randomly to avoid bias using a 10x objective. Neurite density was then calculated by opening each image in Image J and splitting the channels (red and green). The threshold was then worked out for the red channel only to ensure that the amount of SMI-31 reactivity wasn't skewed by over-exposure of the image. Using the histogram function, the software then produced two figures: the number of black pixels (representing neurite coverage) and the number of white pixels (background). To confirm that the programme was running effectively, a simple calculation could be performed to ensure that the number of black pixels + white pixels always equalled 1447680 (total pixels).

The black pixel value was then expressed as a percentage of the total pixel value to give a value denoted as **% neurite density** using Microsoft Excel to perform the calculations:

(Black pixels ÷ total pixels) x 100 = % neurite density

2.3.2 Quantification of the % of Myelinated Axons

Adobe Photoshop Elements 7.0 was used to edit images of myelinating cultures by manually drawing over myelin sheaths using a pure blue (Blue 255, Red 60 and Green 0) with a brush size 9 paint tool. Since the AA3 antibody that recognises PLP also recognises its isoform DM20, which can be expressed by OPCs, staining with this antibody also resulted in the labelling of immature and mature oligodendrocytes, as well as myelin sheaths. Thus, care had to be taken to ensure that only sheaths (and not cell bodies) were highlighted in blue to ensure accurate quantification of myelin. Whilst this method is arguably subjective, since analysis is performed by the same person throughout, it can be assumed that any inconsistencies will be averaged out across all conditions. Once the myelin has been manually drawn on all images, 3-4 representative images from each condition were opened in Image J and the average threshold for the green channel across the images was noted. This measure was taken to ensure, as with calculating neurite density, that the information shown in the green channel was neither over nor under-exposed. A custom made macro, which recognises the pixel value of where the blue (manually drawn sheaths) overlaps with the green channel (PLP staining), was then run on all images for each condition. These values were exported directly into Microsoft Excel, where they were expressed as a percentage of the total black pixel value (SMI-31 reactivity; see section 2.3.1) to give a value denoted as **% myelinated axons.**

(Blue overlying green pixels ÷ Black pixels) x 100 = % myelinated axons

2.3.3 Quantification of Cell Number

As with all experiments, cell counts were performed on duplicate coverslips, taking 10 images from each for analysis. OPCs were cultured as described in section 2.1.5 for approx 5-7 days in Sato + PDGF and FGF before treatment began. After this period, some cells were kept in growth factors, whilst others were switched to Sato alone or Sato + SCM/ CTGF. Staining was then carried out 1 and 5 days after treatment, using a antibody to detect NG2 (a chondroitin sulphate proteoglycan expressed on the surface of OPCs, see Table 2.1) and the O4 antibody (recognises cell surface sulphatides and seminolipids in immature and mature oligodendrocytes). Based upon the number of cells expressing each of these markers, coupled with a brief descriptive of their morphology, some assessment could be made regarding the level of cell differentiation under each condition. Using the same markers, the population of OPCs/oligodendrocytes in myelinating cultures could also be assessed at D12 and D18. Images were taken using a x40 objective and opened in Image J. To give a value for the total cell number, DAPI nuclei were counted and totalled using the Image J cell counter. The number of cells which did not express oligodendrocyte markers (contaminating astrocytes or microglia in OPC preps or astrocytes, neurons or microglia in myelinating cultures) was then subtracted from this value to give an approximation of the total DAPI count for the OPC/ oligodendrocyte population

Each marker was calculated as a % by expressing the number of cells which labelled for the antigen of interest over the OPC/oligodendrocyte DAPI count to give % of cells expressing as a proportion of that population.

(Cells expressing ÷ DAPI count) x 100 = % of cells expressing

2.3.4 Assessing Cell Proliferation

To quantify proliferation, cultures were treated with bromodeoxyuridine (5-bromo-2-deoxyuridine; BrdU), which is an analogue of thymidine. BrdU is used as a marker of cell proliferation due to the fact that it becomes incorporated into the DNA during the S-phase of division. Briefly, astrocytes were cultured onto glass coverslips or PCL scaffolds in 10% FBS, at a cell density of 100,000 cells/ 100 µl for 7 days. After 6 days *in vitro*, BrdU (10 µM) was added into the media and left for approximately 16 hr.

Cultures were then fixed using ice-cold methanol, which also permeabilized the cells. The cells were incubated at -20˚C for 10 min, washed in PBS with Tween (PBST 0.05%), and then further fixed using 0.2% paraformaldehyde for 1 min at room temperature. After washing in PBST 0.5%, 0.07 M sodium hydroxide was added to the cells for 10 min at room temperature. The cells were then washed again as before, and an antibody to BrdU (mouse IgG1, 1:20) was added for 45 min at room temperature. Following this incubation, the cells were washed once again in PBST, and the relevant secondary antibody was added (anti-mouse IgG1) for 30 min at room temperature. The coverslips were mounted in Vectashield with DAPI, as previously described. The number of BrdU $^{+ve}$ nuclei were then counted per image, and 20 images per condition were quantified. Proliferation was given as the number of BrdU $^{+ve}$ cells dived by the total GFAP $^{+ve}$ population, and expressed as a %.

2.4 Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

RNA was extracted using a commercial kit from Qiagen (RNeasy Mini Kit, #74104; Qiagen, United Kingdom) and converted to cDNA using a reverse transcription kit from Primer Design (# RT-nanoscript). Triplicates of each sample were used and the reaction was prepared in a 96 well plate. Mastermix containing SYBR Green®, a synthetic fluorescent dye that binds non-covalently to double stranded DNA emitting light (522 nm), was used to quantify mRNA expression (#Precision-SY; Primer Design, UK). By comparing the fluorescence emission between sample groups, the relative amount of DNA could then be quantified. The reaction was carried out using a 7900HT Fast Real-Time PCR System with SDS 2.3 software (Applied Bioscience, UK). The software was set up to the conditions of the plate using a specific programme of thermocycling as illustrated by Table 2.3, whilst the housekeeping gene GAPDH was used as a reference gene.

	Temperature (˚C)	Time (mm:ss)
Step 1	95	10:00
Step 2 (x40 cycles)	95	00:15
	60	01:00
Step 3	95	00:15
	60	00:15
Step 4 (dissociation)	95	00:15

Table 2.3 - *RT-qPCR methodology*. A 96 well reaction plate was run through a 7900 HT Fast Real-Time PCR System using a programme with 4 distinct stages of thermocycling at temperatures ranging from 60 – 95ºC to amplify the PCR product.

A “cycle threshold” (CT value) is the number of cycles required for the fluorescence to cross the threshold level (background level) and was given as the quantitative measurement. The higher the CT value, the more cycles were required to cross this threshold, indicating that there was a lower amount of the product there initially. An average of the three triplicate wells for each sample was taken and any outliers whereby the CT values differed by more than one cycle were removed. Values of 35 cycles or above were considered to represent little or no mRNA expression.

CT values were expressed relative to a housekeeping gene (glyceraldehye-3-phosphate dehydrogenase, GAPDH) using the comparative CT method (Lival & Shmittgen., 2001) to describe data in terms of a relative quantification of fold change (RQ value), using the following equations:

$$\Delta CT = CT \text{ gene of interest} - CT \text{ internal control (GAPDH)}$$

$$\Delta\Delta CT = \Delta CT - CT \text{ gene of interest value in control sample}$$

$$\underline{RQ \text{ value} = 2^{(-\Delta\Delta CT)}}$$

Forward and reverse primers for connec0tive tissue growth factor (CTGF/CCN2), glial fibrillary acidic protein (GFAP), bone morphogenic protein 4 (BMP4), transforming growth factor ß (TGF-ß) and interleukin beta 1 (IL-ß1) were bought from Primer Design and validated for use in rat cultures.

2.5 Enzyme Linked Immunosorbent Assay (ELISA)

A classic "sandwich" ELISA was used for this study (Figure 2.6), whereby the antigen of interest was specifically immobilised on a 96 well plate, having been bound by a "capture" antibody, which had been allowed to coat the plate for 24 hrs followed by 1-2 hr of a blocking agent and then the addition of the supernatant to be tested. A detection antibody, specific to the antigen of interest, was added to the plate and an isotype-specific antibody conjugated with horseradish peroxidase (HRP), followed by substrate solution was used to initialise the enzymatic reaction required to produce a colour change. For each experiment, a standard curve was produced using known concentrations of the antigen of interest to give a plot depicting the mean absorbance of each standard on the y axis against its known concentration on the x axis (Figure 2.7).

A spectrophotometer (Dynex Technologies MRX 2.02) using Revelation software (version 4.25) was used to read each plate by quantifying the amount of transmitted light from each well following the enzymatic colour change and converting this value to a concentration in pg/ml, based upon the absorbance for each of the known standards. A reliable standard curve was considered to be one where the O.D value did not exceed 0.2 for the "0" standard or 1.2 for the highest standard concentration. The plate was read for absorption at 405 nm, with a correction set at 650 nm.

For all ELISA experiments, media was collected from confluent flasks of OECs, SCs and astrocytes and cells were counted from each flask so that data could be normalised to give a concentration per 100,000,000 cells. Supernatants were collected in the same media for all cell types within an experiment and this media was also tested on the ELISA plate as a control. To gain a more accurate insight into what may be being secreted by each cell type when present within a myelinating culture, supernatants were collected in DM (media used for myelinating cultures). However, it was suggested that the biotin present within the DM may affect the binding of the antibodies; therefore it was omitted for

these ELISA experiments. All antibodies and reagents were diluted according to the manufacturer's protocol.

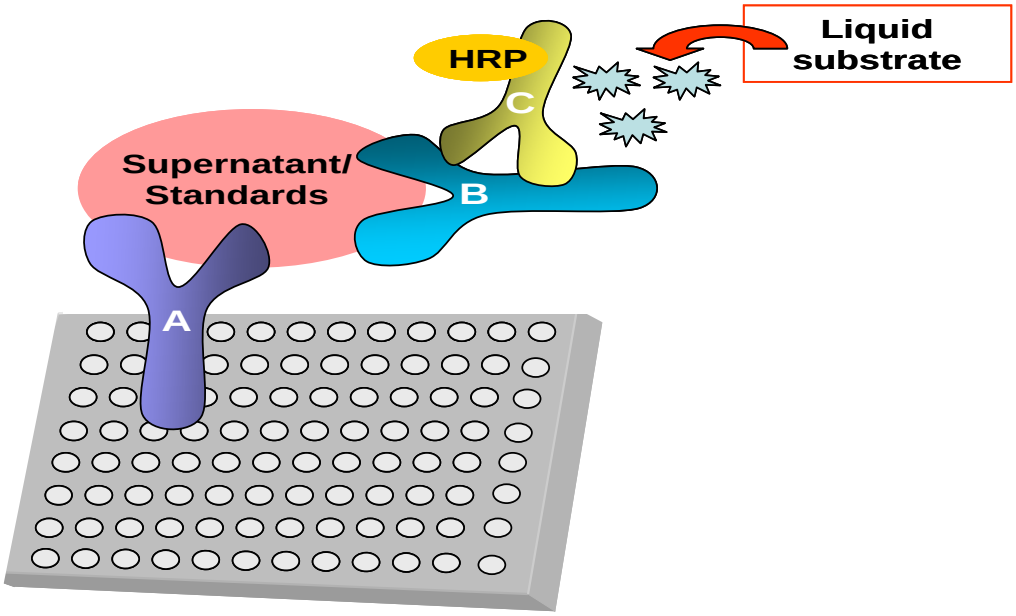


Figure 2.6 – *Enzyme-linked immunosorbent assay methodology.* A "sandwich" ELISA was used, whereby the antigen of interest was specifically immobilised to the plate by pre-coating each well with a capture antibody (A) for 24 hr. The plate was blocked to prevent non-specific binding of the antibodies and then the supernatant was allowed to bind for 1-2 hr at room temperature. Known concentrations of the antigen were also left to bind so that a standard curve could be produced. A specific detection antibody (B) then bound to the antigen and an isotype specific antibody (C) with a horseradish peroxidise (HRP) conjugate labelled the detection antibody. Finally, a liquid substrate was used to initialise an enzymatic colour change which could be quantified by a spectrophotometer. A darker colour indicated a higher concentration of the antigen being assayed.

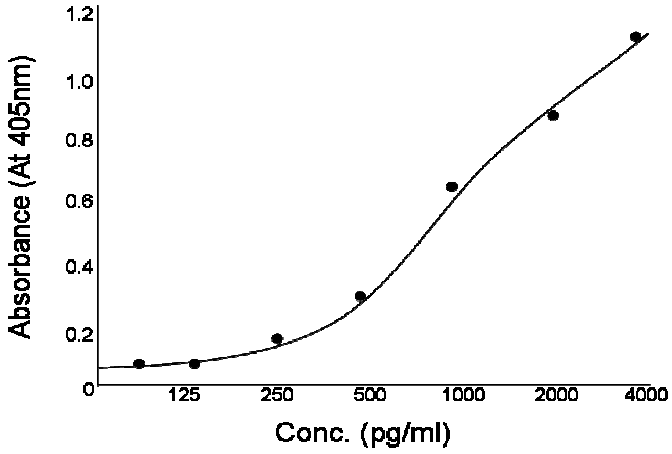


Figure 2.7 – *Typical standard curve produced for ELISA*. By assaying known concentrations of the antigen being tested, a standard curve could be produced, plotting each concentration against its average absorbance (as detected by the enzymatic colour change). The amount of antigen in each sample could then be calculated based upon the absorbance from each well.

2.5.1 Cilliary Neurotrophic Factor (CNTF) Expression by ELISA

To assess the levels of CNTF in SCs, OECs and astrocytes, an enzyme-linked immunosorbent assay (ELISA) kit (Rat CNTF DuoSet ELISA Kit, catalogue No: DY557) was purchased from RayBiotech, Inc. (Insight Biotechnology, Middlesex, UK). Briefly, a 96-well well plate was coated with capture antibody overnight at room temperature, washed with PBS-Tween 0.05% (PBST 0.05%) and then blocked in 1% BSA in PBS for a minimum of 1 hr at room temperature. After each incubation stage, the plate was washed a further 3 times in PBST 0.05%. The standards and samples were added to the wells in duplicate and left at room temperature for 2 hr. Following another wash, Streptavidin-HRP was added to each well and left for 20 min, during which time the plate was placed away from direct light to avoid photoreactivity. Finally, substrate solution (1:1 mixture of Colour Reagent A (H2O2) and Colour Reagent B Tetramethylbenzidine) was added and left for 20 min (R&D Systems Catalogue # DY999) away from direct light before the addition of Stop solution (2N H2SO4). The plate was then read immediately to determine the optical density of each well. The reader was set to 450 nm, with wavelength correction set 420 nm to 570 nm. Readings from the

standards were used to create a standard curve, off which sample readings were read.

2.5.2 Connective Tissue Growth Factor (CTGF) Expression by ELISA

CTGF protein levels were assessed as above by using an ELISA kit from Peprotech (Peprotech EC. Ltd, London, UK; catalogue no: 900-K317) and following the manufacturer's protocol. Unlike the aforementioned ELISA kit, the kit for CTGF did not provide a "Stop" solution. However, the plate was read after 20 min without further readings since we were not looking at the kinetics of CTGF expression.

2.6 Western Blot Analysis

To look at protein expression in astrocytes, monolayers were washed 3 times with PBS and then lysed using CelLytic M Cell Lysis Reagent (Sigma, Dorset, UK) for 15 min at room temperature. The cells were then scraped off the coverslip and spun down to remove debris. The concentration of total protein for each sample was measured using the Nanodrop (Invitrogen, Paisley, UK) so that equal concentrations of protein (5-10 µg) could be loaded for each condition into a NuPage 4-12% Bis-Tris Gel (Invitrogen, Paisley, UK) alongside rainbow molecular-weight markers (Amersham International, Little Chalfont, UK). The gel was run for 45 min in Running Buffer (Invitrogen), with a constant voltage of 200 V. The gel was transferred using the iBlot system (Invitrogen, Paisley UK) and then placed into PBST (0.1%) containing 5% dried milk (Marvel) for at least 2 hr at room temperature. An antibody against GFAP (polyclonal rabbit; see Table 2.1) was diluted in the blocking agent and added to the membrane for 1 hr.

After this period, the membrane was washed with PBST three times for 15 min and then incubated with an anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody (anti rabbit, 1:10000, Santa Cruz;) for 1 hr at room temperature (also diluted in 5% powdered milk in PBST). Following a further 3

washes, the membrane was developed using an enhanced chemiluminescence kit (ECL plus, Amersham Biosciences) and visualised using a Konica Minolta SRX101A imaging system (Tokyo, Japan) and Kodak photo paper in a dark room. Optical densitometry was carried out using Image J, which attributed a pixel value to each band. Expression was then given as a ratio of the densitometry of each band relative to the densitometry of the GAPDH loading control for each condition.

2.7 Preparation of Biomaterials

All scaffolds were plasma etched after fabrication (Harrick Plasma Cleaner; Harrick Plasma, USA) using a Hi setting (740V DC, 40 mA DC, 29.6W) for 5 min to aid sterilisation and to reduce the hydrophobicity of the substrate in order to enhance cell attachment, following an initial period of fabrication where little or no cells survived/remained attached. After plasma etching, the scaffolds were immediately placed into ethanol to avoid contamination before being rinsed thoroughly in ddH_20 in a sterile tissue culture hood and then treated with 13.3 µg/ml PLL, as described in section 2.1. Experiments on biomaterials were cultured under the aforementioned methodology and conditions, unless otherwise stated.

2.7.1 Fabrication of the Poly-ε-caprolactone (PCL) Micropatterned Scaffold

To explore the ways in which spatial parameters such as groove width or depth affected the survival and overall biology of glial cells, PCL scaffolds were prepared by initially washing high molecular weight PCL pellets (MW: 90,000; Sigma, UK) with methanol on a shaker platform, before placing them in a 400-well grid so that each bead was equidistant from its neighbour. The grid was removed without displacing any of the beads, which were then fixed in place between two glass plates held by clips, and placed in an oven at 70 ^oC for 1 hr followed by an overnight cooling period. A small area of approx 1.5 cm^2 was then cut out from the PCL sheet and placed on top of a glass slide on a hotplate at a temperature of 70 ^oC for 3-4 s. Once the PCL had started to soften, a quartz

template containing a micropattern was placed on top with a small amount of even pressure. The molten scaffold was then removed from the hotplate and allowed to cool thoroughly before the quartz template was lifted carefully, thus preserving the integrity of the micropattern, and removed from the glass slide. Each quartz template was pre-embossed with a topography of 5 defined areas of grooves and ridges at widths of 5-100 μm at a constant depth of either 5 or 10 μm (Figure 2.8 b, 2.8 c).

2.7.2 Fabrication of Scaffolds in a Range of Biomaterials

In order to assess which biomaterial was optimal for enabling the survival and differentiation of the myelinating cultures, a range of potential substrates were tested, namely: low molecular weight PCL (MW 45,000; Sigma, UK), Polycarbonate (PCB, Bayer Makrolon OD2015), Polystyrene (PS, Proprietary grade), Poly-L-lactic acid (PLLA, Ingeo^{TM} Biopolymer D3001, Nature Works LLC), Poly(methyl) methacrylate (PMMA; Evonik Degussa Plexiglas® 6N). Each of these was dissolved in chloroform to give a resultant polymer concentration 10% (w/v), which was then fabricated into a membrane overlying a glass coverslip by spin coating.

Briefly, glass coverslips (13 mm diameter) were placed in a spin coater and 150 μl of polymer solution was applied on top before spinning at 2000 rpm for 15 s, with 200 rpm/s acceleration and 200 rpm/s deceleration (Figure 2.8 a). Each membrane was allowed to air-dry on top of the coverslip for approx. 1 min before being taken directly to the plasma cleaner to be treated as previously outlined. Polydimethylsiloxane (PDMS; Dow Corning, Hochheim, Germany) was prepared by mixing Sylgard 184 at a ratio 10:1 of base and curing agent, degassed for 20 min and cast against a fluorinated silicon wafer to achieve a flat substrate.

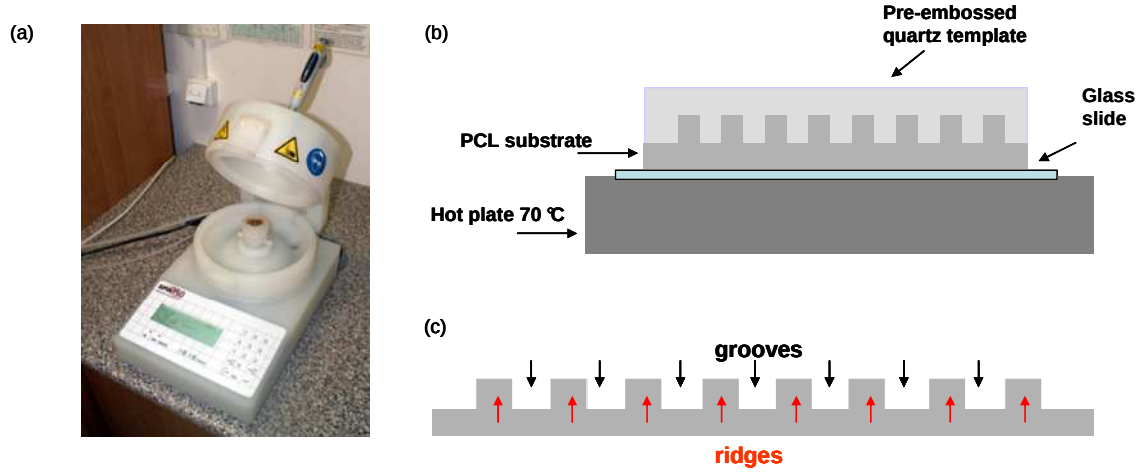


Figure 2.8 – *Manufacturing of biomaterials*. Biodegradable membranes in a range of biomaterials were spun onto glass coverslips using a spin-coater (a). To investigate groove depth and width, scaffolds were hot embossed using a hot plate and a pre-embossed quartz template (b) in order to produce a finished product which contained a micropattern of grooves and ridges of defined dimensions, ranging from 5-100 µm in width, at a constant depth of either 5 µm or 10 µm (c).

2.8 Statistical Analysis

For comparison of values between groups of conditions, data was analysed using paired Student's T-test in Microsoft Excel, with 1 as the null hypothesis of the mean. All values were expressed as means ± the standard error of the mean (SEM). Significance was represented using p-values where values below 0.05 were considered significant and were indicated by the presence of an asterisk. Two asterisks indicated results which were termed "highly significant", since the p value was less than 0.01. For myelinating cultures, the percentage of myelinated neurites was expressed as a ratio of the control to give myelin arbitrary units, where the control value was always given as 1. Statistical analysis was only performed when a minimum of 3 biological replicates (n=3) was carried out. The term "preliminary" refers to data whereby less than 3 biological replicates were carried out; therefore no statistical tests were applied. The number of replicates (n) of an experiment is indicated in each figure legend throughout this thesis.

Results Chapter 3:

Validating the Use of an *In Vitro* System for Studying Myelination and Glial Cell Behaviour

3.1 Introduction

Given that glial cells are known to be implicated in the pathology of almost all diseases and injuries of the CNS, a thorough and comprehensive understanding of their complex interactions is imperative for the development of therapeutic strategies to promote repair. For example, the formation of an astrocytic glial scar has been associated with areas of neuronal loss following trauma or disease progression in Parkinson's disease, amyotrophic lateral sclerosis, stroke and spinal cord injury, whereby the up-regulation of a host of pro-inflammatory cytokines and growth-inhibitory molecules prevents neurite outgrowth, whilst simultaneously containing the damaged area to prevent further cellular loss (described in section 1.4.1.1.) (Wu & Raine, 1992; Rudge et al., 1990; Daginakatte et al., 2008).

Interestingly, some of what we now know about the mechanisms of glial scar formation has been derived from *in vitro* studies, designed to recapitulate one or more dimensions of the damaged CNS. For example, monocultures of astrocytes were used to demonstrate that plating directly onto Aß peptide, the molecule associated with Alzheimer's pathology, induced a hypertrophic, reactive astrocyte phenotype, with up-regulated expression of chondroitin sulphate proteoglycans (CSPG), all of which are typical characteristics of the inhibitory scar *in vivo* (Canning et al., 1993). Furthermore, it is now well-documented through the use of co-cultures of glial cells that astrocytes form a boundary with Schwann cells (Lakatos et al., 2000) via complex mechanisms involving heparin sulphates, FGF9 (Higginson et al., 2012), FGF2 (Santos-Silva et al., 2007) N-Cadherin (Fairless et al., 2005) and the ephrins (Afshari et al., 2010). This phenomenon is also mirrored *in vivo* given that Schwann cells are limited to the periphery by the presence of boundary cap cells at the dorsal root entry zones, and when transplanted into the damaged CNS, rarely integrate extensively with host astrocytes within the graft (Shields et al., 2000; Plant et al., 2001; Lakatos et al., 2003; Li et al., 2012).

More complicated still are the glial/neuronal interactions underlying myelination. Although these mechanisms are not yet fully understood, they

involve concise and timely oligo-axonal contact mediated via numerous signalling pathways, as well as the presence of cytokines and astrocyte-secreted factors, such as cilliary neurotrophic factor (CNTF), which are thought to be crucial in regulating oligodendrocyte survival and differentiation (Talbott et al., 2008; Cao et al., 2010; Nash et al., 2011). Gaining a more precise knowledge of the detailed series of molecular events governing myelination may lead to significant breakthroughs in understanding and overcoming the barrier to competent, long-term remyelination and repair of the diseased or damaged CNS. Given that such events require multi-faceted interplay between neuronal and glial cells, a system which accurately models all components of the CNS *in vitro* so that the behaviour of individual populations of cells could be accurately examined would prove invaluable in developing strategies to promote neurorepair.

One such system, originally developed in murine cultures (Thomson et al., 2006, 2008), makes use of enzymatically dissociated embryonic spinal cords to produce an assay containing a mixed population of neurons, spinal astrocytes, microglia and OPCs, which were plated onto PLL-coated glass coverslips (see section 2.1). Overtime, neurite outgrowth, OPC differentiation and myelination could be followed using immunocytochemistry and fluorescence microscopy for approximately 28 days in culture.

This system was further developed for use in rat cultures (Sørenson et al., 2008), which was highly beneficial given that the majority of *in vivo* models of spinal cord injury are conducted in rats due to the technical challenges faced when conducting the required surgeries for such experiments. However, the availability of transgenic mice has seen an increase in the numbers of mouse models of SCI in recent years (Jacob et al., 2001, 2003; Joshi et al., 2002; Mure et al., 2004; Plemel et al., 2008; Cho et al., 2012). Results from Sørenson and colleagues demonstrated that unlike murine cultures, the survival of cells grown on PLL alone was poor in cultures derived from embryonic rat spinal cord tissue. However, when these cultures were grown on a monolayer of neurosphere-derived astrocytes (Ns-As) cultures remained confluent and healthy for up to 28 days, whilst oligodendrocytes could also be observed wrapping axons in correctly formed myelin sheaths, suggesting the existence of crucial astrocyte-dependent,

contact-mediated mechanisms and/ or secreted factor(s). Conversely, plating directly onto an OEC or SC monolayer resulted in poor neurite density with little or no myelination. However, if the underlying OEC or SC monolayer was dense then neurite density was improved (Sørenson et al., 2008). Although endogenous oligodendrocytes could successfully extend processes on an OEC substrate, they failed to ensheath axons and to form compact myelin. Thus, the methodology was adapted to include the initial seeding of a supportive astrocyte monolayer grown to confluency over a period of approximately 7 days, prior to culturing the embryonic spinal cord directly on top (collectively denoted as "myelinating cultures"; see section 2.1.6).

Since the isolation of purified spinal cord-derived astrocytes is technically challenging, previous studies directly compared monolayers of cortical-derived astrocytes (Co-As) and Ns-As, with results suggesting that whilst there was no significant difference between the level of myelination observed on each, there was more variation on cultures grown on Co-As (Sørenson et al., 2008). This evidence, coupled with the knowledge that a high yield of confluent coverslips of Ns-As could be produced from a single flask of neurospheres (approx 96 coverslips), justifies their use in this system.

The results from Sørenson et al., (2008) may be somewhat unsurprising given that astrocytes have long been shown to influence cells within the oligodendroglial lineage, with astrocyte conditioned media (ACM) previously being utilised for its mitogenic properties on OPCs (Noble & Murray, 1984). More recently, using the myelinating culture system it was demonstrated that the astrocyte monolayer was a crucial source of CNTF, which is a known pro-myelinating cytokine (Nash et al., 2011), amongst other factors. Additional evidence from this study further emphasised the importance of the astrocyte monolayer in use with this myelinating culture system, given that manipulation of the monolayer using reagents to induce astrocyte quiescence and reactivity (such as tenascin-C and CNTF) significantly altered the levels of myelination observed.

Previous characterisation of this system demonstrated that endogenous oligodendrocytes were able to form myelin internodes and nodes of Ranvier, with correctly assembled Na_V channels, neurofascin and axonal contactin-associated protein (Caspr) at the nodes, thus suggesting that the myelinating culture system presents itself as an appropriate system to accurately study myelination. Furthermore, not only can this system be used to study glial/axonal relationships under normal conditions, it has also been used as the basis for modelling the role of autoantibodies in demyelinating disease (Elliot et al., 2012) and to demonstrate the effects of pharmaceutical reagents in their ability to enhance neurite outgrowth and myelination following spinal cord injury (SCI) *in vitro* (Boomkamp et al., 2012). In this manner, the role of any given molecule/ endogenous or exogenously added cell can be studied in a high throughput, low cost manner, which is not always possible *in vivo* without initial data to focus subsequent studies upon.

3.1.1 Aims

The aim of this chapter was to demonstrate the presence of the typical features of myelin formation in the cultures, which have been reported and published, including nodal proteins and a range of oligodendrocyte/ myelin markers. The reproducibility of the system was also explored, with some description given as to the amount of variability observed in the levels of myelination and neurite density in untreated cultures. Although there are endogenous spinal astrocytes present within the myelinating culture system, it may at times be advantageous to work in a system with fewer numbers of astrocytes (ie. no monolayer) so that the exact mechanisms of any factors which influence myelination could be dissected, to some extent, away from astrocyte-dependent effects. Thus, the role of increased cell density was examined as a means of enhancing the survival and differentiation of cultures grown in the absence of an astrocyte monolayer.

3.2 The Myelinating Cultures Express Several Myelin Proteins, with Correctly Formed Internodes and Nodes of Ranvier

As described in section 3.1 and in the materials & methods chapter (section 2.1.6), the myelinating cultures used for this thesis were derived by enzymatically dissociating embryonic tissue from the spinal cords of E15.5 rat pups to produce a culture consisting of several classes of neurons, spinal astrocytes, microglia and OPCs. This cell suspension was then plated down at a density of 150,000 cells/ 100 µl onto a confluent monolayer of neurosphere-derived astrocytes. After approx 22-28 days in culture, a carpet of neurites, along with oligodendrocytes and myelin sheaths, could be visualised using immunofluorescence.

As oligodendrocytes mature, they alter their expression of several proteins in a temporal fashion, such that the appearance of a particular marker can be indicative of the maturation stage of a given cell, though many of these markers are transient, spanning one or more defined stages of differentiation (Figure 3.1). Initial experiments showed the presence of oligodendrocyte 4 (O4) both in the myelin sheath (Figure 3.2 a) and in the cell body of highly branched, non-myelinating oligodendrocytes (Figure 3.2 b). Similarly, both proteolipid protein (PLP) and myelin basic protein (MBP) were highly expressed in the myelin sheaths, with some staining observed in oligodendrocyte cell bodies. This cell body expression was more abundant when using antibodies against PLP since the splice variant encoded by the PLP gene, DM20, is recognised by the same antibody and is commonly expressed in the early differentiation stages of oligodendrocyte maturation. It is hypothesised that as maturation progresses, the ratio of DM20/PLP expression shifts so that PLP becomes the more dominantly expressed protein of the two, present in both pre-myelinating and myelinating oligodendrocytes (Levine et al., 1990).

Further proof that the expression of these myelin markers is indicative of correctly formed myelin internodes is given in Figure 3.3, which depicts both the presence of nodes of Ranvier (3.3 a) and the expression of Caspr positioned at

the paranode (3.3 b). Correct assembly of nodal structures in the appropriate, highly specialised regions of the axon is vital for maintaining axonal integrity and enabling rapid Saltatory conduction. In the unmyelinated fibre, Caspr expression is said to be diffuse and highly distributed throughout the length of the axon and its expression down regulated and redistributed firstly to the juxtaparanode and then to the paranode as myelination commences (Einheber et al., 1997). Thus, the appearance of paranodal Caspr in these cultures would suggest the presence of correctly formed myelin sheaths.

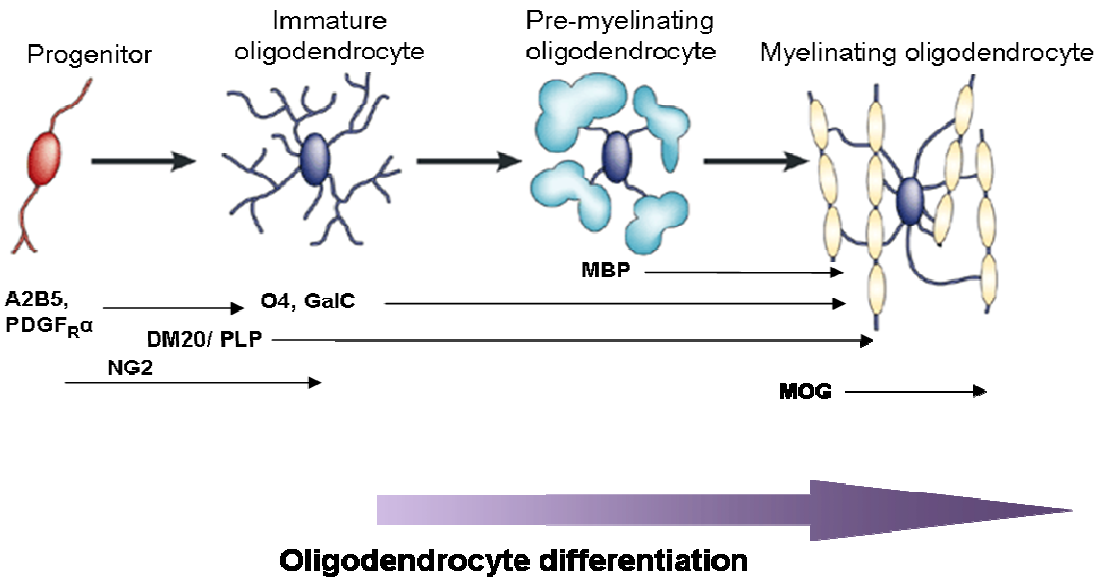


Figure 3.1 – *The maturation stages of oligodendrocyte differentiation, as depicted by their expression of various markers.* The stages of oligodendrocyte maturation can be identified by morphological changes within the cell, ranging from a polarised phenotype into a complex multi-process/ sheath bearing cell, as well as antigenically by using markers which are typically associated with a particular stage of differentiation. Classically, markers such as A2B5 and platelet derived growth factor receptor alpha (PDGF$_{R}\alpha$) are associated with the very earliest oligodendrocyte precursor cells, whilst markers such as proteolipid protein (PLP), myelin basic protein (MBP) and myelin oligodendrocyte glycoprotein (MOG) are used to depict myelinating cells and myelin sheaths. It's important to note, however, that the expression of many of these protein markers is transient, given that the intermediate marker O4 can also be found in mature oligodendrocytes and in the myelin sheath, whilst PLP and its isoform DM20 are also present in non-myelinating and immature oligodendrocytes. (Modified from Zhang (2001) Nature Reviews Neuroscience. **2** 840-843).

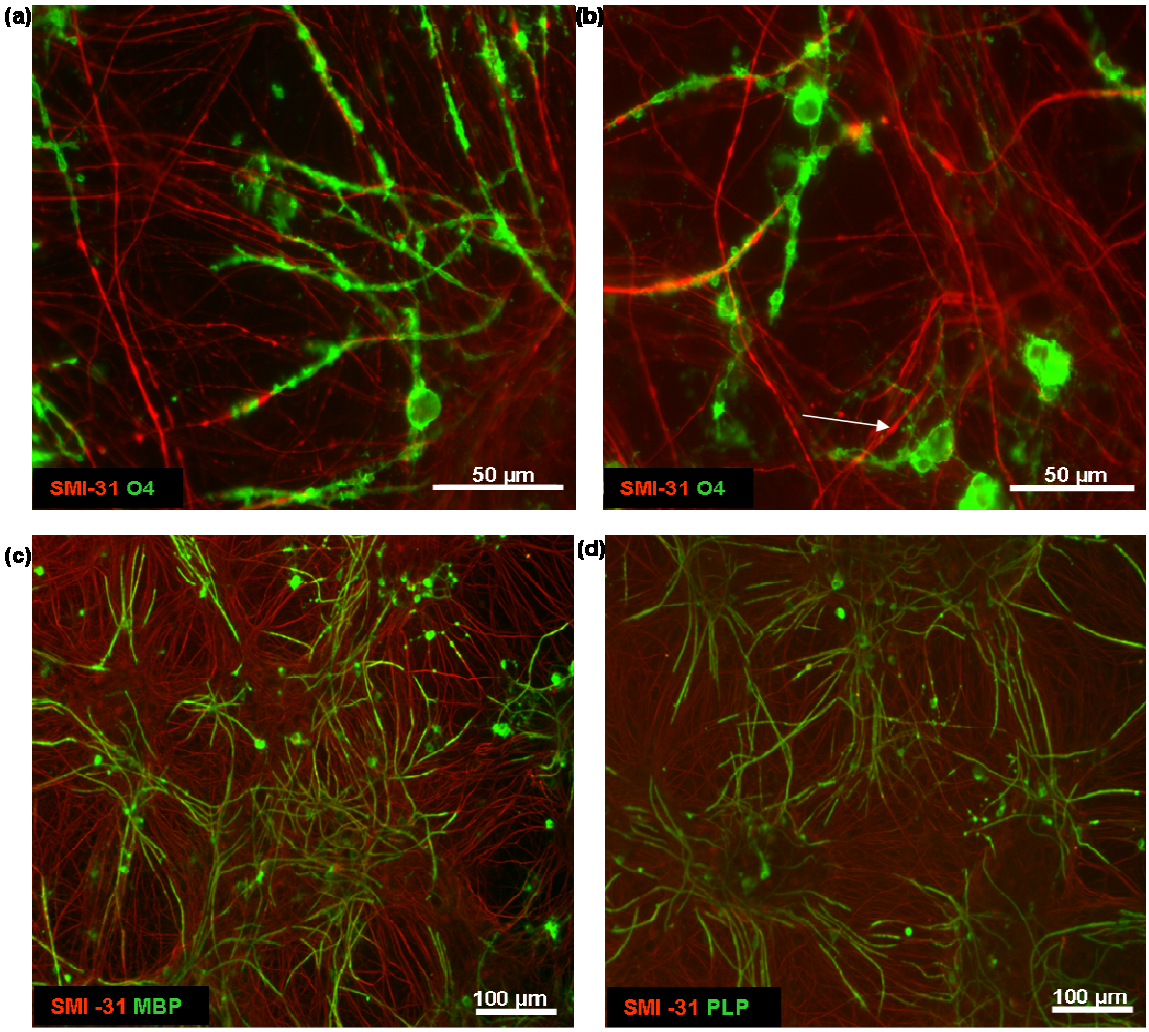


Figure 3.2 –*The myelinating cultures demonstrate both intermediate and late markers of oligodendrocyte maturation and myelin formation*. Embryonic rat spinal cord tissue was enzymatically dissociated to produce a culture of mixed neurons, spinal astrocytes, microglia and OPCs, which was plated directly on top of a supportive astrocyte monolayer (collectively termed "myelinating cultures"). Over a period of up to 28 days, neurite outgrowth and density could be quantified, along with the % of myelinated axons using immunofluorescence and Image J to calculate pixel values for both the red and green channels. By the end stage of culture, oligodendrocyte 4 (O4) antibody was detected in the myelin sheaths (a) as well as in highly branched oligodendrocyte cell bodies (b; white arrow). Evidence of early O4+ ensheathment and initial axonal contact was also observed in the cultures using O4 as a marker (b). Both myelin basic protein (MBP) and proteolipid protein (PLP) were evidenced in abundance in the myelin sheaths, and to some extent, in oligodendrocyte cell bodies. Images were taken using an Olympus BX51 epifluorescence microscope. Scale bars = 50 µm and 100 µm. SMI-31 labelled phosphorylated neurofilament in neurites.

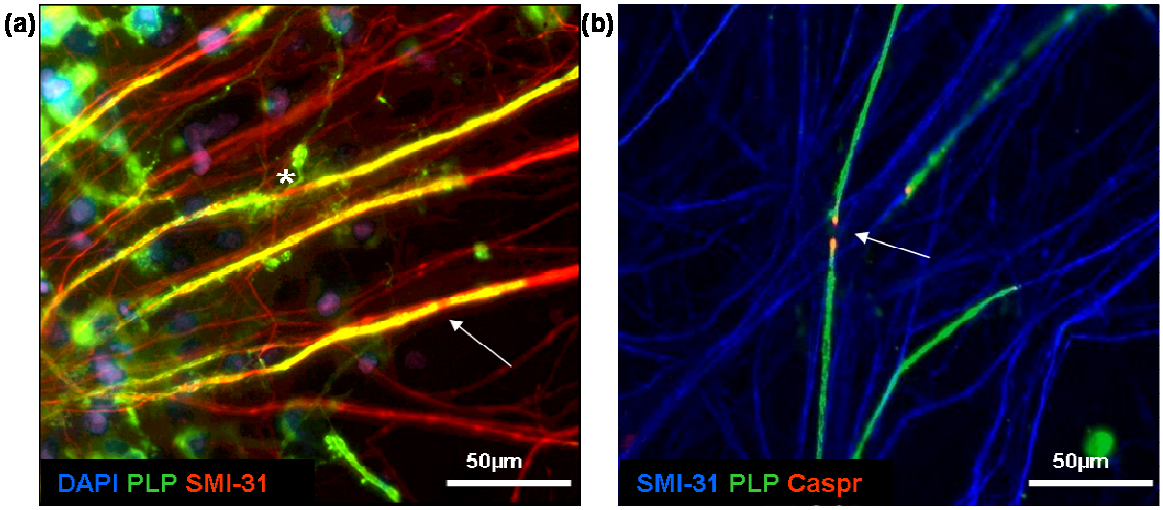


Figure 3.3 – *Nodes of Ranvier were present in the cultures, with correctly positioned paranodal Caspr.* Myelinating cultures were grown as previously described on an astrocyte monolayer for up to 28 days prior to immunofluorescent labelling. Nodes of Ranvier could be observed in cultures, in between myelin internodes (a; white arrow), with the expression of axonal contactin-associated protein (Caspr) present at the paranode, suggesting the presence of correctly formed myelin (b). The asterisk (a) also appears to highlight the initial stages of oligo/axonal contact in between two internodes of myelin. Images were captured with an Olympus BX51 epifluorescence microscope. Scale bars = 50 µm. SMI-31 labelled phosphorylated neurofilament in neurites, an antibody to PLP labelled oligodendrocytes and myelin sheaths, whilst an antibody to Caspr depicted the location of the paranodal regions of the Nodes of Ranvier.

3.3 Natural Variation Occurs in the Levels of Myelination and Neurite Density Observed in each Untreated Culture

To allow for multiple comparisons to be made at later stages across independent experiments, some thought was given as to how reproducible the myelinating cultures were, based upon the amount of variation there appeared to be in the levels of myelination and neurite density in each culture. Neurite density was calculated by immunostaining with SMI-31 followed by a TRITC-conjugated secondary antibody to label phosphorylated neurofilament and then using Image J to calculate the number of red pixels. This value was then expressed as a % over the total pixel number in each image; whist myelination was calculated by manually drawing over each myelin sheath using Adobe Photoshop. The number of pixels overlying the green myelin sheaths (by manually drawing) was then expressed over neurite density to give a % myelination (explained in more detail in section 2.3.1 and 2.3.2). The average values from 20 images were taken from 20 biological replicates to deduce the average of the average, the standard deviation (SD), standard error of the mean (SE) and the co-efficient of variance of the mean (CV).

Myelination between biological replicate was subject to some variability, with the lowest level of myelination being 2.78%, and the highest being 8.28% (Figure 3.4 c), as illustrated in Figure 3.4 a and 3.4 b, respectively. The average % myelination across all experiments was 4.47%. Since SD and SE tend to increase proportionally with the mean, the CV was also calculated to give a more accurate indication of the degree of variance as follows:

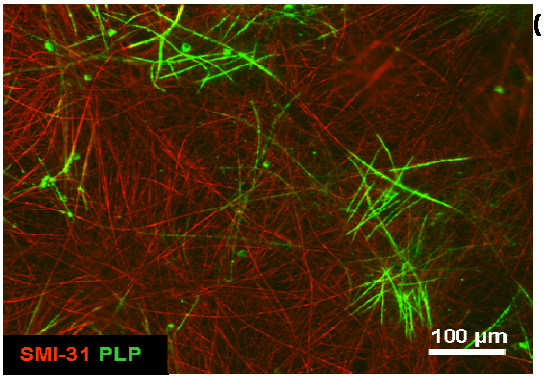
(SD/ average) x 100 = % CV

Whilst a CV value of 27.69% indicated some variation in the levels of myelination observed throughout the experiments (Figure 3.4 c), generally CV values of less than 30% can be considered to be indicative of biological reproducibility. Furthermore, throughout this thesis the trend between each experiment was

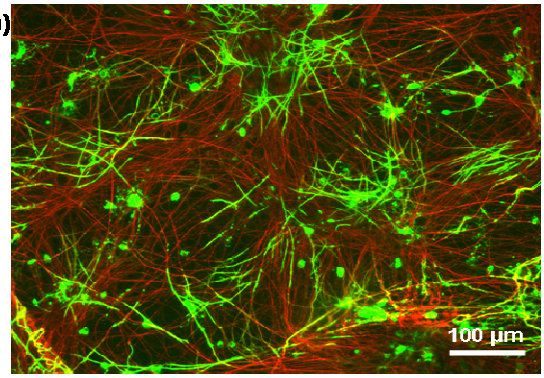
always the same in that if myelination was low, then the control was also low, however the relative changes between conditions was reproducible. Therefore, in order to make comparisons between experiments, myelination data was standardised so that the % of myelination in control cultures was denoted as 1 and each condition was represented as a proportion of 1, based upon their % of myelination. (eg. control of 4% and treatment at 2% becomes 1 and 0.5 in myelin arbitrary units, respectively).

Conversely, the level of neurite density observed across experiments was highly reproducible, as evidenced in the CV value of 5.26%. Typical values for neurite density ranged from 69.69–80.97%, with an average value of 75.76% (figure 3.4 c).

(a)



(b)



(c)

	Lowest to highest values	Standard deviation	Standard error of the mean	Co-efficient of variance of the mean	Average value
% Neurite density	69.69 - 80.87%	3.98	0.89±	5.26%	75.76%
% Myelinated axons	2.78 - 8.28%	1.24	0.28 ±	27.69%	4.47%

Figure 3.4 – *Analysis of multiple biological replicates indicates the level of variation throughout the myelinating culture system.* The average of the average level of myelination and neurite density was calculated from 20 biological replicates of untreated myelinating cultures grown on an astrocyte monolayer. Whilst the CV value for myelination was much higher than that for neurite density (c), this value was considered to be within the confines of reproducibility (<30%). Typical myelin values ranged from 2.78–8.28%, as illustrated in (a) and (b), respectively. Images were taken using an Olympus bx 51 epifluorescence microscope. Scale bars = 100 µm. SMI-31 labelled phoshorylated neurofilament in neurites, whilst an antibody against PLP labelled myelin sheaths and oligodendrocytes. n=20.

3.4 Preliminary Results Suggest that Increasing the Initial Seeding Density Enhanced Survival, though these Results were not Reproducible

Previous data from Sørenson et al., (2008) indicated that the survival of dissociated cells from embryonic rat spinal cord tissue was poor when plating onto PLL alone in the absence of an astrocyte monolayer. By varying the initial seeding density from 150,000 cells/ 100 µl to 100,000, 200,000 and 300,000 cells/ 100 µl, it was hypothesised that we may be able to improve the overall survival of the cultures without an astrocyte monolayer, perhaps by improving the likelihood of initial attachment. Since these cultures are a tool primarily for the investigation of myelination, it was hoped that a comparable level of ensheathment could be achieved by enhancing the survivability of the culture, negating the requirement for the presence of an astrocyte monolayer.

Although there are many contact-mediated mechanisms and secreted factors which mediate oligodendrocyte behaviour, it has also been demonstrated that oligodendrocyte differentiation can be governed, to some extent, by intrinsic, temporal mechanisms in the absence of axonal influences (Abney et al., 1981; Raff et al., 1985). Furthermore, Rosenberg at al., (2008) demonstrated that the micro-environment in which oligodendrocytes were cultured could enhance their maturation in that a more dense culture resulted in enhanced differentiation of oligodendrocytes. The use of micro-beads bound to the surface of fixed axons to mimic a dense culture was sufficient in enhancing differentiation and myelin production in that particular study, concluding that geometrical constraints also appeared to play a role. Thus, it was hypothesised that increasing the initial cell density may serve to reproduce a dense enough micro-environment to stimulate oligodendrocyte differentiation in the absence of the astrocyte monolayer.

Phase microscopy of the cultures grown without an astrocyte monolayer illustrated an apparent increase in neuronal density and the appearance of fasiculations with increased seeding density at day 9 (Figure 3.5 a-d). Whilst the maximum neurite density for cell densities of 100,000, 150,000 and 300,000 cells/ 100 µl was reached at day 18, it continued to increase until around day 23

when 200,000 cells/ 100 µl were initially seeded (Figure 3.5 e-h). Although there were virtually no neurites left after 28 days in cultures when using 100,000 or 150,000 cells on PLL alone, a neurite density of around 60% was observed at this time-point in cultures which were initially seeded with 200,00 cells/ 100 µl, whilst the seeding of 300,000 cells correlated with a neurite density of approximately 30% (Figure 3.5 i).

Similarly, whilst there was no myelin observed in cultures seeded with 100,000, 150,000 or 300,000 cells/ 100 µl, myelination was observed in cultures with an initial plating density of 200,000 cells (Figure 3.6 d). However, when compared to control cultures of 150,000 cells/ 100 µl grown on an astrocyte monolayer (Figure 3.6 a), both the level of myelination and neurite density appeared to be greatly reduced (Figure 3.6 c and d, respectively), though statistical significance could not be deduced on an n=2, given that repeats of this experiment were not reproducible.

Interestingly, in higher density cultures where no axonal myelination was evidenced and neurite density was relatively low, PLP $^{+ve}$ oligodendrocytes were observed depositing flat sheets of myelin, thus suggesting their ability to differentiate in the absence of at least some axonal cues (Figure 3.7 a and b). In other areas, isolated clusters of confluent spinal astrocytes remained, though these were not in sufficient abundance to form a dense monolayer of astrocytes devoid of any gaps (Figure 3.7 c).

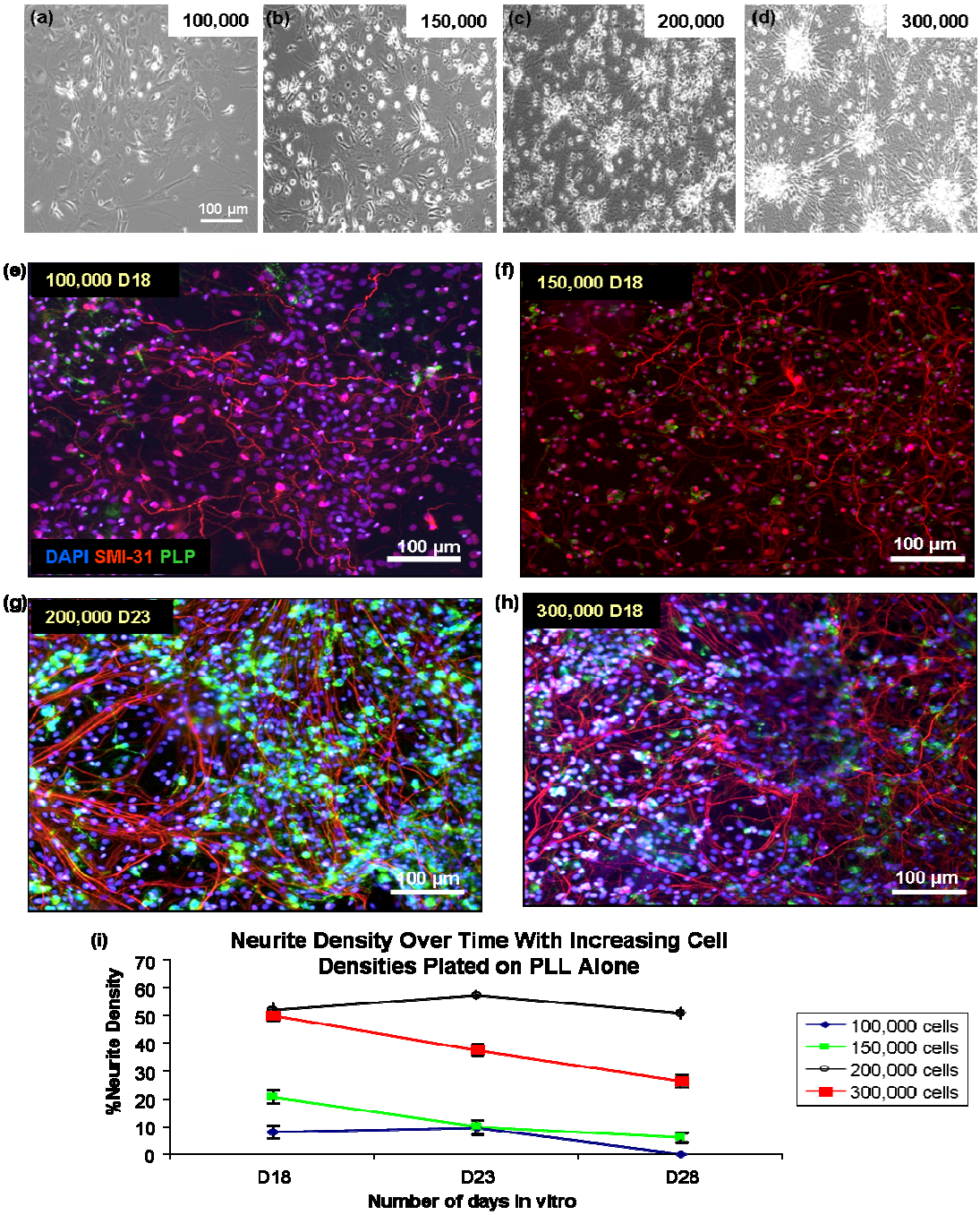


Figure 3.5 - *Neurite density was greatest in the absence of an astrocyte monolayer when an initial seeding density of 200,000 cells/ 100 µl was used.* Embryonic rat dissociated spinal cord cells were plated at a range of densities onto PLL coated glass coverslips in the absence of an astrocyte monolayer. Staining was carried out on days 18, 23 and 28 to assess levels of neurite density. Initial phase/ contrast images demonstrated an increase in neurite density with increased seeding density from as early as day 9 (a-d). Whilst maximum neurite density was reached at day 18 for all other cultures (e,f,h), it continued to increase until day 23 when 200,000 cells/ 100 µl were initially plated down (g). After 28 days in culture, neurite density was approximately 60% at 200,000 cells, though there were relatively little or no neurites present in lower density cultures. (i). Both phase/ contrast and fluorescence images were taken on an Olympus BX51 epifluorescence microscope. Scale bars=100 µm. n=2. SMI-31 labelled phosphorylated neurofilament in neurites, whilst an antibody against PLP labelled myelin and oligodendrocytes.

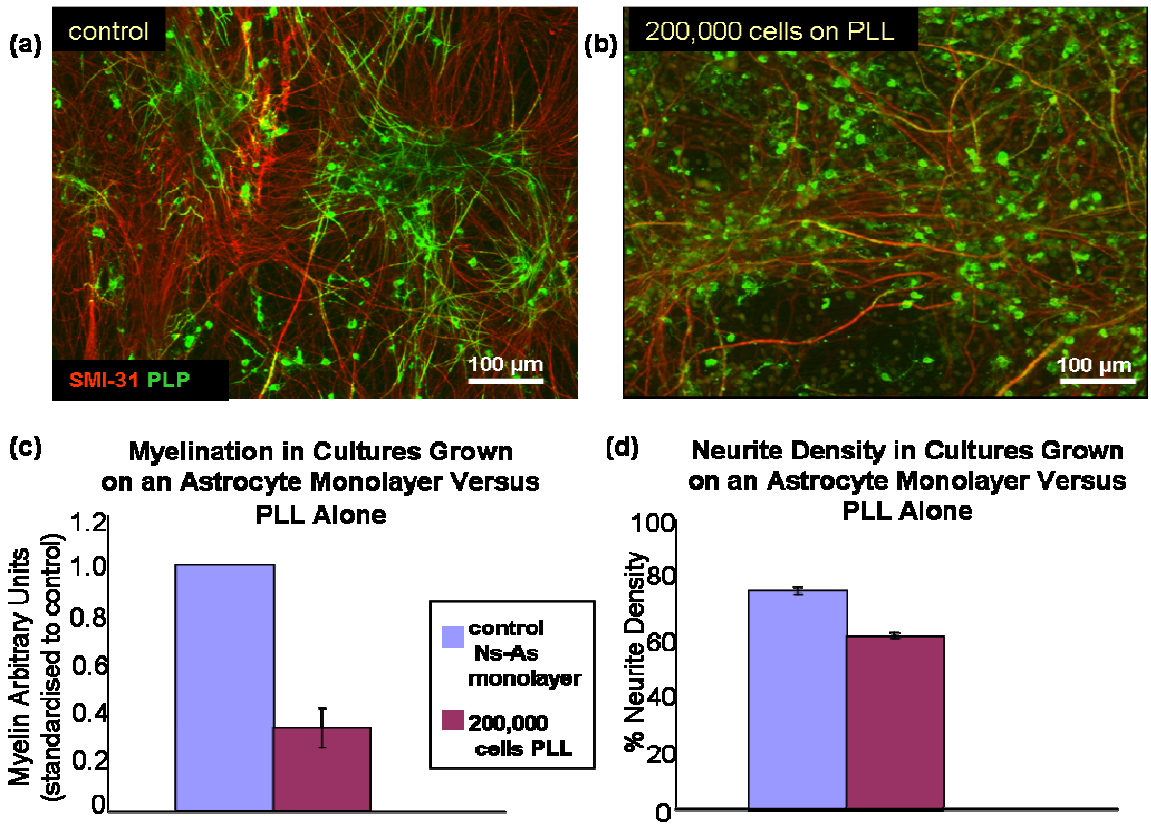


Figure 3.6 - *Myelination appeared to be greatly reduced in cultures grown in the absence of an astrocyte monolayer*. Embryonic rat dissociated spinal cord cells were plated at a density of 200,000 cells/ 100 µl in the absence of an astrocyte monolayer. Comparisons were made to controls seeded at an initial density of 150,000 cells/ 100 µl on an astrocyte monolayer. Though a small amount of myelin was present in cultures plated directly onto PLL at a density of 200,000 cells/ 100 µl (b,c), this was at least 4 times less than the amount of myelin observed in control cultures (a,c). Neurite density also appeared to be reduced in these cultures (d). Images were taken on an Olympus BX51 epifluorescence microscope. Scale bars = 100 µm. SMI-31 labelled phosphorylated neurofilament in neurites, whilst an antibody against PLP labelled myelin sheaths and oligodendrocytes. n=2.

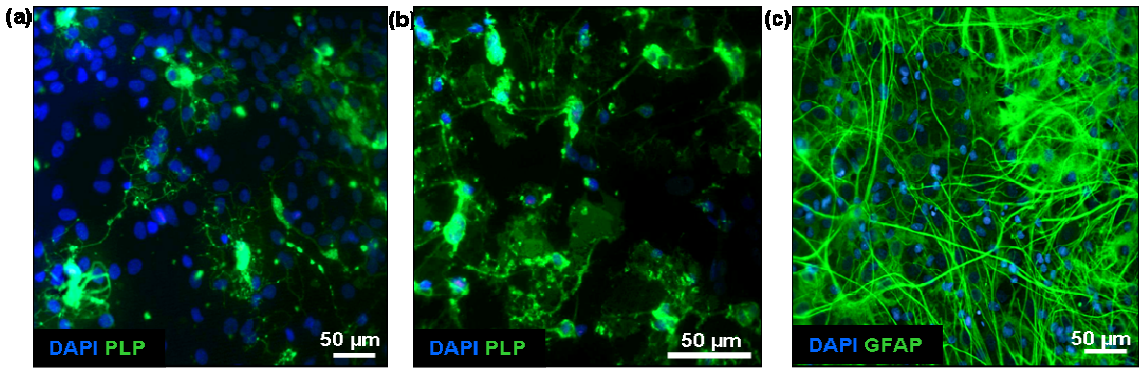


Figure 3.7 – *Astrocytes and myelin-forming oligodendrocytes remained after 28 days in vitro in spinal cord cultures initially seeded without an astrocyte monolayer*. Embryonic rat dissociated spinal cord cells were plated at 300,000 cells/ 100 µl onto PLL coated glass coverslips in the absence of an astrocyte monolayer and immunolabelling was carried out after 28 days *in vitro*. Although neurite density was greatly reduced and no myelin sheaths were observed under these conditions, surviving oligodendrocytes produced flat sheets of PLP+ve myelin membrane (a, b). Areas of dense spinal astrocytes were also present (c), though these were isolated and not confluent enough to create an intact monolayer. Images were taken using an Olympus BX51 epifluorescence microscope. Scale bars = 50 µm. Antibodies against glial acidic fibrillary protein (GFAP) were used to label astrocyte intermediate filaments, whist myelin and oligodendrocytes were stained with an antibody against PLP. DAPI labelled nuclei. n=2.

3.5 Discussion

Although the myelinating culture system generated from rat dissociated spinal cord cells is a published method (Thomson et al., 2006, 2008; Sørenson et al., 2008, Nash et al., 2011, Boomkamp et al., 2012; Elliot et al., 2012), this chapter has justified that the techniques used throughout this thesis are indicative of an *in vitro* system which correctly represents multiple features of oligodendrocyte differentiation and myelin formation.

The use of antibodies against myelin proteins such as PLP and MBP can be used to label myelin sheaths and oligodendrocyte cell bodies, whilst the O4 antibody labelled multi-process bearing oligodendrocytes at the end stage of culture, as well as myelin sheaths (Figure 3.2). Furthermore, the presence of nodes of Ranvier and Caspr at the paranodes suggested that the myelin observed was not an artefact of *in vitro* culture (Figure 3.3). Demyelination studies have demonstrated aggregates of Caspr at the juxtaparanode immediately prior to its redistribution to the paranode with the onset of remyelination, suggesting its importance in facilitating essential oligo-axonal adhesions, which are required for maintaining myelin formation and compact ensheathment (Wolswijk et al., 2003; Coman et al., 2006). Thus, these findings further illustrate the significance of demonstrating the presence of Caspr, with the correct paranodal location, within the myelinating cultures. Furthermore, previous electron microscopy studies reported that the myelination observed in these cultures was compact (Thomson et al., 2008).

Statistical analysis of the myelinating culture system based upon findings from 20 independent biological replicates, suggested that they are biologically reproducible, though there appears to be more natural variance in the levels of myelination observed across experiments, compared to neurite density (CV = 27.69% versus 5.26%, respectively) (Figure 3.4). The reasons for this biological variability could be multiple; for example, natural variation in the abundance and availability of endogenous OPCs between different spinal cord dissections could be one plausible explanation. To minimise these effects, a strict protocol

was followed for each dissection, ensuring that at least 4-5 mm of each spinal cord (including the cervical plexus, which is rich in OPCs) was taken from the same number of cords each time. The bioavailability of secreted pro-myelinating factors may also be a contributing factor in the fluctuating levels of myelination observed. Indeed, the health and condition of the underlying astrocyte monolayer is highly influential on culture survival and myelination and may also vary from prep to prep. Ensuring that confluent, age-matched monolayers were used for each experiment should minimise this influence to some extent.

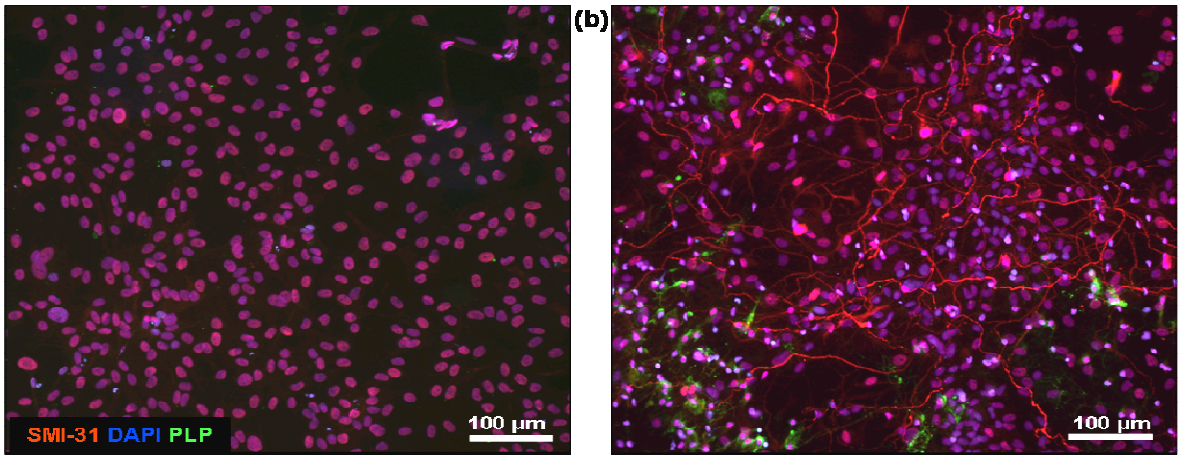
Experiments designed to try to negate the need for an astrocyte monolayer, so that any effects on myelination which were non-astrocyte dependent could be studied in more detail, further demonstrated its importance. Though previous studies had suggested that the survival of the myelinating cultures on PLL alone was poor (Sørenson et al., 2008), it was hypothesised in light of the literature that cell-intrinsic mechanisms for differentiation and geometrical constraints may also play a role in oligodendrocyte maturation. Therefore, it was hypothesised that by increasing the initial seeding density and plating directly onto PLL, that the survival of the culture could be improved, allowing myelination to proceed as normal. Preliminary results suggested that whilst survival was limited in almost all other conditions, when seeding at a starting density of 200,000 cells/ 100 µl neurite density remained around 60% (Figure 3.5; n=2), which was an improvement from previous reports of little or no survival in the absence of a monolayer of astrocytes (Sørenson et al., 2008). However, neurite density, along with myelination, appeared to be considerably lower under these conditions when compared with control cultures, where 150,000 cells/ 100 µl were plated onto an astrocyte monolayer (Figure 3.6, n=2). Unfortunately, data from these experiments was not reproducible, thus modification of the myelinating culture system in this way does not appear to be a viable option. Nonetheless, the presence of flat, myelin sheets in higher density cultures with reduced neurite density suggested that certain mechanisms underlying myelin formation may be governed by oligodendrocyte cell-autologous means, and perhaps to some extent, by the confines of the microenvironment in our system (Figure 3.7).

When utilising the myelinating cultures, there are a few technical caveats which require consideration. Firstly, though labelling with the SMI-31 antibody appears to specifically label phosphorylated neurofilament in neurites in control cultures, it appears to co-localise with DAPI in low density cultures. This staining pattern is unlikely to be true given that there seems to be an almost 100% nuclear staining with SMI-31 under these conditions. To offset this, *only* in cultures which were plated without an astrocyte monolayer, the level of SMI-31 reactivity (red pixels per image) was calculated for an image containing no neurites and given to be 8.08% (Figure 3.8). This value was then deducted from all images for those particular experiments to prevent erroneously high values for neurite density due to the presence of non-specific SMI-31 reactivity.

It is advantageous to use immunocytochemistry to quantify changes within the myelinating culture assay, given that it allows the user to visualise individual cells, cell contacts and myelin sheath formation; as opposed to Western blot analysis or PCR studies where only the expression of total proteins and transcripts are considered. With the latter techniques, the presence of late myelin markers may be misleading when assessing myelination given that several of these markers (PLP, MBP, O4) are present in abundance throughout oligodendrocyte cell bodies even in the absence of ensheathment. However, care must be taken when manually analysing data following immunolabelling to ensure that cell bodies and/or cell processes are not included as myelin (since antibodies to PLP and other myelin markers label both cell bodies and sheaths) (Figure 3.9). Though human error will occur, it is hoped that high repetition will lessen the likelihood of this skewing data. Furthermore, since data is standardised to myelin arbitrary units, the emphasis is upon relative *change*s in the amount of myelinated axons, as opposed to a definitive value of the % of myelinated axons per condition.

Taken together, these data highlight the usefulness of the myelinating cultures as an optimised system for modelling and studying myelination and glial development *in vitro*. Given these findings, these cultures have been used for all subsequent experiments carried out during this thesis to assess the ways in which myelination can be influenced by exogenous glial cells. Furthermore, it

was hoped that their use may help to dissect out the precise mechanisms underpinning the ways in which myelination can be influenced, with the view to providing useful data to the field of cell transplant-mediated repair of the damaged CNS



(c) **(Black pixels ÷ total pixels) x 100 = red pixels**

(117039 ÷ 1447680) x 100 = 8.08% SMI-31 reactivity

Figure 3.8 - *Non-specific SMI-31 reactivity can be offset with a simple calculation.* Rat embryonic spinal cord cells were plated onto PLL-coated glass coverslips in the absence of an astrocyte monolayer for up to 28 days prior to immunolabelling. In cultures with few axons, SMI-31 reactivity can be highly non-specific (a,b). By working out the average SMI-31 reactivity using the same calculation as is used for calculating neurite density, a value of 8.08% was given and could be deducted from all subsequent images where neurite density was low in the absence of the astrocyte monolayer (c). SMI-31 was used to label neurites, whilst an antibody to PLP labelled oligodendrocytes and myelin and DAPI labelled nuclei. Scale bars = 100 µm.

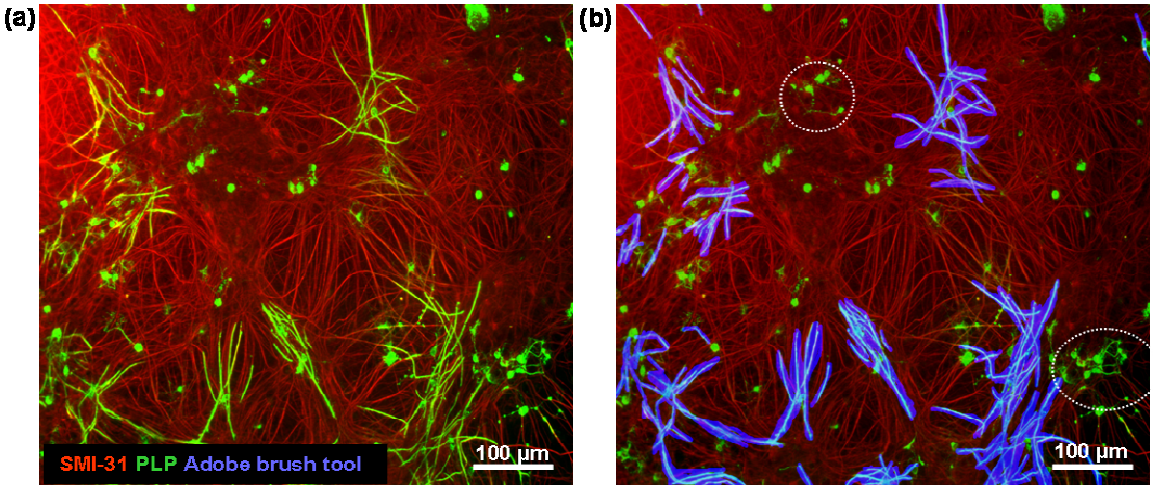


Figure 3.9 - *Manual quantification of myelin should ensure that only myelin sheaths are drawn over, at the exclusion of processes and cell bodies.* Myelinating cultures were plated down onto an astrocyte monolayer on PLL-coated glass coverslips. After approximately 26 days, cultures were immunolabelled and analysed using Image J and Adobe Photoshop to manually draw over myelin sheaths using the brush tool (b; shown in blue). Since antibodies against PLP label myelin sheaths, mature and immature oligodendrocytes (b, dotted circles) and the DM20 splice variant, care had to be taken to ensure that only myelin sheaths were drawn over. Though this system requires a degree of subjectivism from the user, it was hoped that by maintaining this standard throughout that relative changes across conditions would still be easily observed. SMI-31 labelled phosphorylated neurofilament and an antibody to PLP labelled myelin sheaths and oligodendrocytes. Scale bar=100 µm.

Results Chapter 4:

Investigating the Effects of Exogenously Added Glial Cells on Endogenous Myelination by Oligodendrocytes *In Vitro*

4.1 Introduction

As described throughout this thesis, cell-mediated repair strategies have long been considered to be potentially beneficial in aiding functional recovery following damage to the CNS. Previous studies have perhaps focused more on the capacity of exogenously transplanted cells to promote neurite outgrowth and their ability to remyelinate demyelinated axons. However, very little research has been directed at understanding the ways in which cell transplantation into the CNS may affect the ability of *endogenous* oligodendrocytes to ensheath axons in central myelin.

Both SCs and OECs have demonstrated an ability to modulate repair in several *in vivo* models of spinal cord injury (SCI), with varying degrees of success. For example, Ramon-Cueto and colleagues initially reported the ability of what they termed "ensheathing glia" to promote the regeneration of dorsal root axons after transplantation of these cells at the dorsal root entry zones (Ramon-Cueto & Nieto-Sampedro., 1994; Navarro et al., 1999), though these findings have been contradicted to some extent by others, as discussed in Chapter 1.

More recent studies have expanded upon the early findings of Ramon-Cueto; such as the study by Verdú and colleagues (2003), who demonstrated through use of a photochemical lesion induced in adult rats that transplantation of adult olfactory bulb-derived OECs (OB-OECs) into the lesion site resulted in improved functional recovery. Furthermore, they reported reduced symptoms of pain, as assessed by behavioural testing, as well as an increase in the area of preserved spinal cord, thus suggesting the ability of OECs to exert neuroprotective effects following transplantation into the CNS. Similarly, transplants of SCs derived from sciatic nerves have been reported to promote axonal regeneration following transplantation into the damaged spinal cord (Kromer & Cornbrooks., 1985; Guénard et al., 1993; Harvey et al., 1994; Xu et al., 1995, 1997). For example, Pearse et al., (2007) reported that the transplantation of SCs into a thoracic contusion injury model produced moderate improvements in locomotor skills and hind limb co-ordination in injured rats (Pearse et al., 2007).

Although transplantation of neither OECs nor SCs has thus far managed to promote significant neurite outgrowth beyond the region of the glial scar, these previously reported small gains in functional recovery could be attributed to the ability of both SCs (Blakemore 1977; 1985; Brierly, 2001) and OECs (Franklin et al., 1998, Smith et al., 2001,2002) to remyelinate demyelinated CNS axons with peripheral myelin. Following ethidium bromide-induced demyelination of the dorsal columns, endogenous SCs were demonstrated to infiltrate the lesion site and remyelinate denuded axons with myelin protein zero (MPZ/ P0) positive peripheral myelin. Furthermore, both cell types were reported to induce a similar pattern of distribution of both potassium and sodium channels at the paranodal regions and nodes of Ranvier, respectively, as observed in intact axons which had been myelinated by oligodendrocytes (Black et al., 2006). Similar findings were also reported following transplantation of OB-OECs into areas of demyelination in the spinal cord, with specific nodal clustering of the sodium channel, $Na_V1.6$, which is the dominant class present at the nodes of Ranvier in endogenously myelinated CNS axons. In addition, the correct assembly of nodal components following OEC transplantation also coincided with an increased conduction velocity in remyelinated axons (Dombrowski et al., 2006; Sasaki et al., 2006).

Whilst these studies highlight the ability of both SCs and OECs to produce internodes of myelin with correctly assembled nodes of Ranvier in the CNS, it is unclear how subtle differences in the composition of peripheral versus central myelin may affect the long-term conductivity and integrity of peripherally-myelinated CNS axons. For example, the presence of P0, peripheral myelin proteins 1 and 22 (PMP1, PMP22) and the lack of the CNS myelin protein, myelin oligodendrocyte glycoprotein (MOG), in peripheral myelin may have consequences for CNS axons remyelinated by OECs or SCs.

When considering glial cell transplantation as a strategy to promote remyelination in demyelinating inflammatory diseases such as multiple sclerosis (MS), whereby auto-antibodies to CNS myelin surface proteins such as MOG are thought to play a role in pathogenesis (Elliot et al., 2012), a lack of such proteins in the myelin sheath may be beneficial in conferring additional neuroprotection to CNS axons undergoing immune-mediated demyelination.

However, as discussed in Chapter 1, a study by Pagany et al., (2003) also reported the presence of MOG mRNA throughout the periphery in rats and primates, hypothesised to be associated with SCs. Immunolabelling failed to detect MOG at the protein level in SCs *in vivo*, although its presence was demonstrated in the cytoplasm of non-myelinating SCs *in vitro*, but absent from the plasma membrane where it would be abundantly expressed by oligodendrocytes. This data therefore suggests that the expression of MOG may be differentially regulated in SCs and oligodendrocytes.

In the case of SCI, these differences in myelin protein composition, along with variations in the lipid content of myelin, such as the presence of the ganglioside LM1 found exclusively in peripheral myelin sheaths (Chou et al., 1985), may be important when considering cell-mediated therapies to induce repair, whereby remyelination is an important consideration for adequate functional recovery. It could therefore be suggested that the most efficacious repair strategy long-term would be one that not only enhanced neurite outgrowth but facilitated the role of endogenous oligodendrocytes to remyelinate CNS axons with characteristic central myelin.

Thus, it was hypothesised that by elucidating the ways in which both SCs and OECs may differ in their ability to affect the myelinating capability of endogenous oligodendrocytes, that those findings could be used to advocate the preferential use of one cell type over the other as being potentially more effective for cell-mediated repair of the CNS.

4.1.1 Aims

The aim of this chapter was to investigate the ways in which OECs and SCs affected the ability of endogenous oligodendrocytes present within our CNS culture system to myelinate axons. Furthermore, this chapter aimed to assess whether these influences, if present, were due to contact-dependent mechanisms or the presence of factors which were being secreted by either cell type.

4.2 Exogenous OECs Enhanced Endogenous Myelination *In Vitro*, Whilst SCs Reduced Oligodendrocyte Myelination

Initially, purified cell suspensions of OECs were trypsinised, spun down and resuspended in plating media to calculate cell densities. Either 5,000 or 10,000 OECs were then seeded onto the myelinating coverslips at day 12; a process which involved using forceps to lift coverslips containing the myelinating culture into a dry Petri-dish and seeding the OECs directly on top in a meniscus. After an incubation period of 1 hr at 37 °C to allow the OECs to attach to the coverslip, the myelinating cultures were flooded with media composed of 50% of their current media (containing any secreted factors) and 50% fresh media.

However, several repeats of this experiment using this methodology (n=4) demonstrated limited survival of the cultures after 26 days (Figure 4.1 a-c). In some instances where few axons remained there was evidence of OECs lining up alongside, and even ensheathing, axons (Figure 4.1 d, e), though there was no co-localisation with PLP. Thereafter, the protocol was modified so that purified cell suspensions of OECs or SCs were added into the mixed embryonic spinal cord cell suspension and plated directly on top of an astrocyte monolayer from day 0, as described in section 2.1.6. Control cultures did not receive any exogenously added cells.

After 26 days in culture, the level of myelination was significantly higher after the addition of 10,000 OECs (Figure 4.2 c, f) compared with control cultures (Figure 4.2 a, f) or myelinating cultures containing 5,000 OECs (Figure 4.2 b, f) (n=3, p values=0.04 and 0.03, respectively). Although there appeared to be a slight decrease in the level of myelination observed with the addition of 5,000 OECs compared with control, this trend was not significant (p value=0.29). Furthermore, the survival of OECs throughout the culture period was demonstrated by the presence of $p75^{NTR\ +ve}$ cell bodies (Figure 4.2 d, e), which were not observed in control cultures. However, the presence of $p75^{NTR}$ did not co-localise with PLP in the myelin sheaths. Neurite density was not affected by the exogenous addition of OECs.

Conversely the addition of SCs to the myelinating cultures reduced the level of myelination detected after 26 days in culture. Whilst myelination was not completely abolished under these conditions, the addition of 5,000 SCs or 10,000 SCs significantly reduced the level of myelination compared to controls (Figure 4.3 a, b, c, f) (n=3, p values <0.01). The % of myelinated fibres observed in cultures containing 10,000 exogenous SC versus cultures where 5,000 SCs were added was also significantly lower (p value=0.04). As was the case in cultures where OECs were added, SCs were still present at the end point of the myelinating cultures at a similar density to remaining OECs (Figure 4.3 d, e). Neurite density remained consistent for all conditions, at approximately 75%.

Higher cell densities of OECs and SCs were also tested (15,000 and 20,000 exogenously added cells) to investigate whether or not the effects of OECs or SCs on the culture system could be titrated out. However, these cultures showed limited survival, possibly due to over-confluency and subsequent depletion of vital growth factors within the culture media.

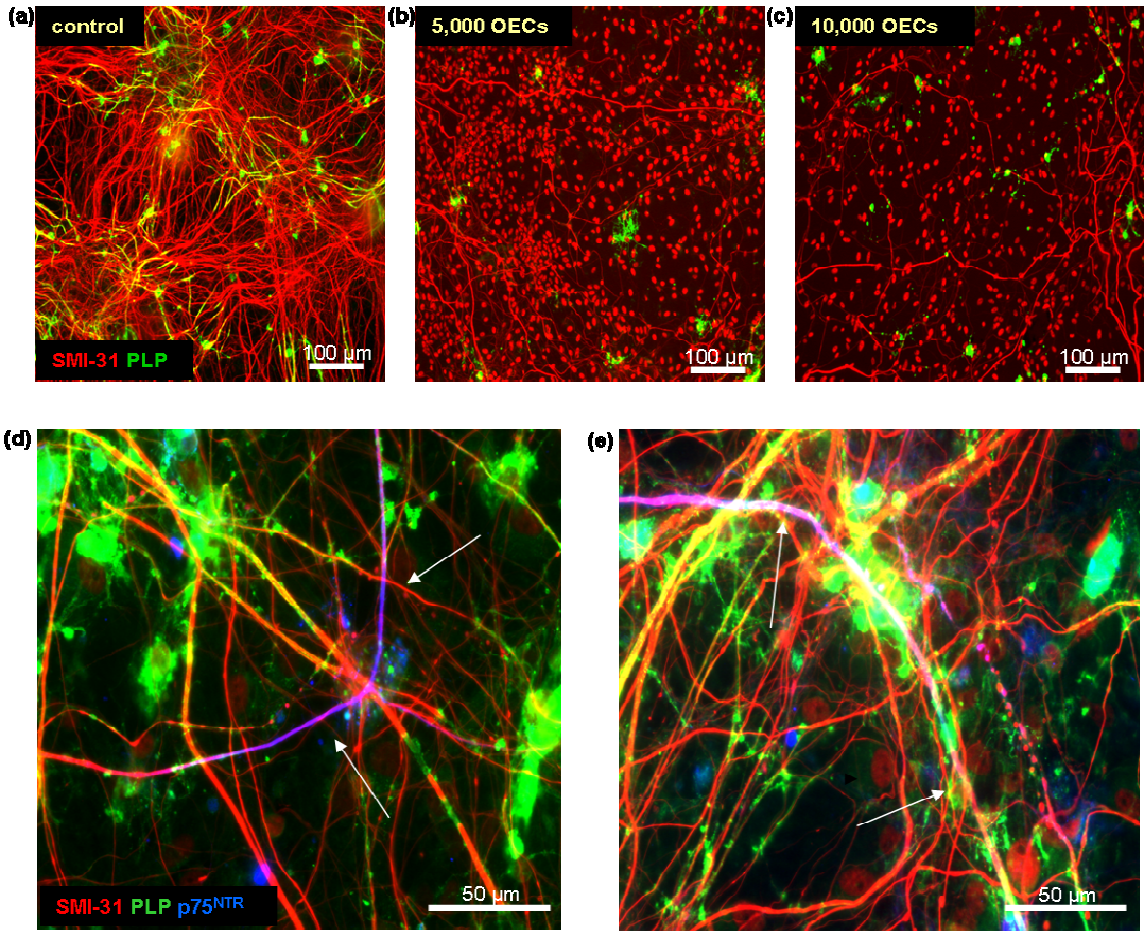


Figure 4.1 – *Seeding OECs onto established myelinating cultures from day 12 limited the survival of the cultures.* Purified OECs were seeded on top of established myelinating cultures on an astrocyte monolayer at day 12 by fishing out the coverslips of myelinating cultures and placing them into a dry Petri dish. Either 5,000 or 10,000 OECs were then seeded directly on top of the myelinating cultures in a miniscus. After 1 hr to enable attachment the cultures were fed and maintained for a further 14 days as usual, prior to immunolabelling. Mechanically fishing out coverslips in order to seed 5,000 or 10,000 OECs resulted in poor survival of the myelinating cultures (b, c). In some cultures where few fibres remained, however, OECs were observed extending along neurites (d, e; white arrows), thought they did not co-localise with PLP. Images were taken using an Olympus BX51 epifluorescence microscope. Scale bars=100 µm and 50 µm, respectively. An antibody to $p75^{NTR}$ labelled OECs, an antibody to PLP labelled myelin sheaths and oligodendrocytes and SMI-31 labelled phosphorylated neurofilament in neurites. n=4.

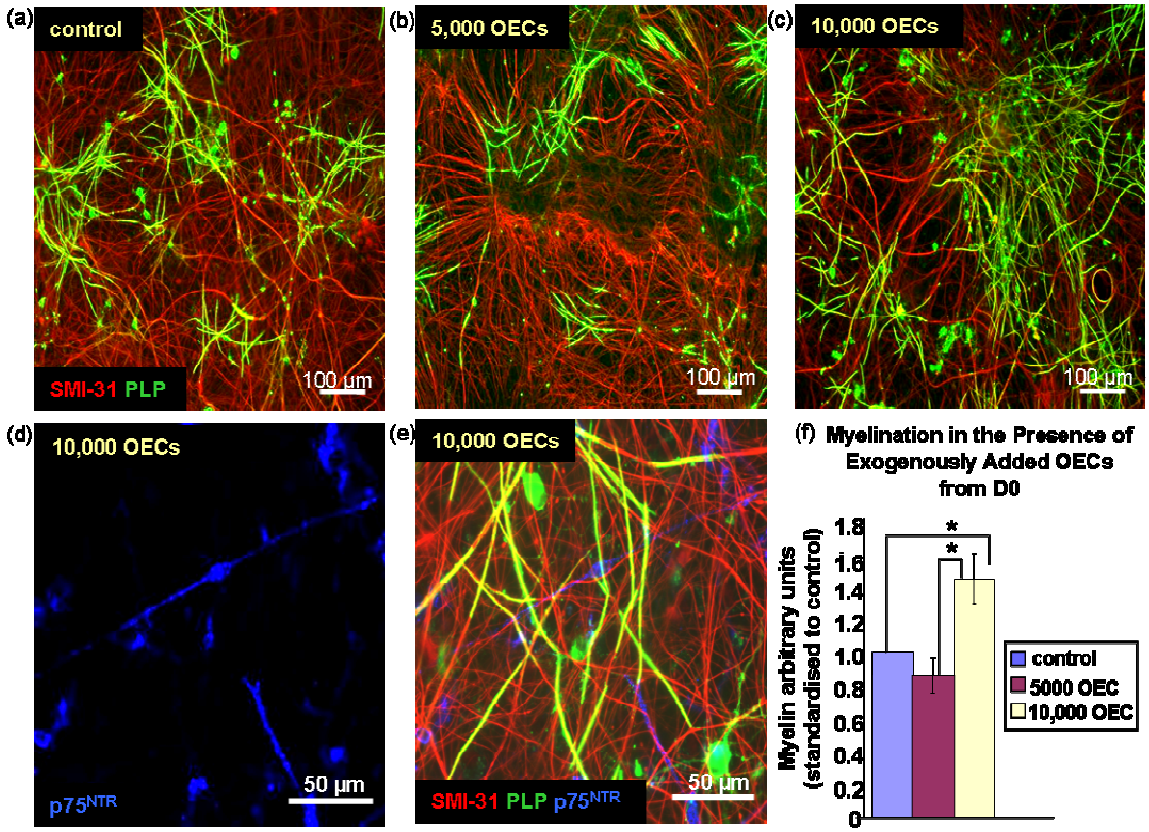


Figure 4.2 – *The addition of 10,000 OECs significantly enhanced the level of myelination in cultures.* 5,000 or 10,000 OECs were added to the mixed embryonic spinal cord cell suspension and plated down onto a confluent monolayer of neurosphere-derived astrocytes. After 26 days, the level of myelination in control cultures (a, f) or cultures where 5,000 OECs had been added (b, f) was significantly less than that of cultures containing 10,000 OECs (c, f). Whilst there appeared to be a slight reduction in the level of myelination between control cultures and cultures with 5,000 exogenous OECs, this difference was not significant. OECs were present at the end stage of culture (d, e) but did not appear to associate with the myelin sheaths. SMI-31 was used to label phosphorylated neurofilament in neurites, an antibody to PLP labelled oligodendrocytes and myelin sheaths and an antibody to $p75^{NTR}$ labelled OECs. Scale bars=100 µm and 50 µm. n=3.* = p values <0.05.

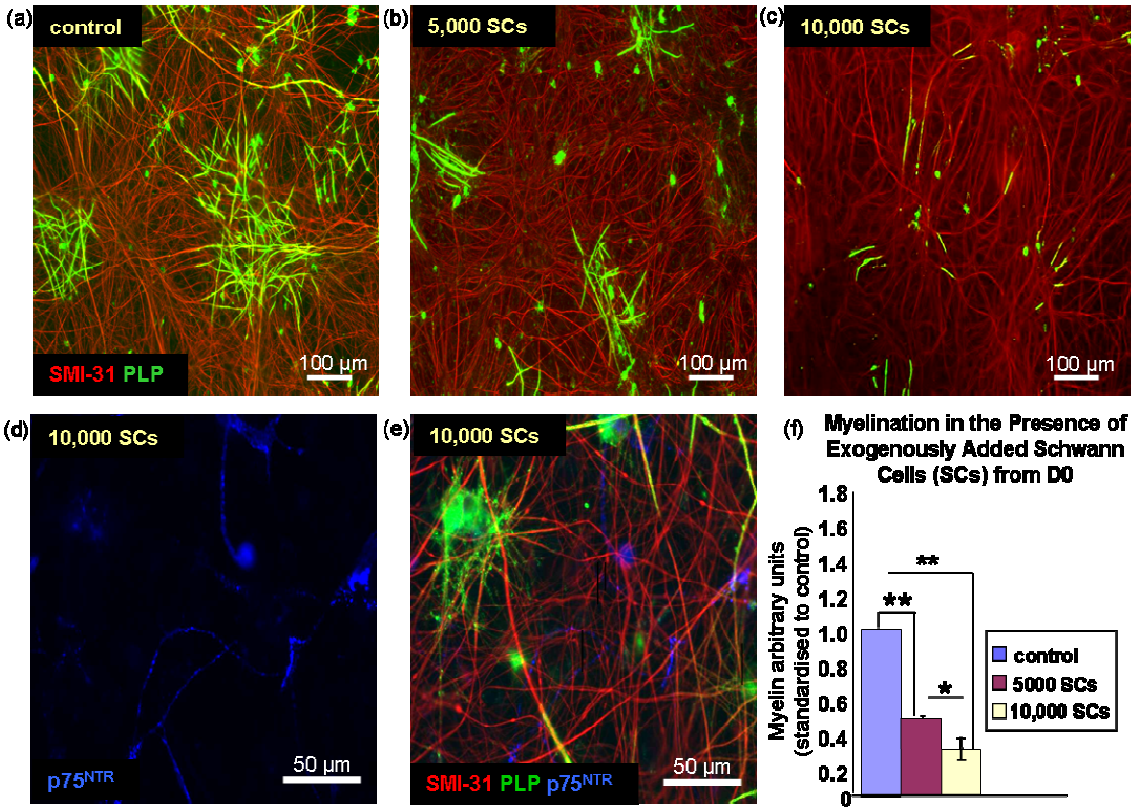


Figure 4.3 - *The addition of either 5,000 or 10,000 SCs significantly reduced the level of myelination in cultures.* 5,000 or 10,000 SCs were added to the mixed embryonic spinal cord suspension and plated down onto a confluent monolayer of neurosphere-derived astrocytes. After 26 days, the level of myelination in cultures containing either 5,000 (b, f) or 10,000 SCs (c, f) was highly significantly less than that of control cultures (a, f). Myelination was also significantly lower in cultures containing 10,000 SCs, compared to those where only 5,000 SCs were added (f). SCs could still be detected after 26 days in culture using an antibody to $p75^{NTR}$ (d, e) but did not appear to associate with the myelin sheaths. SMI-31 was used to label phosphorylated neurofilament in neurites, whilst an antibody to PLP labelled oligodendrocytes and myelin sheaths. An Olympus BX51 epifluorescence microscope was used to capture all images. Scale bars = 100 µm and 50 µm. n=3.* = p values <0.05, ** = P values <0.01.

4.3 The Positive Effects of OECs on Endogenous Myelination may be Dominant to the Negative Effects Exerted by SCs

As in section 4.2, purified suspensions of OECs and SCs were trypsinised, resuspended and counted so that 5,000 or 10,000 of each cell type were added *together* into the mixed embryonic spinal cord suspension and plated onto an astrocyte monolayer. Controls included the addition of each cell type to the myelinating cultures on their own, as well as no exogenous cells being added, as seen in previous experiments.

Preliminary results suggested that the mechanisms which induced the increase in myelination observed when 10,000 OECs were added to the myelinating cultures may be dominant to the negative effects exerted by SCs, given that the addition of a combination of both cell types appeared to increase the level of myelination almost two-fold above control levels, and marginally more than the addition of 10,000 OECs alone (Figure 4.4 a, f, g, h).(n=1). As previously shown (Figure 4.2), the addition of 10,000 OECs alone (Figure 4.4 c) significantly increased myelination (n=4, p value=0.04), whilst 5,000 or 10,000 SCs (Figure 4.4 d, e) alone significantly reduced the level of myelination compared to control (n=4, p values=0.04 and <0.01, respectively). Neurite density was not affected by any treatment.

Furthermore, it appeared likely that this increase in myelination was in fact due to an enhancement of the endogenous myelinating capacity of the culture system as opposed to the likelihood that either SCs or OECs were contributing to this phenomenon by exogenously myelinating axons in peripheral myelin, since labelling with an antibody to detect P0 in cultures containing both cell types demonstrated a lack of this abundant peripheral myelin protein in the myelin sheaths (Figure 4.5 b). Interestingly, however, punctate P0 staining was evident in these cultures in the cell bodies of bi-polar cells with typical morphological characteristics of both OECs and SCs. There was no evidence of this staining in control cultures which did not contain exogenously added glial cells, suggesting

that this pattern of staining is unlikely to be non-specific and that control cultures are not likely to contain contaminating peripheral glia (Figure 4.5 a).

Observations from co-culture assays of GFP labelled OECs and non-labelled SCs, whereby each cell type was cultured in an adjacent strip on a glass coverslip and allowed to migrate towards each other for 5 days (adapted from confrontation assays, Lakatos et al., 2000) suggested a mingling of both cell types, as demonstrated by the presence of GFP-OECs on either side of the seeding "boundary" (Figure 4.6 b, denoted by dotted yellow line). Since the culture of GFP-OECs was only approximately 70% GFP^{+ve}, (Figure 4.6 a) it is also possible that $p75^{NTR+ve}$ cells on either side of the border could be OECs *or* SCs. Although this data in no way suggests that either or both cell type was still present after 26 days in the myelinating cultures, it may offer some interesting insight into the ways in which OECs and SCs could interact with each other in culture, resulting in synergistic effects on endogenous myelination.

Whilst these results are inconclusive and preliminary, since subsequent repeats resulted in the "sloughing off" of cultures from coverslips, perhaps due to the increased presence of proliferative factors, it may be worth considering them for future experiments when investigating the implications of an OEC mediated repair strategy, since SCs often migrate into the injury site via the spinal roots and surrounding blood vessels when the glia limitans is breached following CNS injury (Franklin and Blakemore., 1985, Baron-Van Evercooren et al., 1993). Thus, it would be useful to confirm these findings in order to demonstrate whether or not OECs can still exert beneficial effects on endogenous myelination in the presence of SCs *in vitro*.

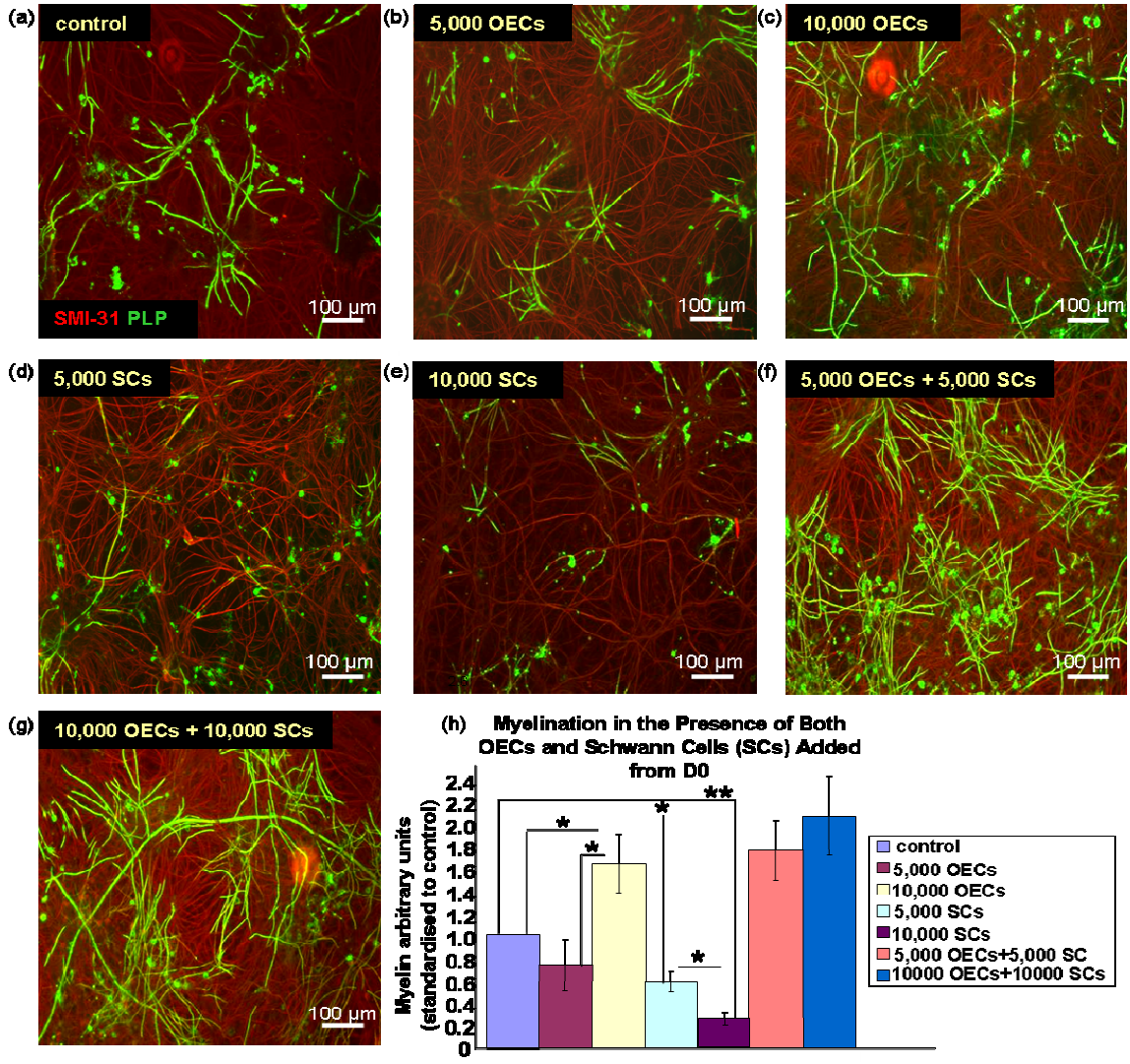


Figure 4.4 - *The addition of a combination of both SCs and OECs appeared to enhance myelination above control levels.* Combinations of 5,000 OECs plus 5,000 SCs, or 10,000 OECs plus 10,000 SCs were added to the mixed embryonic spinal cord suspension and plated down onto an astrocyte monolayer. Control cultures contained no exogenously added glial cells, whilst additional controls included adding either OECs *or* SCs alone to the myelinating cultures. Preliminary results suggested an increase in myelination compared to controls after 26 days in culture when combinations of both cell types were added, resulting in an almost two-fold increase in myelination with the addition of 10,000 OECs + 10,000 SCs (a, f, g, h) (n=1). 10,000 OECs alone (c, h) significantly increased the level of myelination compared to controls and cultures containing 5,000 OECs (b, h) (n=4), whilst the addition of 5,000 SCs or 10,000 SCs (d, e) significantly decreased the level of myelination versus control cultures (a, h) (n=4). Neurites were labelled with SMI-31 to detect phosphorylated neurofilament and an antibody to PLP labelled myelin sheaths and oligodendrocytes. Images were taken on an Olympus BX51 epifluorescence microscope. Scale bars=100 µm. * = p values < 0.05, ** = p values <0.01.

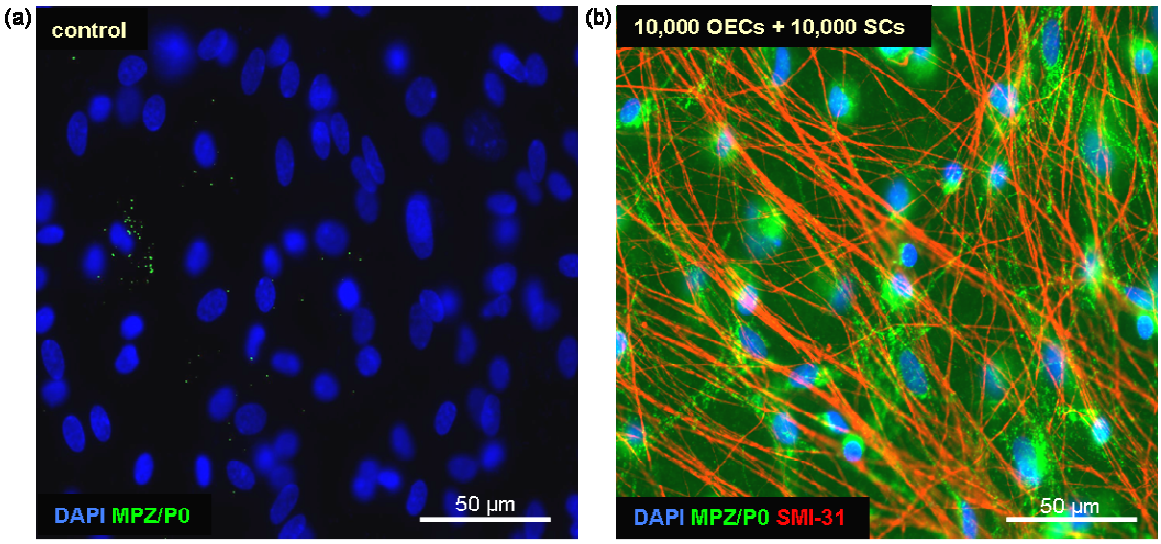


Figure 4.5 - *No MPZ/P0 staining was detected in the myelin sheaths in cultures containing a combination of OECs and SCs.* 10,000 OECs plus 10,000 SCs were added together to the mixed embryonic spinal cord cell suspension and plated down onto an astrocyte monolayer. Control cultures contained no exogenously added glial cells. Preliminary observations suggested that OECs and SCs were not forming peripheral myelin after 26 days in culture, since there was no detectable presence of MPZ/P0 ensheathing axons (b). There was, however, punctate MPZ/P0 staining in the cell bodies of cells with similar morphologies to both OECs and SCs. This staining pattern appears to be true given that there was little evidence of non-specific staining using this antibody in control cultures where no exogenous glial cells were added (a). Images were taken using an Olympus BX51 epifluorescence microscope. Scale bars=50 µm. DAPI labelled nuclei, SMI-31 labelled neurites and an antibody to MPZ/P0 was used to detect the peripheral myelin protein, myelin protein zero.

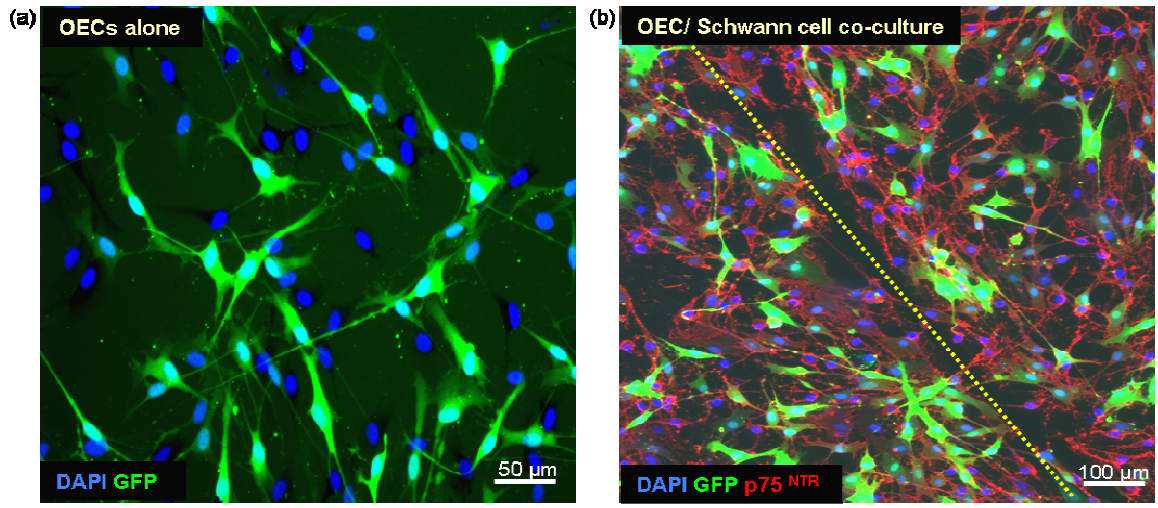


Figure 4.6 - *GFP-OECs appear to mingle with SCs in co-cultures.* GFP-OECs were plated into a strip directly adjacent to, but not touching, an identical strip of SCs on 2 PLL-coated 13 mm^2 glass coverslips. After 5 days, GFP^{+ve} cells could be observed on either side of the boundary where each cell type was initially seeded (denoted by broken yellow line), suggesting a mingling of both OECs and SCs (b). Cultures of OECs were only approximately 70% GFP^{+ve}, (a) thus, cells which were positive for $p75^{NTR}$ could represent either SCs or the remaining 30% of the OEC population. Images were taken using an Olympus BX51 epifluorescence microscope. Scale bars=50 µm (a) and 100 µm (b). DAPI labelled nuclei, whilst an antibody to $p75^{NTR}$ labelled OECs and SCs. GFP denotes green fluorescent protein, which OECs were labelled with using a lenti-virus. n=1.

4.4 The Negative Effects Mediated by SCs on Endogenous Myelination Were Induced by Secreted Factors, as Opposed to Contact-Dependent Mechanisms

Conditioned media (CM) was collected in differentiation media (DM; used to maintain myelinating cultures) from equally confluent flasks of both SCs and OECs over a 3 day period and filtered sterile before use (see also section 2.1.4). It was then added to myelinating cultures thrice weekly from day 12 onwards at a dilution of 1:4 with fresh DM, allowing an initial period whereby the cultures could become established. Control cultures were fed only with DM.

After 26 days, the level of myelination observed in cultures whereby Schwann cell conditioned media (SCM) was added was highly significantly lower than in control cultures (Figure 4.7 a, b, d) (n=4, p value< 0.01). A lack of Caspr staining in these cultures, compared to controls, demonstrated the unlikelihood that internodes of myelin lacking in PLP or immunoreactivity to the O4 antibody had formed under these conditions (Figure 4.8).

The addition of OEC conditioned media (OCM), however, produced variable results, with cultures often showing poor survival following this treatment (Figure 4.7 c). It was hypothesised that this may be due to an over-growth and subsequent sloughing off of the cultures due to the mitogenic effects of OECs. Thus, CM was also diluted down to 1:8 and tested in the same way as above. Under these conditions, though not completely diminished, myelination was still significantly reduced compared to controls with the addition of SCM (Figure 4.9 a, b, d) (n=3, p value=0.03). Furthermore, OCM that had been diluted down to 1:8 prior to use was no longer detrimental to the survival of the myelinating cultures, with myelination under these conditions comparable to controls but significantly more than in cultures which were treated with SCM at a dilution of 1:8 (Figure 4.9 c, d) (n=3, p value=0.04).

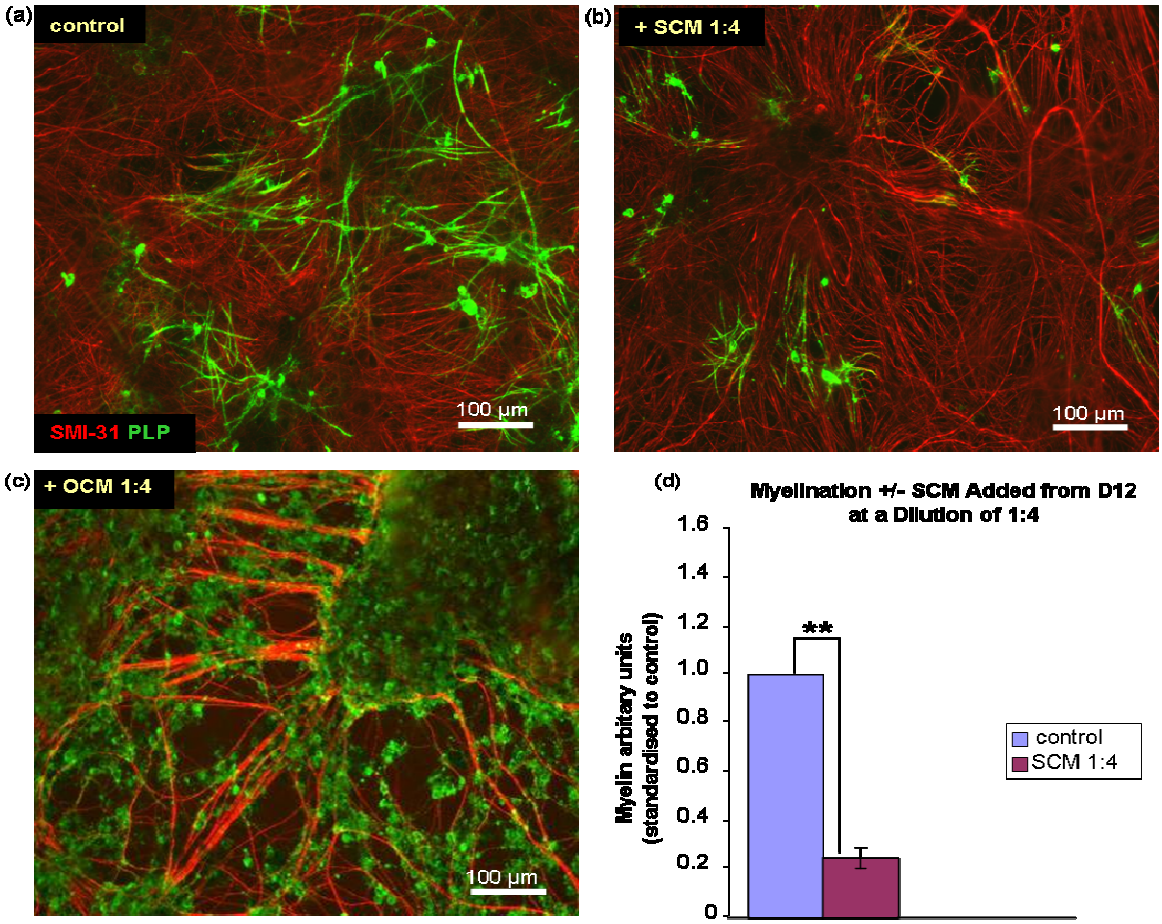


Figure 4.7 – *SCM at 1:4 significantly reduced the level of myelination compared to control cultures, whilst OCM was detrimental to the survival of myelinating cultures.* Conditioned media was collected from SCs (SCM) or OECs (OCM), diluted with fresh differentiation media (1:4) and added to myelinating cultures every other day from day 12. After 26 days, the level of myelination following SCM treatment was significantly lower compared to controls (a, b, d). Overall, the survival of cultures treated with OCM was poor and thus, myelination could not be quantified (c). Images were taken on an Olympus BX51 epifluorescence microscope. Scale bars=100 µm. Myelin sheaths and oligodendrocytes were labelled with an antibody against PLP and SMI-31 labelled phosphorylated neurofilament in neurites. n=4. ** = p value < 0.01.

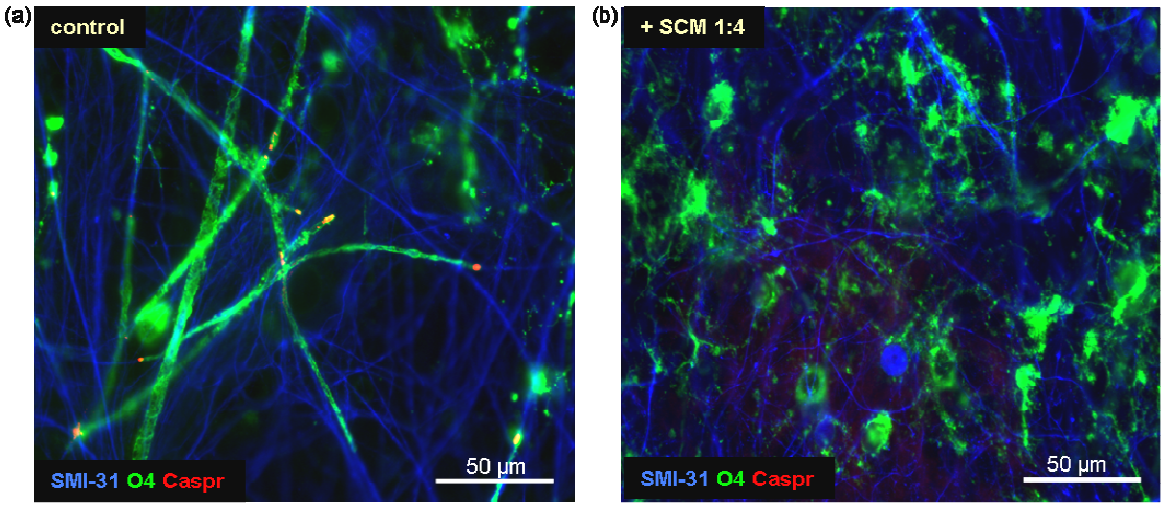


Figure 4.8 – *There was no evidence of Caspr staining in cultures which were treated with SCM, indicating a lack of correctly formed nodes of Ranvier*. SC conditioned media (SCM) was collected in differentiation media and added to myelinating cultures from day 12. After 26 days, immunolabelling with an antibody against the intermediate oligodendroglial marker, oligodendrocyte 4 (O4), demonstrated a lack of O4+ve myelin sheaths in SCM treated cultures (b) compared to untreated controls (a). Furthermore, in control cultures axonal contactin associated protein (Caspr) was located at the paranode, indicating the presence of correctly formed myelin internodes (a), and absent from cultures which had been treated with SCM, suggesting a lack of myelin formation in these cultures. An Olympus BX51 epifluorescence microscope was used to capture images. Scale bar=50 µm. An antibody to O4 was used to label oligodendrocytes and myelin sheaths, whilst SMI-31 labelled phosphorylated neurofilament in neurites.

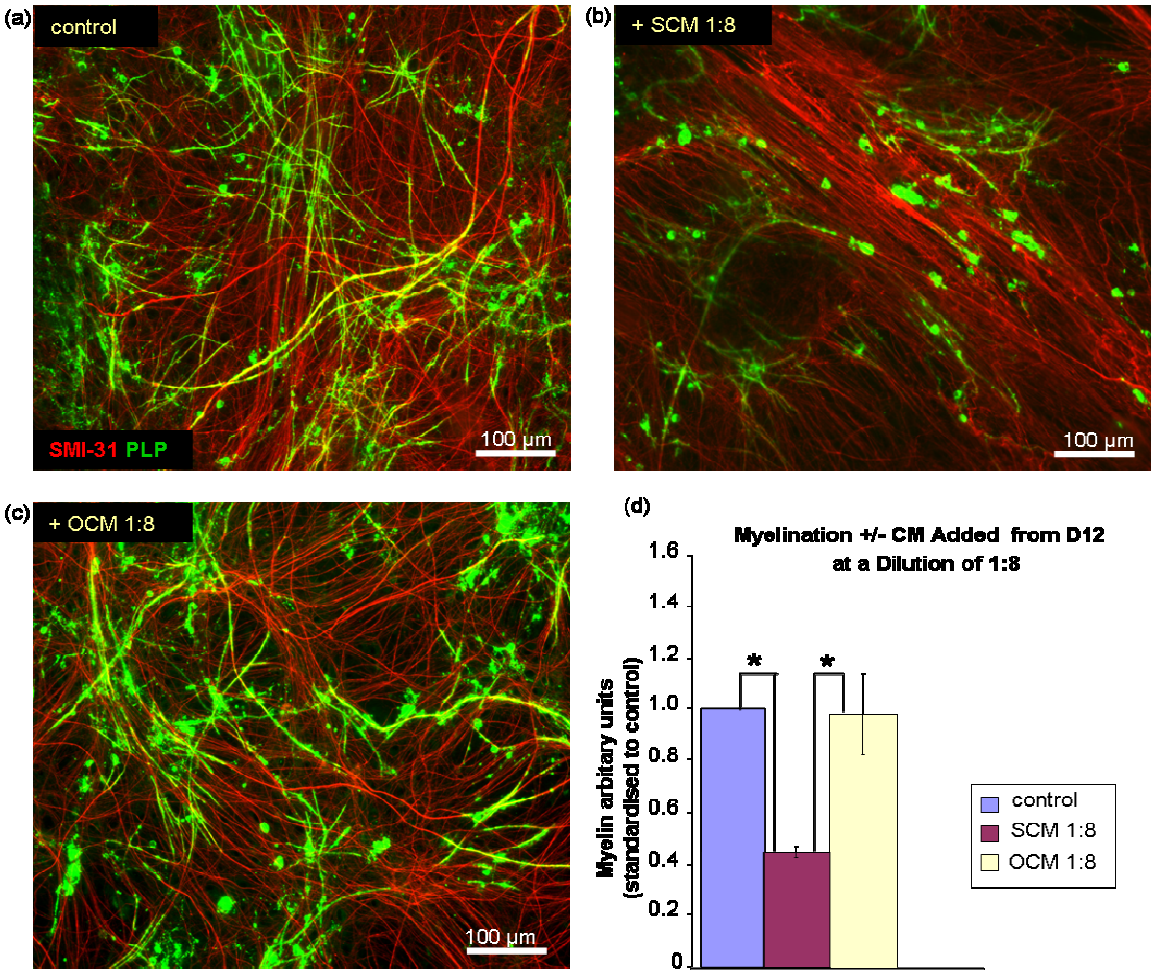


Figure 4.9 – *SCM at a dilution of 1:8 was still negative for endogenous myelination, whilst OCM was comparable to control.* Conditioned media was collected from SCs (SCM) or OECs (OCM), diluted with fresh differentiation media (1:8) and added to myelinating cultures every other day from day 12. The level of myelination following SCM treatment at 1:8 was significantly lower compared to control cultures (a, b, d). In cultures treated with OCM, myelination was comparable to controls but significantly higher than in SCM treated cultures. (c, d). Images were taken on an Olympus BX51 epifluorescence microscope. Scale bars=100 µm. Myelin sheaths and oligodendrocytes were labelled with an antibody against PLP, whilst SMI-31 labelled phosphorylated neurofilament in neurites. n=3. *= p value <0.05.

4.4.1 If the Conditioning Paradigm Involved Cross-Talk Between SCs and the Myelinating Cultures, Endogenous Myelination Still Appeared to be Reduced.

In order to assess whether or not the secreted factors from SCs promoted paracrine signalling of factors from endogenous axons/glia in the myelinating cultures, thus affecting myelination, 2 coverslips of SCs were placed in a Petri dish and allowed to continually condition one coverslip containing a myelinating culture from day 12 onwards. Coverslips of SCs were replaced every other day (as described in section 2.1.7.2). Unlike when conditioning with SCM generated directly from flasks of SCs, conditioning in this way enabled "cross-talk" between SCs and the cells present within the myelinating cultures, which could potentially have influenced the secretion of factors from both SCs and endogenous CNS cells in the cultures..

Previously this experiment was carried out in the Barnett lab by Besma Nash (Nash et al., 2011) using OECs on coverslips to condition myelinating cultures instead of SCs. Results from those experiments indicated that OEC coverslip conditioning resulted in a significant increase in the level of myelination compared to unconditioned controls (n=3, p value < 0.05). Initial results using coverslips of SCs appeared to demonstrate that conditioning in this way, as opposed to using SCM collected in the absence of cross-talk, *still* resulted in a reduction in the level of myelination, compared to controls (Figure 4.10). Furthermore, conditioning in this manner resulted in the almost complete ablation of myelination in the cultures along with a slight decrease in neurite density (Figure 4.10 b, c), though PLP^{+ve} oligodendrocytes were still evident in SC-conditioned cultures (n=2).

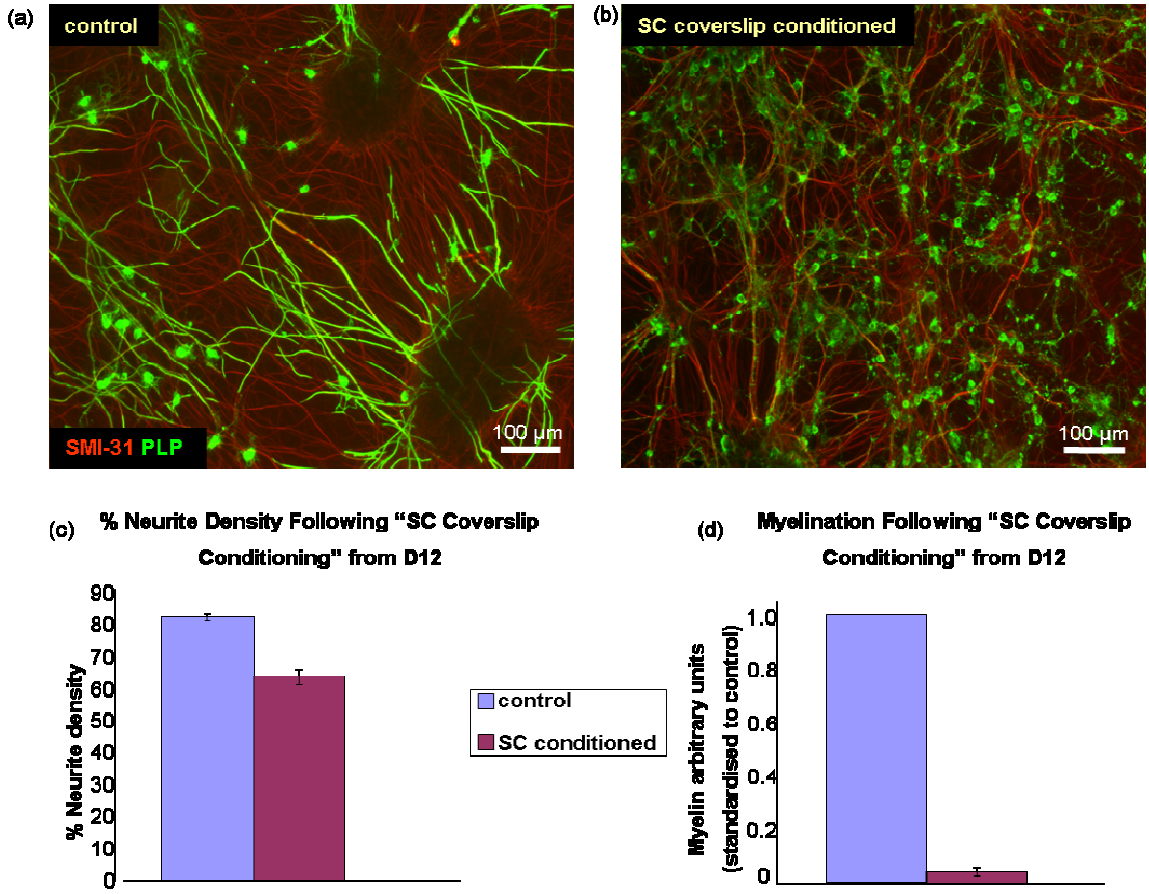


Figure 4.10 - *Conditioning the myelinating cultures using 2 confluent coverslips of SCs ablated myelination in experimental cultures versus control cultures.* 2 coverslips of SCs, which were replaced every other day, were used to condition 1 myelinating culture coverslip in a petri dish from day 12. Preliminary results suggested that there was virtually no myelin in conditioned cultures versus controls, though PLP $^{+ve}$ oligodendrocytes were present (b, d). Neurite density was also BX51 epifluorescence reduced in SC conditioned cultures (c). Images were captured on an Olympus microscope. Scale bars = 100 µm. Neurites were labelled with SMI-31 to detect phosphorylated neurfilament and an antibody against PLP labelled myelin sheaths and oligodendrocytes. n=2.

4.5 The Reduction in Myelination Observed in SC/ SCM Treated Cultures was not Due to Demyelination

Though this chapter has demonstrated that exogenously added SCs and treatment with SCM significantly reduced the level of myelination compared to control cultures, it was unclear whether or not this decrease was due to (i) myelinated fibres forming and then demyelination occurring (due to the presence of SC-secreted factors), or (ii) the inhibition of the endogenous myelinating capacity of the cultures.

To test these two hypotheses, myelinating cultures were allowed to develop as normal until day 26, and then SCM was subsequently added every day for 4 days, before immunofluorescent labelling was carried out at day 30. Untreated cultures were stained at both day 26 and day 30. Results demonstrated that the addition of SCM to established (myelinated) cultures was not detrimental to the level of myelination observed (Figure 4.11). By day 30, there was a slight increase in the level of myelination in both untreated (Figure 4.11 b) and SCM treated cultures (Figure 4.11 c) versus day 26 cultures, and though a temporal increase in the amount of myelination is to be expected, neither increase was significant compared to control (n=3, p values=0.18, 0.29). This data therefore suggests that SCM *prevents* the formation of myelin as opposed to causing demyelination to occur in established CNS cultures.

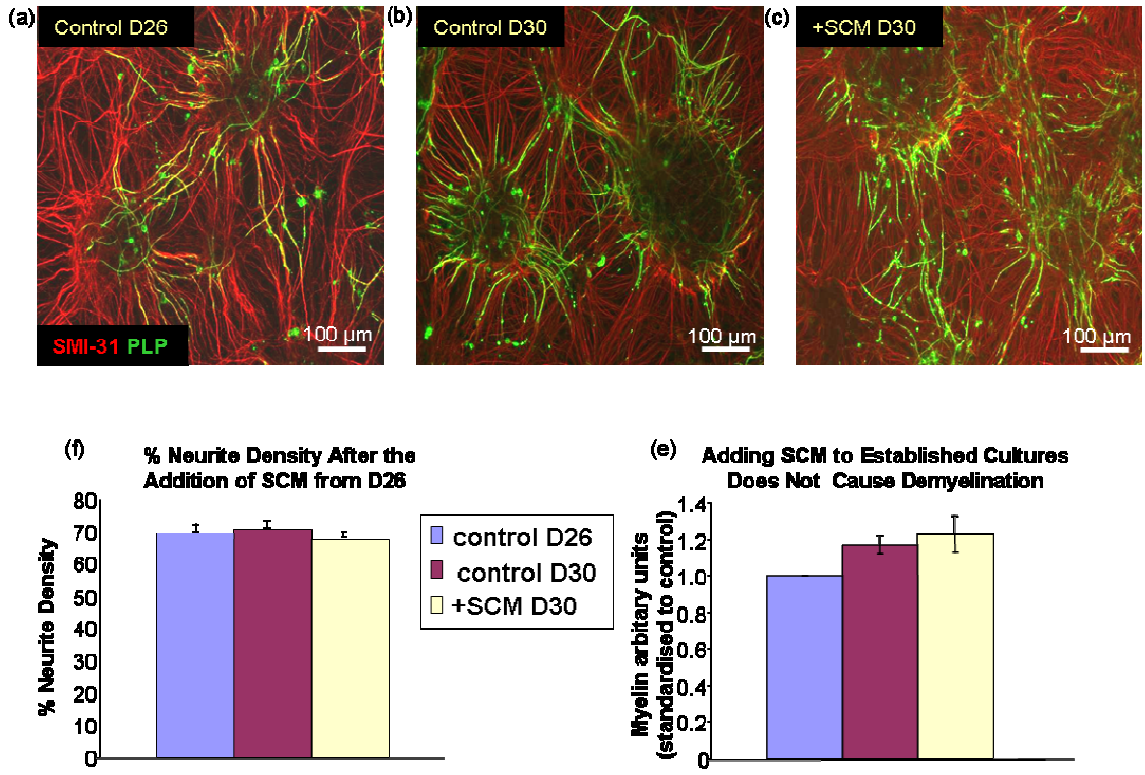


Figure 4.11 - *SCM does not cause demyelination in established myelinating cultures.* SCM was added to cultures every day for 4 days from day 26 before immunolabelling was carried out. Untreated cultures were stained on day 26 and day 30. Whilst the addition of SCM (c) appeared to slightly increase myelination compared to untreated controls (a), this increase was not significant. Myelination appeared to increase slightly with time regardless of treatment though this change was also non-significant (b). Neurite denstity was unaffected by treatment. Images were taken using an Olympus BX51 epifluorescence microscope. Scale bars = 100 µm. An antibody to PLP labelled myelin sheaths and oligodendrocytes and SMI-31 labelled phosphorylated neurofilament in neurites. n=3.

4.6 Discussion

Data from this chapter demonstrated that 10,000 exogenously added OECs significantly increased the level of endogenous oligodendrocyte myelination compared to controls in a myelinating culture system (Figure 4.2). The addition of 5,000 or 10,000 SCs, however, significantly decreased the level of myelination versus cultures which lacked the addition of exogenous cells (Figure 4.3). Furthermore, preliminary data suggested that the combination of OECs and SCs together resulted in an increase in myelination above the level of control and marginally higher than that observed when 10,000 OECs were added alone, which may imply that the positive effects of OECs on endogenous myelination are dominant to the inhibitory effects of SCs (Figure 4.4). Data from CM studies showed that the significant effects of SCs on endogenous myelination were mediated via secreted factors, even when these factors were considerably diluted (Figures 4.7, 4.9). In addition, when exposed to paracrine/autocrine signalling from endogenous cells in the myelinating cultures, SCs still exerted a negative effect on myelination suggested that their expression of inhibitory factors was not mediated via cross-talk. Indeed, in this particular experimental paradigm myelination was almost completely ablated, suggesting that the SC-secreted factors which negatively affect myelination were possibly at higher concentrations compared to when conditioning with SCM derived from SCs grown in monoculture.

Whilst this data shows that SCs are continually negative in every paradigm, with evidence suggesting that these negative effects are mediated via secreted factors, OECs have a positive influence on myelination when exogenously added to cultures and *only* when conditioning in a scenario which directly exposes them to factors being secreted by the myelinating cultures (2 coverslip conditioning method, Nash et al., 2011). These findings suggest that the effects of OECs on endogenous myelination may be partly contact-dependent and partly attributable to cross-talk with resident CNS neuronal and glial cells. Thus, it is likely that the mechanisms that are involved with each cell type and the ways in which they influence oligodendrocyte myelination are highly complex and possibly entirely distinct from one another.

Whilst initial experiments suggested that the addition of OECs to the myelinating cultures from day 12 was detrimental to the survival of the cultures, this is not necessarily an indication that OECs were in some way inhibitory. A more plausible explanation is that the mechanical strain of lifting delicate cultures from one dish to another at what is arguably a critical point in their development resulted in limited survival of the cultures. Indeed, subsequent experiments whereby the control coverslips were also "fished" out at day 12 using forceps resulted in little or no cells remaining at day 26. Thus, the protocol was adapted to negate the need to fish coverslips from dish to dish by adding OECs or SCs from day 0 in a mixed cell suspension with the embryonic spinal cord cells.

Though the results are only preliminary, the combination of both OECs and SCs together mediating an increase in myelination suggests the possibility of a synergistic dynamic between these two distinct populations of glial cells (Figure 4.4). Indeed, Au et al., (2007) speculated at this relationship by demonstrating the ability of OECs to enhance the capacity of SCs to-mediate the outgrowth of dorsal root ganglia (DRG) via the OEC expression of secreted protein rich in cysteine (SPARC) and it's interactions with laminin-1 and transforming growth factor-β (TGF β). Furthermore, the ability of OECs to promote migration of SCs *in vitro*, even on an astrocyte monolayer, was also demonstrated by Cao and colleagues (2007). Several repeats of this experiment were carried out to no avail, with most of the cultures sloughing off before the end stage. The protocol was adapted to incorporate feeding on a daily basis in a bid to combat over-expenditure of vital nutrients due to the confluent nature of these cultures however, this did not improve their durability. Perhaps future considerations should include lowering the density at which the myelinating cultures are initially seeded to, for example, 100,000 cells/ 100 µl instead of 150,000 cells/ 100 µl.

Furthermore, it was virtually impossible to determine whether or not both the exogenously added OECs and SCs survived for 26 days in the myelinating cultures, given that both cell types express $p75^{NTR}$ and there is currently no known marker which can distinguish one cell type from the other. In order to

address this, OECs (or SCs) could be labelled using a lenti-virus encoding green fluorescent protein (GFP) prior to adding them to the myelinating cultures. However, this strategy may not be entirely useful, given that any labelled $p75^{NTR}$ cells, which were not GFP^{+ve}, could also represent a population of non-infected OECs, as opposed to surviving SCs.

It was not surprising that neither cell type appeared to myelinate in our cultures, as evidenced by a lack of P0 staining in the myelin sheaths (Figure 4.5), given that previous work has demonstrated the requirement for ascorbic acid and serum in the culture media to induce basal lamina formation and subsequent myelination by SCs (Eldridge et al., 1987); both of which are absent from the media used to maintain myelinating cultures. Though Devon and Doucette (1995) reported the myelination of DRG neurites by OB-OECs *in vitro* in the absence of ascorbic acid, the issue surrounding the myelinating potential of OECs in culture has remained controversial until recently (Babiarz et al.,2011; see also chapter 1).

However, the observation of P0 staining in the cell bodies of what appeared to be SCs and/or OECs exogenously added to the myelinating cultures may be at odds with reports in the literature. For example, Brockes et al., (1981) demonstrated that fixed cultures of non-myelinating SCs did not express P0 and that its induction was only mediated upon close contact with an axon (Brockes et al., 1980). Mirsky et al., (1980) also demonstrated that whilst freshly dissociated SCs expressed P0, this expression was quickly down-regulated in culture, suggesting the importance of a continual axon/glial interaction. This observation could therefore suggest that remaining OECs and SCs in these cultures had up-regulated their expression of P0 in response to close interactions with axons within the culture, perhaps signifying their myelinating potential. It may also be a phenomenon produced by the culturing of a combination of OECs and SCs alongside endogenous neuronal/glial influences, therefore further studies would be necessary to confirm these observations.

The results from this chapter seem to demonstrate that the ability of OECs to enhance endogenous myelination are somewhat dependent upon direct interactions with endogenous glia/neurons within the culture (Figure 4.2), as well as via the possible modulation of the OEC secretome induced by paracrine signalling, as evidenced in the 2 coverslip conditioning paradigm (see also Nash et al., 2011). OECs are known for their plasticity, acting as a source of trophic support by secreting factors such as nerve growth factor (NGF) to guide regenerating olfactory receptor neurons back to the olfactory bulb, as well as modulating their interactions with axons to induce some aspects of repair following transplantation into the damaged CNS (Graziadei & Graziadei., 1979 a,b; Graziadei et al., 1979; Ramon-Cueto et al., 1998; Keyvan-Fouladi et al., 2003, Li et al., 2003; Toft et al., 2007).

It appears, however, that the factors secreted by SCs are sufficient in limiting the myelinating potential of endogenous oligodendrocytes *in vitro*, regardless of external influences, such as cross-talk with axons and glia (Figure 4.7, 4.9). Though there is currently little evidence to suggest that this is the case *in vivo,* a report by Shields et al., (2000) demonstrated that whilst endogenous oligodendrocyte myelination still occurred in ethidium bromide-induced demyelinating lesions within the brain, SC transplantation altered the repair dynamics such that there was a shift towards peripheral myelin being the dominant type in lesioned areas, with oligodendrocytes tending to be limited to areas which were heavily populated by astrocytes and devoid of SCs around the lesion centre.

Taken together, data from this chapter has suggested that the transplantation of OECs, and possibly a combination of both OECs and SCs together, may be optimal in promoting competent functional recovery of the CNS, given their ability to promote neurite outgrowth, remyelinate axons *in vivo* and enhance endogenous CNS myelination *in vitro*. Although SCs also present several promising attributes for enhancing CNS repair, the observation that they appear to limit the formation of CNS myelin *in vitro* may need to be considered when optimising transplantation studies to provide maximum efficacy.

Results Chapter 5:

Identifying the SC-Secreted Factors, Which Negatively Affect Endogenous Myelination *In Vitro*

5.1 Introduction

Data presented in Chapter 4 demonstrated the novel findings that SCs negatively affected the formation of endogenous myelination by oligodendrocytes *in vitro*. Although the mechanisms which regulate oligodendrocyte myelination are complex and as yet not fully elucidated, several factors have been implicated for their role in oligodendrocyte survival and maturation, such as PDGF (Noble et al., 1988; Raff et al., 1988; Richardson et al., 1988) and IGFs (McMorris et al., 1986; Ye et al., 1995, 2002; Wood et al., 2007) (see Chapter 1).

Recently, connective tissue growth factor (CTGF/ CCN2) has also emerged as a possible negative regulator of oligodendrocyte myelination. Stritt et al., (2009) demonstrated using adenovirus-mediated CTGF expression that CTGF blocked the excessive differentiation of oligodendrocytes *in vitro* and *in vivo*. Though CTGF does not have one clear receptor, it contains various binding domains and interaction sites for heparin sulphate proteoglycans, integrins and IGFs, amongst others (Figure 5.1). By pre-incubating IGF-containing media with CTGF prior to feeding mixed neuronal cultures, Stritt and colleagues postulated that the resulting reduction in the maturation of oligodendrocytes in culture was due to the sequestering of essential IGFs by CTGF.

CTGF is a ubiquitous 38kD secreted protein, which is encoded by a gene belonging to the immediate early gene family, known as the CCN (Cyr61/CTGF/nov) family. It is involved in various physiological functions, such as cell adhesion, migration, proliferation and angiogenesis. Furthermore, its role in fibrogenesis and wound healing has been extensively studied (Chen et al., 2000, 2001; Wang et al., 2003; Minhas et al., 2011; Seher et al., 2011). For example, exogenously added CTGF increased fibroblast proliferation and migration, as well as collagen deposition in a wound healing model (Alfaro et al., 2012); whilst CTGF expression was also significantly up-regulated during the repair of corneal wounds *in vivo* (Robinson et al., 2012; Shi et al., 2012), thus suggesting a vital role in repair. CTGF has also been implicated in pathologies, such as liver fibrosis in that patients with non-alcoholic fatty acid liver disease showed an increased expression of CTGF compared with healthy controls (Colak

et al., 2012), although the expression of CTGF in these patients was not thought to be directly associated with disease progression or residual liver function, since CTGF levels remained elevated in patients even when clinical scores improved (Bauer et al., 2012). However, increased levels of CTGF can be used as biomarkers to predict the onset of liver cirrhosis (Kovalenko et al., 2009; Zhang et al., 2010). An understanding of its role in the CNS is somewhat limited; however, recent studies have demonstrated a correlation between increased CTGF expression and neurodegenerative diseases.

Spliet et al., (2003) reported that CTGF expression was increased in patients with amyotrophic lateral sclerosis (ALS) compared with healthy controls. Furthermore, these authors described a significant up-regulation of CTGF in motor neurons, the targets of ALS, suggesting a possible role for CTGF in neurodegeneration in this particular disease. In rat models of Parkinson's disease (PD), whereby pathology was induced by administering a neurotoxin to stimulate degeneration of dopaminergic neurons, CTGF was also significantly increased in the acute stages of the disease onset (McClain et al., 2009). It has also been reported using patient-derived glioma tumour cells that a micro-environment rich in CTGF may increase the invasiveness of tumours. The aforementioned studies illustrate the fact that CTGF could be a potential therapeutic target in several neurodegenerative diseases. To date, however, the work carried out by Stritt & colleagues (2009) is the only direct indication that CTGF may also be involved in regulating the myelinating potential of oligodendrocytes, making it an interesting candidate for further study.

In summary, the mechanisms underlying oligodendroglial survival and maturation are highly complex. They are likely to be mediated, however, via a delicate balance of those factors which promote survival and proliferation and those which enhance differentiation to ensure the timely maturation of oligodendrocytes and to control cell numbers during normal development. Therefore, altering these signalling dynamics via over-expression or suppression of one or more of these factors directly or indirectly could greatly alter several aspects of oligodendroglial cell behaviour with varying consequences.

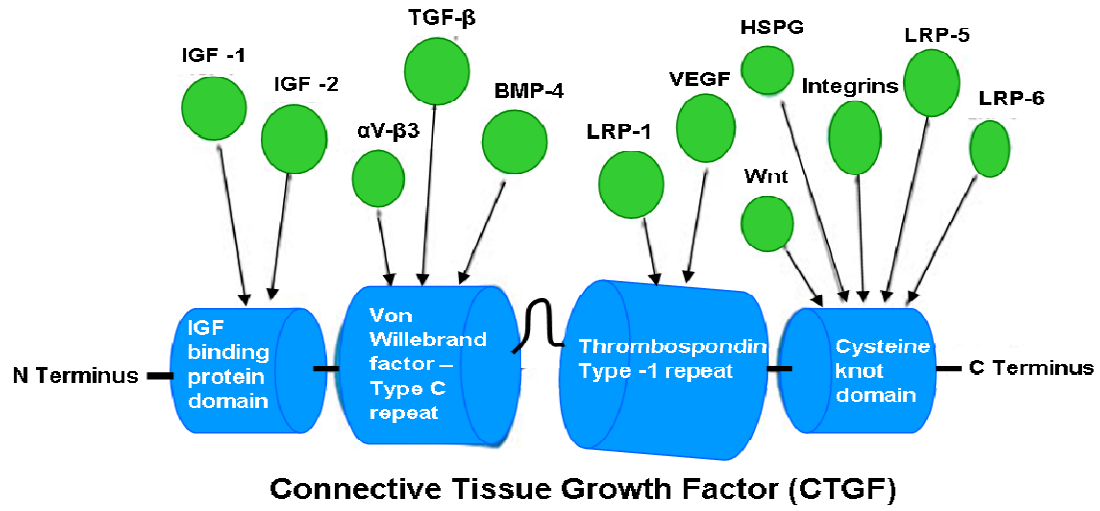


Figure 5.1 - *Structure of Connective tissue growth factor*. CTGF (CCN2) is a 38 kD peptide which has been implicated in cell functions such as cell migration, proliferation and angiogenesis throughout the body. It contains several characteristic binding domains, such as the insulin growth factor (IGF) binding domain at its N-terminus and a heparin sulphate proteoglycan (HSPG) at its C-terminus, as well as several other interaction sites thus, mediating its diverse range of functions.

5.1.1 Aims

Further to the findings that SCs inhibit endogenous myelination via secreted factors, the overriding aim of this chapter was to identify this candidate. This was carried out by i) studying the differences in the expression of known factors which affect myelination in SCs compared with OECs and Ns-astrocytes; and ii) assessing whether these factors, if differentially expressed by SCs, negatively affected oligodendrocyte myelination *in vitro*.

5.2 Both OECs and SCs Secrete Comparable Levels of the Pro-Myelinating Factor CNTF

Previous data from the lab had suggested a role for CNTF in promoting myelination in our culture system, therefore we decided to examine if OECs and SCs secreted different levels of this trophic factor by carrying out an ELISA. Conditioned media (CM) was collected from confluent flasks of OECs and SCs and from 2 coverslips of neurosphere-derived astrocytes (Ns-astrocytes), prior to their use as a monolayer for the embryonic spinal cord mix. CM was collected in DM (myelinating culture media) and concentrations were normalised to give a value/ 10^6 cells. Independent batches of CM in quadruplet were taken from each cell type and each sample was run in triplicate on the ELISA plate (see section 2.5 for more details).

From the ELISA data, it was shown that Ns-astrocytes secreted significantly more CNTF protein than OECs or SCs, at around 700 pg/ml versus 250 pg/ml or 180 pg/ml, respectively (Figure 5.2) (n=4; p values <0.01). Although OECs appeared to secrete slightly more CNTF than SCs, this value was not significant (p value=0.24). Interestingly, there appeared to be more variation in the amount of CNTF secreted by different batches of Ns-astrocytes than by biological replicates of OECs or SCs, with values ranging from 500 pg/ml up to 1 ng/ml.

In light of these findings, it was hypothesised that SCs were also likely to secrete a factor/ factors which were inhibitory to myelination and perhaps dominant in their effects over the pro-myelinating actions of CTGF.

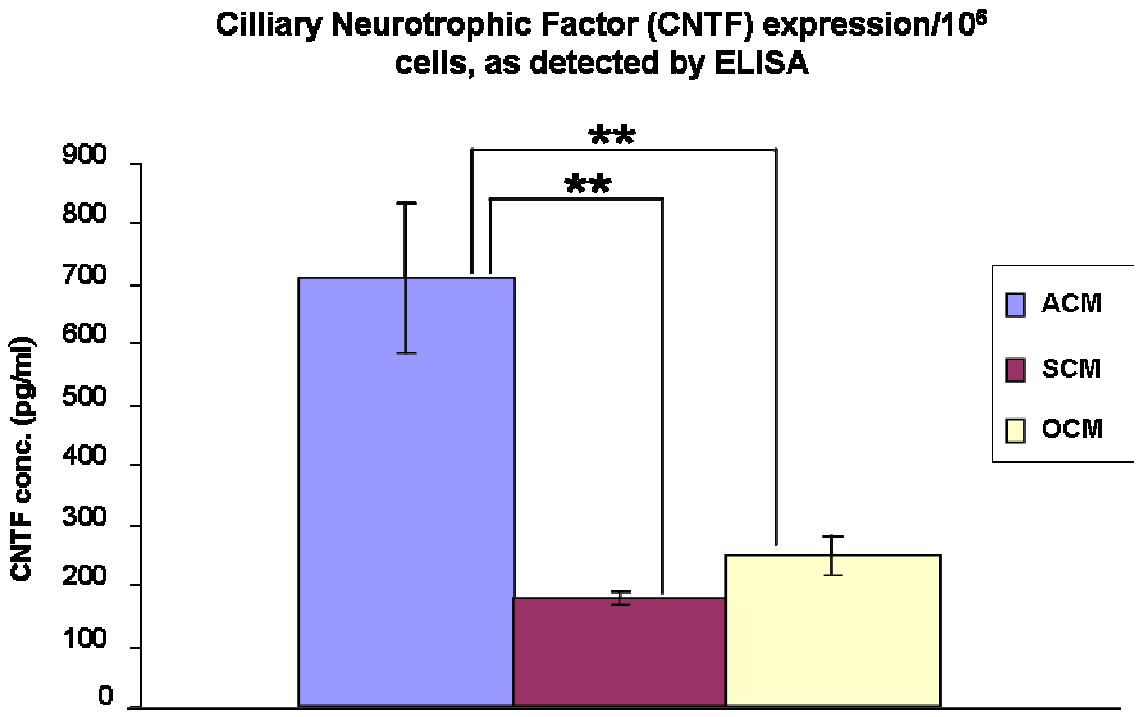


Figure 5.2 - *Ns-astrocytes secrete significantly more CNTF than OECs or SCs.* CM was collected from confluent flasks of OECs (OCM) and SCs (SCM) and from coverslips of Ns-astrocytes (ACM) prior to their use as a monolayer for myelinating cultures. Values were normalised to give concentrations/ 10^6 cells. Using a rat ELISA kit for CNTF it was found that significantly more CNTF protein was present in ACM, than in OCM or SCM. Whilst there appeared to be a slight increase in the concentration of CNTF secreted by OECs compared with SCs, this difference was not significant. n=4 batches of CM. ** = p values < 0.01.

5.3 The SC-Secreted Factor(s) which Negatively Affect Endogenous Myelination are Proteinaceous

To further investigate the nature of the SC-secreted factors which negatively affect oligodendrocyte myelination, SCM was heat treated at 55 °C for 1 hr to alter the tertiary/ quaternary structure of secreted proteins. Denaturing in this way is usually sufficient in changing the conformation of a protein such that its binding affinity and function can be reduced. SCM and heat-treated SCM (h.SCM) were mixed, as before, with fresh DM media (unheated to ensure the presence of essential growth factors) at a dilution of 1:4.

As shown previously in Figure 4.6, SCM added to the cultures from day 12 onwards significantly reduced the level of myelination compared to controls. However, heat treatment of the same batches of CM significantly restored the level of myelination compared to SCM treatment alone (Figure 5.3) ($n=4$, p values < 0.01). Although this increase appeared to supersede the amount of myelin observed in control cultures, it was not statistically significant (p value$=0.07$). Neurite density was unaffected by treatment.

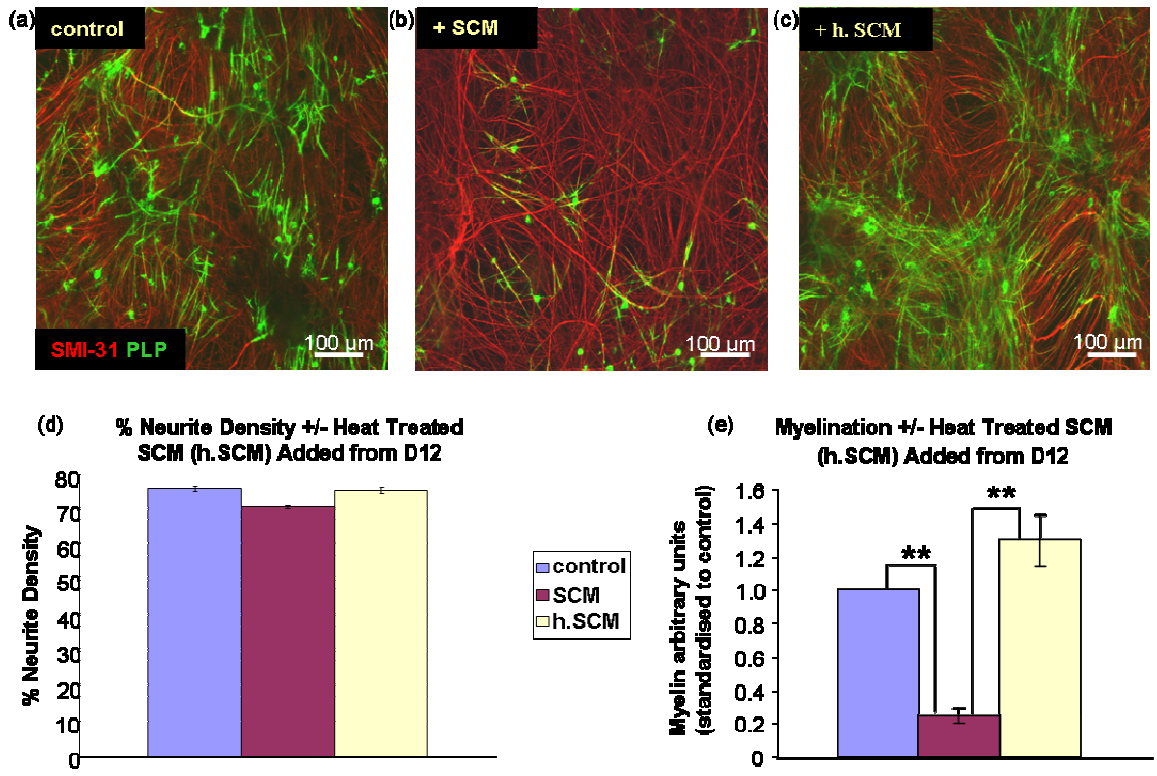


Figure 5.3 - *Heat treatment of SCM significantly restored the level of myelination compared to the addition of SCM alone*. SCM was collected as before in DM and added to the cultures every other day from day 12. Each batch of CM was also heat treated at 55 °C for 1 hr (h.SCM), before being diluted with fresh media (1:4, as with SCM). As previously shown, SCM significantly reduced the level of myelination compared to control values (a, b, e), whilst h.SCM treatment (c) significantly restored the level of myelination compared to treatment with SCM alone (b). Although the increase in myelination with h.SCM appeared to surpass the level of myelination in controls, this was not significant. Neurite density was not significantly affected by any of the treatments (d). Images were captured using an Olympus BX51 epifluorescence microscope. SMI-31 labelled phosphorylated neurofilament and an antibody against PLP labelled oligodendrocytes and myelin sheaths. Scale bar=100 µm. n=4. ** = p values < 0.01.

5.4 SCs Express Significantly More Connective Tissue Growth Factor (CTGF) mRNA and Protein than OECs

As discussed in the introduction to this chapter, CTGF has recently emerged as a possible negative regulator of oligodendrocyte myelination (Stritt et al., 2009). To determine if there was a difference in the expression of CTGF mRNA between OECs and SCs, RNA was extracted from 3 biological replicates of purified cultures of comparably confluent cells and converted to cDNA using a commercial kit. The relative amount of CTGF mRNA expressed by each cell type was then assessed by RT-qPCR, with each sample being run in triplicate (described in section 2.4). Supernatant was also collected from confluent flasks of OECs and SCs in DM and from coverslips of Ns-astrocytes and assessed for CTGF protein content using an ELISA kit (section 2.5.2). In addition, h.SCM was analysed to assess the levels of CTGF which could be detected after heat treatment. As with previous ELISA experiments, concentrations were given as a concentration per 10^6 cells.

Although RT-qPCR only gives a relative indication of changes in expression standardised to an internal housekeeping gene, as opposed to information relating directly to the specific amount of gene expression, data from these experiments demonstrated that there was ap aproximately a 20-fold increase in CTGF mRNA in SCs than in OECs. (Figure 5.4) (n=3, p values <0.01). These observations were confirmed at the protein level using a CTGF ELISA kit. An average value of approximately 500 pg/ml of CTGF was present in SCM, which was significantly greater than the amount secreted by astrocytes by approximately 40% (p value=0.03). Similarly, SCM contained approximately 3 times and 5 times as much CTGF than OCM and h.SCM, respectively. (p values=0.02 and <0.01) (Figure 5.4 b) (n=4 batches of CM). Although ACM appeared to contain more CTGF than OCM and h.SCM, this increase was not significant.

Taken together, these data demonstrate the presence of a factor which has been reported to be inhibitory for myelination, CTGF, at significantly higher levels in SCM than in the CM from other glial cells.

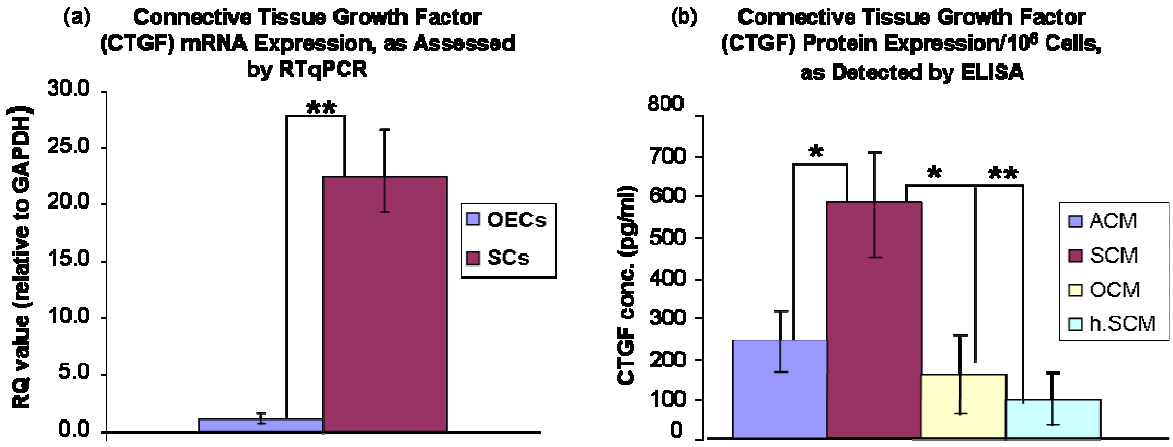


Figure 5.4 - *SCs express significantly more CTGF at both the gene and protein level than other glial cells.* RNA was extracted from purified SCs and OECs, converted to cDNA and analysed using RT-qPCR to give a relative fold change in CTGF expression, normalised to the house keeping gene GAPDH (a). To confirm these findings at the protein level, CM was collected from OECs, SCs and Ns-astrocytes in DM and tested using a CTGF ELISA kit. Heat treated SCM (h.SCM) was also analysed. Values were normalised to give a concentration/ 10^6 cells (b). SCs expressed almost a 20-fold increase in CTGF mRNA expression, compared with OECs (a). Similarly, SCM contained significantly greater levels of CTGF protein than ACM, OCM or h.SCM (b). There was no significant difference between the amounts of secreted CTGF when comparing ACM, OCM and h.SCM.(b). ***** = p values <0.05; ****** = p values <0.01. n=3 biological replicates for RT-qPCR; n=4 batches of CM for ELISA.

5.5 Exogenously Added CTGF Significantly Reduced the Level of Endogenous Myelination *In Vitro*

Since previous data from this thesis demonstrated that SCs secrete a factor/ factors which negatively affect myelination and CTGF expression was higher in SCs than in OECs or astrocytes, exogenous CTGF was added to the myelinating cultures from day 12 onwards to assess its affects on oligodendrocyte myelination. As previously described, CTGF has been reported to be inhibitory for myelination both *in vitro* and *in vivo* by sequestering IGFs, which are essential for oligodendrocyte maturation, at its N-terminus insulin growth factor binding domain (Stritt et al., 2009).

Initially, full length human recombinant CTGF protein (38kD; GenWay Biotech) with cross-reactivity in rats was added to the cultures every other feed day at concentrations ranging from 500 pg/ml (the average detectable amount in SCM by ELISA) up to 100 ng/ml. Preliminary results demonstrated that the addition of CTGF at all concentrations reduced the levels of myelination compared with controls by at least 50% or more (Figure 5.5; n=2). However, further repeats using this peptide proved inconclusive.

Another human recombinant CTGF peptide containing only the C-terminus of the molecule (Figure 5.6) was subsequently tested (11 kD; Peprotech). This peptide was also said to have cross-reactivity in rats. Results from these studies showed that the addition of 10 ng/ml of CTGF from day 12 onwards (Figure 5.7 b) significantly reduced the level of myelination compared to controls (Figure 5.7 a) (n=3, p value <0.01). Neurite density was not significantly affected by the addition of CTGF. No other concentrations were tested using this reagent.

As observed in previous experiments, PLP immunoreactivity in cultures treated with CTGF demonstrated that PLP^{+ve} oligodendrocytes were present even although myelination was significantly reduced. Thus, these results suggest that myelination is inhibited in spite of the presence of oligodendrocytes.

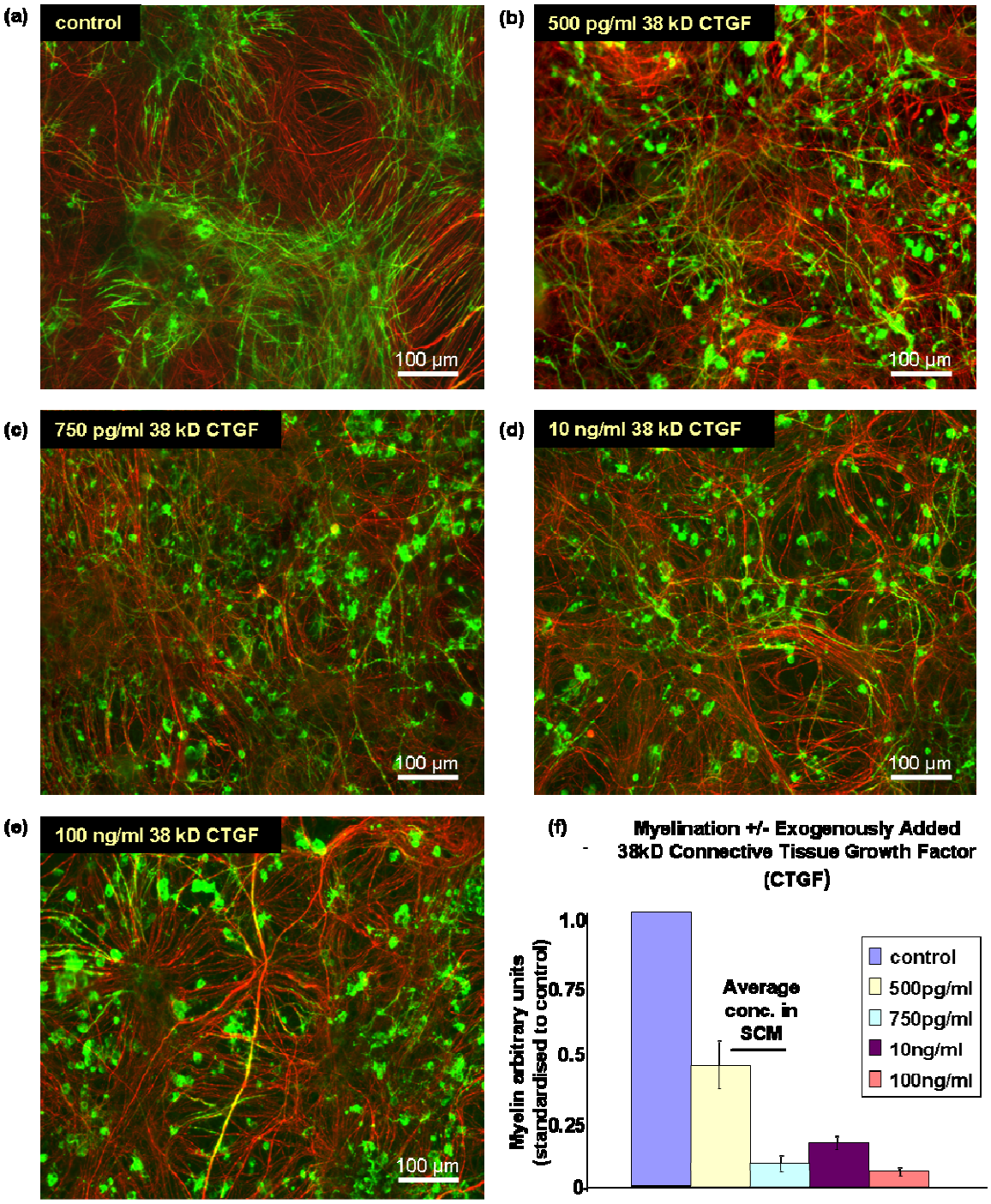


Figure 5.5 - *Preliminary results from the addition of a full-length CTGF peptide to the myelinating cultures demonstrated a decrease in the level of myelination compared with control.* A 38kD CTGF peptide was added to the myelinating cultures every other day from day 12. Myelination appeared to be reduced by at least 50% or more for all conditions compared with controls (f). Though myelination was reduced, PLP^{+ve} oligodendrocytes were evidenced across all conditions (a-e). The average concentration of CTGF present in SCM as detected by ELISA was denoted on the graph, at approximately 500 pg/ml (f). Oligodendrocytes and myelin sheaths were labelled with an antibody against PLP and SMI-31 labelled phosphorylated neurofilament in neurites. n=2. Scale bars=100 µm.

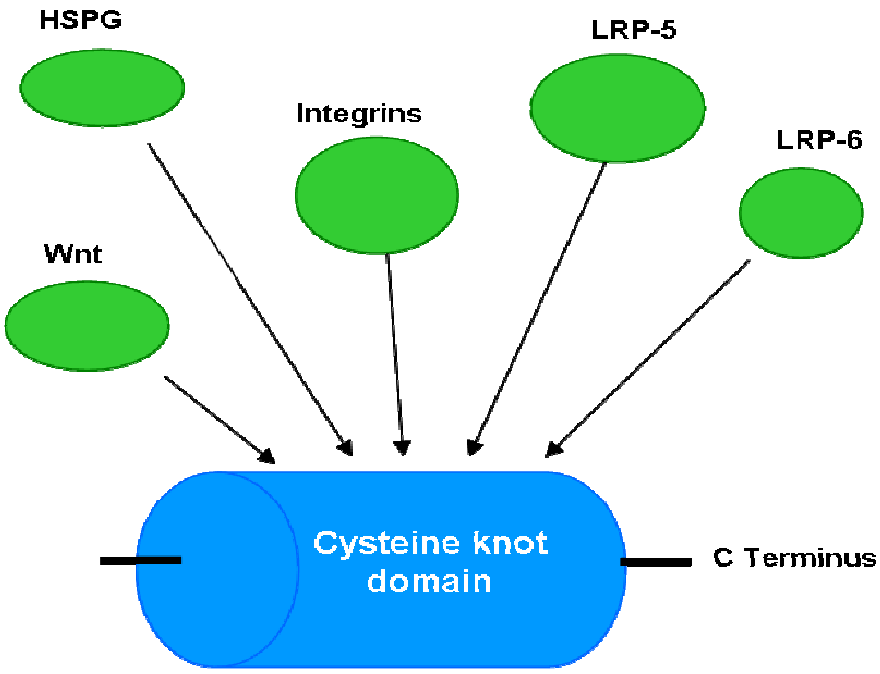


Figure 5.6 – *Shorter length CTGF peptide*. An 11kD human recombinant CTGF peptide from Peprotech was also tested for its effects on endogenous myelination in the cultures. This particular peptide contained only the C-terminus and all of its binding domains. Unlike the full-length CTGF, it lacked, amongst other things, the insulin growth factor binding domain, said to underpin the mechanisms by which CTGF inhibits oligodendrocyte maturation.

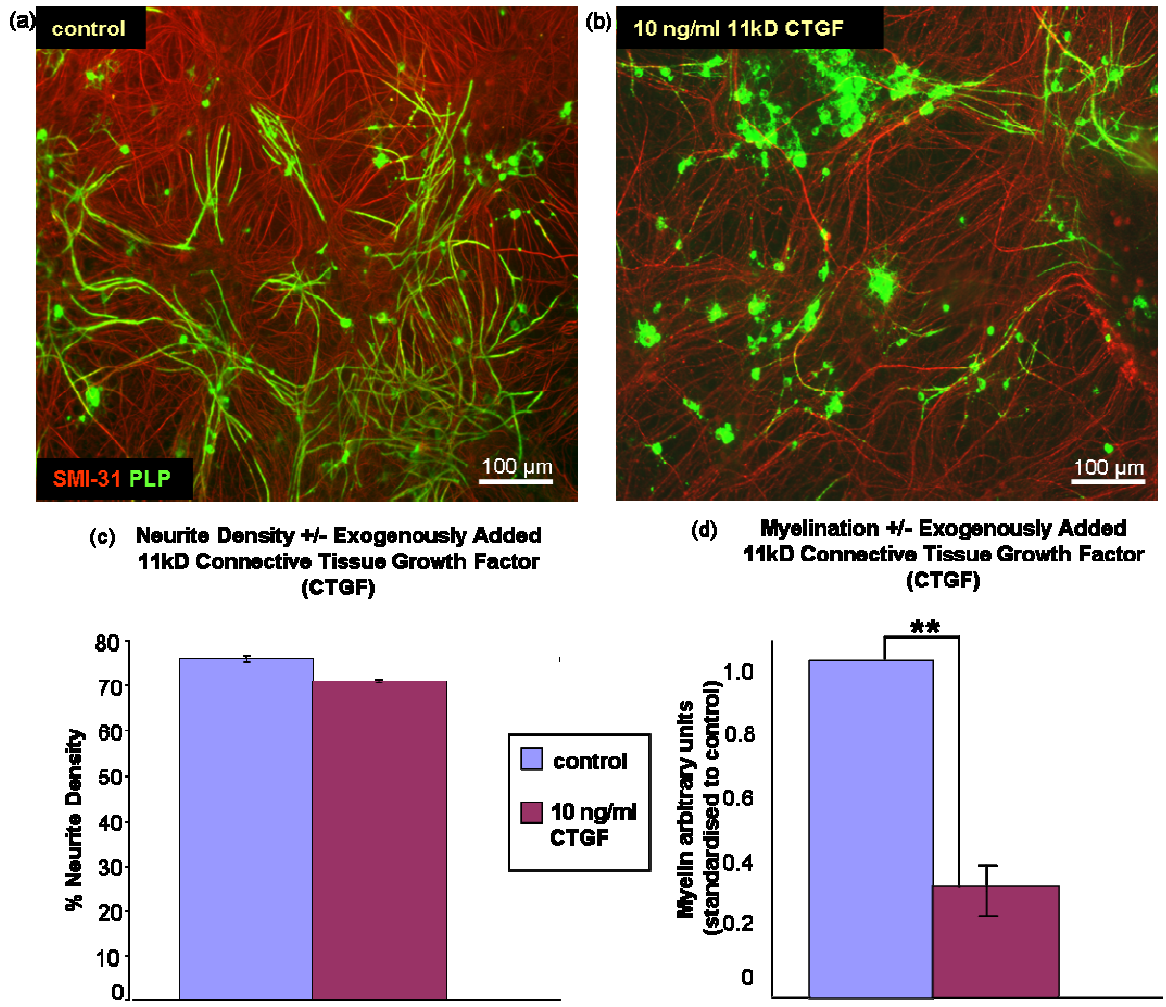


Figure 5.7 - *The addition of a shorter CTGF peptide to the myelinating cultures from day 12 significantly reduced the level of myelination compared with controls.* An 11 kD CTGF peptide containing only the C-terminus of the molecule was added to the cultures at a concentration of 10 ng/ml every other day from day 12. After 26 days in culture, myelination was significantly reduced in CTGF treated cultures (b, d), compared with controls (a, d), though there still appeared to be oligodendrocytes present in the cultures. Neurite density was not significantly affected by CTGF treatment (c). Images were captured using an Olympus BX51 epifluorescence microscope and an antibody against PLP labelled myelin sheaths and oligodendrocytes, whilst SMI-31 labelled phosphorylated neurofilament in neurites. Scale bars=100 µm. n=3. ** = p values <0.01.

5.6 Neutralising CTGF in SCM Restored the Level of Myelination, Compared to SCM Treatment Alone

To further confirm that the inhibitory nature of SCs on endogenous myelination by oligodendrocytes *in vitro* was at least in part due to the presence of SC-secreted CTGF, a neutralising antibody to CTGF was used.

A rabbit polyclonal neutralising antibody to CTGF (LsBio, see also section 2.1.6.4) was added to SCM at a concentration of 10 ng/ml (based upon a calculation of the effective neutralisation of up to 1 ng/ml of CTGF). As with all CM experiments, SCM was mixed with fresh DM at a dilution of 1:4 prior to the addition of the antibody and then added to the myelinating cultures every other day. To ensure that the SCM being tested was inhibitory to myelination as in previous experiments, the same batches of CM which received the neutralising antibody were also tested on the myelinating cultures without antibody. As a further control, the antibody was also added to DM alone from day 12 onwards.

As previously demonstrated, SCM significantly reduced the level of myelination compared with control cultures (Figure 5.8 b) (p values < 0.01). Adding a neutralising antibody against CTGF to SCM before adding it to the cultures significantly restored the level of myelination compared with SCM treatment (c, e) (p values < 0.01), though this increase was not significantly greater than the level of myelination in controls. Treatment with the antibody alone also did not significantly affect the level of myelination compared with controls, though there was some variability with this treatment (e). Neurite density was not significantly affected by the addition of a neutralising antibody to CTGF. $n=3$ throughout.

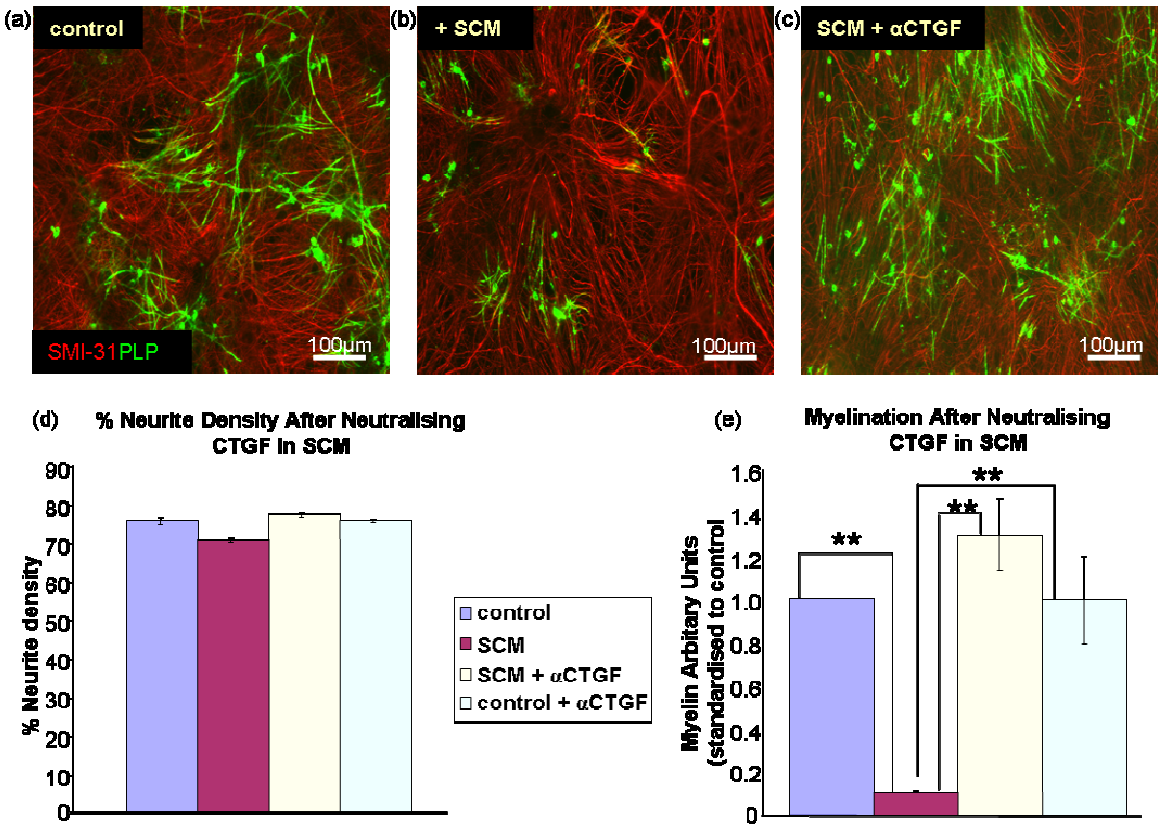


Figure 5.8 - *Neutralising CTGF in SCM significantly increased the level of myelination compared with compared with SCM treatment alone*. A mouse monoclonal neutralising antibody to CTGF was added to SCM or DM at a concentration of 10 ng/ml. SCM was then diluted with fresh media (1:4) and added to cultures every other day from day 12. As positive controls the same batches of SCM minus neutralising antibody were also added to cultures to ensure that they exerted inhibitory effects on endogenous myelination (b). As previously demonstrated, SCM significantly reduced the level of myelination compared with controls (a, c, e). Treatment of SCM with the neutralising antibody to CTGF significantly restored the level of myelination versus treatment with SCM. However, although this increase appeared to exceed the level of myelination observed in controls, this trend was not significant. The addition of the antibody alone to DM did not significantly alter the level of myelination compared with controls, though this amount was significantly greater than myelination following SCM treatment (e). Neurite density was not significantly affected by any of the treatments. Images were taken using an Olympus BX51 epifluorescence microscope. SMI-31 labelled phosphorylated neurofilament in neurites and an antibody against PLP was used to detect oligodendrocytes and myelin sheaths. Scale bar=100 µm. n=3. ** = p values <0.01.

5.7 Discussion

Data from this chapter has demonstrated that whilst Ns-astrocytes secreted significantly more CNTF than OECs or SCs, the amount of this pro-myelinating factor expressed by the latter cell types was comparable (Figure 5.2), thus suggesting that SCs may also secrete dominant factors which are inhibitory to myelination. Heat treatment of SCM to 55 °C for 1 hr significantly restored myelination compared with SCM treatment alone, therefore suggesting that the myelin-inhibitory factors within SCM were proteinaceous and likely denatured during this process (Figure 5.3).

Results from RT-qPCR studies comparing purified SCs and OECs demonstrated that SCs expressed significantly higher levels of the peptide CTGF, which has been reported to be inhibitory for oligodendrocyte myelination, than OECs (Figure 5.4 a). These findings were confirmed at the protein level by ELISA analysis of SCM, OCM, Ns-ACM and h.SCM, which revealed that SCM contained significantly higher amounts of CTGF protein than was present in all other CM (Figure 5.4 b).

Although results from the exogenous addition of a full-length CTGF peptide to the cultures were inconclusive (Figure 5.5), 10 ng/ml of an 11kD human recombinant CTGF containing only the C-terminus (Figure 5.6) demonstrated that myelination was significantly reduced after CTGF treatment compared with controls (Figure 5.7). The likelihood that SC-secreted CTGF was at least partly responsible for the SC-mediated inhibition of oligodendrocyte myelination was given further weight by data obtained from studies whereby a neutralising antibody to CTGF was added to SCM. These data showed that the addition of the antibody into SCM, prior to its administration to the myelinating cultures, resulted in significant restoration of myelination compared to SCM treatment alone. An additional indication that this increase in myelination was due to competent neutralisation of CTGF, as opposed to the non-specific action of the antibody itself, was demonstrated in that the presence of the antibody in control media given to cultures did not significantly alter the level of myelination compared with controls without antibody (Figure 5.8).

Although I have not investigated the mechanisms governing the ability of OECs to increase endogenous myelination, as aforementioned ELISA data demonstrated that OECs secrete the pro-myelinating factor CNTF. Thus, OECs may enhance oligodendrocyte myelination by potentiating the pro-myelinating effects of the astrocyte monolayer (Nash et al., 2011) by contributing to the bioavailability of CNTF in the cultures. Furthermore, OECs could also secrete a plethora of additional factors which contribute to their actions on endogenous myelination. Although SCs were shown by ELISA to secrete CNTF, they were also a source of relatively high concentrations of CTGF; the negative effects of which appeared to be dominant to the pro-myelinating effects of CNTF. An interesting observation was the degree of variability in the concentration of CNTF expressed across different batches of ACM, ranging from 500 pg/ml to 1 ng/ml (Figure 5.2). These findings could to some extent explain why the level of myelination is variable across experiments, given that the availability of pro-myelinating factors can increase or decrease 2-fold with different batches of astrocytes. These results also highlight the importance of the astrocyte monolayer in our culture system, given their increased expression of CNTF compared with other glial cells.

The expression of CTGF by glial cells is a relatively novel field, however a microarray study by Vincent et al., 2005 reported that neonatal rat OECs expressed a higher level of CTGF mRNA than SCs; results which appear to directly contradict our findings (Figure 5.4 a). However, these findings were not said to be significant. Furthermore, they reported that CTGF expression appeared to be slightly greater in cultured OECs than in SCs, although this difference was less apparent *in vivo* in SC-rich tissues in the adult rat. The apparent discrepancy between my findings and those of Vincent et al., (2005) may reflect differences in the preparation of OECs for each study. For example, OECs used for this thesis were isolated and purified from the olfactory bulbs by positive selection based upon their expression of $p75^{NTR}$, using a system of immunomagnetic nano-beads. In contrast, the methods employed by Vincent and colleagues differed somewhat. They combined cells from the (peripheral) olfactory mucosa, a tissue rich in multiple cell types (Lindsay et al., 2010), with olfactory nerve fibre layer (ONFL) tissue. Arabinoside C (AraC) was then added to cultures to eradicate contaminating fibroblasts. Thus, their tissue may have

contained both peripherally and centrally-derived OECs with the possibility of other contaminating cell types. It has previously been reported that OECs from the two tissue sources may have different properties, in that LP-OECs were reported to be more migratory *in vivo* after transplantation and to exert differential growth-promoting properties than OB-OECs (Richter et al., 2005). Thus, this data may also highlight the possibility that lamina propria-derived OECs (LP-OECs) and olfactory bulb-derived OECs (OB-OECs) could differ in their expression of CTGF, amongst other factors.

In addition to demonstrating an increase in CTGF expression at the protein level in SCM compared with OCM or ACM, ELISA data from this chapter also highlighted that heat treatment of SCM significantly reduced the level of detectable CTGF compared to untreated SCM (Figure 5.4 b). Given that the addition of h.SCM to the myelinating cultures significantly restored the level of myelination compared to SCM treatment alone (Figure 5.2), this observation suggests that the activity of CTGF was abolished in h.SCM, possibly via heat denaturing of the protein to alter its binding structure. Whilst the protein would still have been present in h.SCM, its tertiary/ quaternary structure could have been modulated such that its function was impaired, along with its ability to bind to a sandwich ELISA, thus reducing the amount of CTGF detected.

Perhaps the most novel and interesting findings from this chapter allude to the possible mechanisms by which CTGF exerts its inhibitory effects on myelination. Although it was previously reported that CTGF inhibited oligodendrocyte myelination *in vitro* by sequestering IGFs (Stritt et al., 2009), data from my studies using an 11kD CTGF, which lacked the IGF binding domain, also demonstrated its ability to significantly reduce myelination in our cultures, compared with controls (Figure 5.7). Whilst there is no denying the importance of IGF signalling on oligodendrocyte maturation (McMorris et al., 1986; Bartlett et al., 1991; Mozell & McMorris., 1991; Goddard et al., 1999) and the likelihood that its sequestration would impair this process, results from this chapter open up the possibility that other mechanisms may also underpin the actions of the CTGF-mediated inhibition of myelination. Furthermore, Stritt and colleagues do not confirm their findings by adding CTGF to cultures which were maintained

with pro-myelinating factors other than IGF-1 to further validate their proposed mechanism. The importance of integrins on oligodendrocyte differentiation has been discussed in Chapter 1, thus it could be hypothesised that the presence of the integrin interacting site at the C-terminus of CTGF could also be involved in its dysregulation of myelination. Similarly, HSPGs have been reported to play a role in the adhesion of oligodendrocytes and the possible mediation of their polarisation, the morphological change which precedes process extension and subsequent differentiation (Yim et al., 1993). Therefore, the interaction of oligodendrocyte HSPGs with the HSPG binding domain of CTGF could also conceivably alter oligodendrocyte behaviour. However, these suggestions are purely speculative and would require confirmation via thorough experimentation.

The notion that SCs are likely to secrete a host of pro-myelinating factors in addition to CTGF may be apparent when considering data from both the heat treatment of SCM (Figure 5.3) and the antibody neutralisation of CTGF in SCM (Figure 5.8). Whilst both of these treatments significantly restored the level of myelination compared to SCM alone, both also demonstrated a trend to increase myelination beyond the level of control. This data could suggest that by negating the inhibitory actions of CTGF, other factors may be able to exert positive effects on endogenous myelination within the cultures.

Thus, data from this chapter has highlighted the novel findings that mature SCs can inhibit CNS myelination *in vitro* via the secretion of CTGF. Whilst these effects can be overcome, these results further demonstrate the need for careful selection of the most appropriate candidate when considering cell-based therapies for repair, given that remyelination is likely to be an important consideration for functional recovery (Murray et al., 2001; Duncan et al., 2009). These results have also drawn attention to the fact that CTGF signalling in the context of myelination may be more complex than previously reported. What remains unclear is whether or not CTGF mediates its effects by acting directly upon the oligodendrocyte or via an indirect mechanism. For example, CTGF may target other endogenous CNS cells, which are then triggered to affect myelination. However, it is possible that a combination of both mechanisms is at

play. Identifying these means may aid the development of novel therapeutic strategies for the treatment of CNS injury and demyelinating diseases.

Results Chapter 6:

Investigating the Mechanisms Involved in the SC-Mediated Inhibition of Endogenous Myelination *In Vitro*

6.1 Introduction

Thus far, work carried out for this thesis has demonstrated that SCs expressed more of the myelin inhibitory growth factor, CTGF, than OECs and that neutralisation of this factor in SCM negated the SC-mediated reduction of myelination in our cultures. Taken together, these results strongly suggest that the secretion of CTGF by SCs is at least partly responsible for their inhibitory effects on myelination *in vitro*, although the precise cellular targets of CTGF are unknown. On the one hand, CTGF may act directly upon the oligodendrocyte to alter its differentiation state and ultimately its myelinating capacity by interfering with the bio-availability of important mediators of myelination; or by affecting the signalling of several factors which influence oligodendroglial maturation (as discussed in Chapter 5).

On the other hand, CTGF could also alter the biology of other endogenous glial/neuronal cells, such as the astrocytes within the culture, causing them to up or down-regulate vital factors which affect oligodendrocyte behaviour. It appeared less likely that the axons were the target of CTGF, since neurite density was largely unaffected by the majority of CTGF/SCM treatments reported throughout this thesis. However, given that previous studies have demonstrated the importance of an astrocyte substrate *in vitro* (Sørenson et al., 2008) as well as the effect that modulating the astrocyte phenotype had on oligodendrocyte myelination (Nash et al., 2011); it seemed plausible that astrocytes could be a potential target of CTGF. Furthermore, since the structure of CTGF is such that it contains several distinct binding domains and interaction sites (see Figure 5.1), which can mediate the induction of multiple signalling pathways, it is also possible that CTGF could negatively regulate oligodendrocyte myelination via both direct *and* indirect mechanisms.

Early evidence that SCs may affect the ability of endogenous oligodendrocytes to myelinate axons and that astrocytes may be involved in this mechanism has previously been reported *in vivo*, although the data was not necessarily interpreted in this way at the time. For example, Blakemore (1975) demonstrated that following lysolecithin-mediated demyelination of the spinal

cord in adult rats, infiltrating SCs and endogenous oligodendrocytes remyelinated axons. However, central and peripheral myelin were segregated by the reconstitution of the glia limitans/ glial limiting membrane, a thin membrane comprised of astrocyte foot processes which acts as a protective barrier to the CNS, after approximately 3 months. In this lesion, SCs tended to myelinate axons out-with the glia limitans and oligodendrocytes predominantly ensheathed those within.

Conversely, in X-irradiation studies of the dorsal columns of neonatal rats, whereby the glia limitans did not reform so competently it was demonstrated that SCs and oligodendrocytes were able to mingle and remyelinate within close proximity in areas devoid of astrocyte processes and to some extent in astrocyte-rich areas where the astrocytic basement membrane was lacking (Blakemore & Patterson, 1975; Sims & Gilmore., 1983). In addition, Blakemore was able to determine that the extent of SC remyelination was directly related to the degree of astrocyte depletion by using a 6-aminonicotinamide (6-AC) induced lesion. This method resulted in a more wide-spread and extensive loss of endogenous glial cells, and demonstrated an even greater level of SC remyelination in the place of endogenous oligodendrocyte myelination (Blakemore, 1975). Furthermore, in demyelinating lesions induced by the glio-toxin, ethidium bromide (EB), which also resulted in the loss of local endogenous glial cells, remyelination was predominantly carried out by infiltrating SCs whilst oligodendrocyte myelination was limited to the edge of the lesion, which was typically devoid of SCs but rich in astrocytes (Blakemore, 1982; Graça & Blakemore, 1986). Similar studies in EB-induced lesions of the rat brain have also reported that whilst SC transplants extensively remyelinated denuded axons, their migration and myelinating capacity was limited to astrocyte free areas (Shields et al., 2000). The findings of Harrison (1985) may slightly contradict the aforementioned studies as they reported that SC remyelination in the spinal cords of irradiated and lysolecithin treated rats was limited in some instances, even in areas where the glia limitans *was* disrupted. However, they postulate that these findings may have been due to whether or not the perivascular glia limitans was intact, thus altering the root of entry for infiltrating SCs and possibly limiting their accessibility to areas of demyelination.

Studies which demonstrated that X-irradiation with a critical dose of 40 Grays prevented spontaneous endogenous remyelination (Blakemore & Crang, 1985; 1988) allowed researchers to investigate the cellular interactions between transplanted oligodendrocytes and other glial cell populations in demyelinated lesions. Blakemore & Crang (1989) reported that following the aforementioned lesion and subsequent transplantation of a mixed non-shaken culture of oligodendrocytes, OPCs and Type-1 cortical astrocytes, oligodendrocytes were able to remyelinate the lesion extensively in the non-irradiated cord. With transplantations of *shaken* cortical cultures (thus removing O-2A/OPC lineage cells), oligodendrocytes failed to successfully remyelinate and invading SCs became the dominant myelinating cell within the lesion. Most compellingly, when the myelinating potential of infiltrating SCs was reduced by the critical dose of X-irradiation (4O Grays), oligodendrocyte remyelination was similar following transplantation of shaken *and* unshaken cells, suggesting that the failure of oligodendrocytes within shaken cultures to remyelinate extensively was mediated by the presence of invading SCs. Franklin et al., (1992) added to these findings by reporting that in transplants containing SCs and Type-1 astrocytes which were devoid of OPCs, SC remyelination in the presence of astrocytes was not impeded as in previous studies. Thus, these findings suggested a specific role for transplanted or endogenous OPCs in mediating the astrocyte-dependent inhibition of SC remyelination in CNS lesions.

In summary, the above studies appear to highlight that in several models of demyelination, central and peripheral myelin were predominantly segregated by the presence of astrocyte processes within the glia limitans and that when this membrane was intact, SC infiltration within the lesion was limited. However, in astrocyte-free zones or in areas where the glia limitans was disrupted, infiltrating SCs extensively remyelinated denuded CNS axons to a greater extent than endogenous oligodendrocytes. Similarly, oligodendrocyte remyelination in the presence of astrocytes appeared to be dependent upon the exclusion of infiltrating SCs, thus alluding to an inhibitory relationship between SCs and oligodendrocytes i*n vivo*. Although these results appear to indicate that SC-remyelination in the damaged CNS may be hindered by the *physica*l barrier represented by endogenous astrocytes, which may limit their entry into the CNS, it may also be worth exploring whether or not SCs are capable of impeding

oligodendrocyte myelination in an astrocyte-dependent manner via biochemical/ molecular mechanisms.

As discussed throughout this thesis, astrocytes are a source of vital factors for neuronal and glial cell survival, proliferation and differentiation, such as PDGF (Noble et al., 1988; Richardson et al.,1988) and CNTF (Power et al., 2002; Nash et al., 2011). However, following disease or injury to the CNS, astrocytes undergo gliosis and are known to up-regulate their expression of chondroiton sulphate proteoglycans (CSPGs), which are inhibitory to axonal outgrowth (Dow et al., 1993, DeWitt et al., 1994; Lemons et al., 1999; Asher et al., 2000). Interestingly CSPGs have also been shown to impair oligodendrocyte process extension *in vitro,* whilst inhibition of CSPGs with xyloside following demyelination (Lau et al., 2012), or chondroitinase treatment in a spinal contusion model resulted in improved remyelination and OPC migration, respectively.

Other astrocyte-associated factors such as TGF-β have also been reported to play a role in oligodendroglial behaviour, though TGF-β has been reported to *enhance* oligodendrocyte differentiation by ceasing the proliferative signalling mechanisms induced by PDGF (McKinnon et al., 1993). TGF-β is a member of the TGF-β super-family, which also contains bone morphogenic proteins (BMPs), growth and differentiation factors (GDFs), activins/ inhibins, glial cell line derived neurotrophins (GDNFs). It consists of 3 isotypes, 1, 2 and 3, and is a ubiquitously expressed cytokine with anti-proliferative effects throughout the body, whilst also playing a major role in fibrosis and inflammation. TGF-β expression has been reported to be up-regulated in astrocytes within the extracellular protein-rich glial scar following CNS injury (Unsicker et al., 1991; Lagord et al., 2002) and in chronically active and inactive demyelinating lesions in MS (De Groot et al., 1999). Furthermore, TGF-β1 has been reported to induce astrocyte hypertrophy and cause up-regulation of ECM components such as laminin and fibronectin *in vitro*, suggesting that it may play a role in glial scar formation. Furthermore, it has also been shown to activate CTGF (Grotendorst et al., 1996) via interactions with the ERK and JNK signalling pathways (Xie et al., 2004).

As aforementioned, BMPs (BMP2-BMP20) are members of the TGF-ß super-family, and were originally characterised by their ability to regulate ectopic bone and cartilage formation (Chen et al., 1991; Nakase et al., 1994). However, they have also been reported for their integral role in regulating polarity in the neural tube during embryonic development, thus confining oligodendrogliogenesis to the ventral ventricular zone; a process which is regulated by BMP4 and its antagonists, noggin and chordin (Takahashi et al., 1996; Monsoro-Burq et al., 1996; Huang et al., 2004; Meulemans et al., 2004). BMP and BMP receptor expression (BMPR1A, BMPR1B and BMPRII) has been detected in astrocytes, oligodendrocytes, microglia and axons within the adult rat spinal cord (Miyagi et al., 2012). In addition, Sabo et al., (2011) reported that following cuprizone-mediated demyelination of the corpus callosum, BMP4 expression was up-regulated in astrocytes and oligodendrocytes, whereby it was said to induce proliferation of both cell types, which correlated with a reduction in oligodendrocyte maturation. However, administration of the BMP4 antagonist, noggin, resulted in an increase in oligodendroglial differentiation and in increase in myelin production. In models of experimental autoimmune encephalomyelitis (EAE), BMP4 expression was also said to increase with the onset of disease and peak with severity (Ara et al., 2008). It has been postulated that the actions of BMPs influence the fate of bi-potential cells, particularly in the case of OPCs, which showed an increased predisposition to differentiate into astrocytes rather than oligodendroglial cells following treatment with BMPs, though this effect could be reversed by FGF treatment (Mabie et al., 1997; Grinspan et al., 2000).

Whilst data from this thesis appears to strongly indicate that SC-secreted CTGF induces a reduction in endogenous oligodendrocyte myelination *in* vitro, it has thus far been unclear which cellular targets and signalling mechanisms were involved in mediating this effect. Re-examining previous published data from *in vivo* studies of demyelination could indicate that oligodendrocyte remyelination may be inhibited to some extent by SCs via astrocyte-dependent mechanisms. As astrocytes are key components of the glial scar and a rich source of both pro and inhibitory factors for myelination, it could be postulated that modification of their secretory profile through paracrine signalling could have significant effects on oligodendrocyte behaviour. Equally, given the physiologically diverse structure of CTGF, it could also be possible that its effects on inhibiting

endogenous CNS myelination are mediated via direct interactions with oligodendroglial cells; or via a combination of both mechanisms.

6.1.1 Aims

The aim of this chapter was to determine if astrocytes were the target of CTGF in the inhibition of myelination. Experiments were carried out in which astrocyte monolayers were incubated with SCM or CTGF prior to their use in the myelinating culture system, and myelination was assessed as normal after 26 days. Further aims included assessing transcriptional changes in astrocytes of factors specifically relating to scar formation or the inhibition of myelination following CTGF/SCM pre-treatment. In addition, this chapter aimed to investigate whether or not CTGF was able to influence the differentiation state of OPCs in a non-astrocyte dependent manner.

6.2 Pre-treatment of the Astrocyte Monolayer with SCM or CTGF Reduced Endogenous Myelination and Oligodendrocyte Differentiation *In Vitro*

As stated previously in Chapter 5, CTGF does not appear to have one distinct receptor however; integrins have emerged as a major class of possible receptors which can interact with CTGF (Babic et al., 1999; Gao et al., 2004, 2005; Schober et al., 2001). Consisting of 20 alpha subunits and 8 beta subunits, the primary role of integrins is to respond to environmental cues by interacting with components of the ECM to modulate several aspects of cell behaviour, such as adhesion and migration. In primary cultures of neurosphere-derived astrocytes (Ns-As), punctate α1 and α2 staining were both evidenced. Commonly associated with collagen or laminin binding, this kind of staining indicated the possibility for CTGF to interact with astrocytes via integrin receptors. (Cambier et al., 2005; Hirota et al., 2011) (Figure 6.1). Whilst it is highly unlikely that these are the only integrins expressed by astrocytes *in vitro*, as demonstrated in the literature (Milner et al., 1999, 2001, 2006; Gladson et al., 2000), the expression pattern evidenced appeared to be valid in that isotype controls showed little or nor background staining (Figure 6.1).

Initially, astrocytes were pre-incubated with SCs according to the methodology described in Chapter 2.5, namely that 2 confluent coverslips of SCs were placed in a Petri dish and allowed to condition a confluent coverslip of Ns-As for 4 days. During this time, cultures were fed once by removing half the media and replacing with fresh 10% FBS. Following pre-conditioning, the astrocyte coverslip was removed, rinsed gently in PBS and used as a monolayer for the mixed embryonic spinal cord cells as per the myelinating culture methodology. Some of the Ns-As coverslips were also retained for further analysis (see also section 6.3).

After 26 days in culture, myelination appeared greatly reduced, as did neurite density, though to a lesser extent (Figure 6.2; n=2). Furthermore, astrocytes which had been pre-conditioned by SCs displayed characteristics that were typical of gliosis, such as hypertrophy and an apparent increase in the intensity of GFAP immunoreactivity (as assessed by fluorescence) prior to their use in the

myelinating cultures. This phenotype would typically be observed *in vitro* during astrocyte and SC boundary formation (Lakatos et al., 2000; Fairless et al., 2005, De Silva et al., 2007, Higginson et al., 2012) (Figure 6.2 c, d).

To assess whether or not this reduction in myelination was simply due to the survival of fewer axons with the potential to become myelinated, astrocytes were also pre-treated every day for 4 days with SCM (in 10% FBS) and CTGF (11 kD peptide, 10 ng/ml), as well as with SCM and CTGF containing a neutralising antibody to CTGF at a concentration of 100 ng/ml (estimated to neutralise approximately 10 ng/ml CTGF) (see Figure 6.3 for more details). It was hypothesised that pre-treatment in this way would be less severe than the previous methodology, given that SC-secreted factors, including CTGF, would likely be at lower concentrations in diluted CM. Furthermore, there would be no direct competition for nutrients from SCs being co-incubated with astrocytes (as in Figure 6.2), which could impair the ability of the monolayer to support a myelinating culture.

Results from pre-treatment in this manner indicated that both CTGF and SCM pre-treatment of the astrocyte prior to its use in a myelinating culture resulted in a great reduction in the level of myelination observed after 26 days, compared with controls (Figure 6.4; n=2). Furthermore, the addition of a neutralising antibody to CTGF in cultures where the astrocyte was pre-treated with SCM or CTGF (Figure 6.4 d and f, respectively) appeared to restore myelination to a similar level as in control. The addition of anti-CTGF to astrocyte monolayers which were not pre-treated with CTGF or SCM did not appear to alter the level of myelination compared with control cultures (Figure 6.4 b). Neurite density did not appear to be affected by any of the treatments (Figure 6.4 g).

Although this preliminary data seemed to indicate the involvement of the astrocyte in mediating the CTGF-induced inhibition of endogenous myelination, it was unclear if the reduction in myelination correlated with impaired oligodendroglial cell differentiation within the cultures. To investigate this, astrocytes were pre-treated with CTGF and then set up in a myelinating culture

as previously described, and immunostaining was carried out after 16 days *in vitro* using antibodies to detect NG2 and the expression of the O4 antibody, as a means of assessing oligodendrocyte maturation. Preliminary results from these studies indicated that whilst the majority of the oligodendroglial cells within the culture expressed O4 in the absence of the earlier marker NG2, this figure was reduced by approximately 10% following CTGF pre-treatment of the astrocyte (Figure 6.5; n=2). These results seem to indicate that oligodendrocyte maturation was impaired by this treatment, given that CTGF pre-treatment also resulted in approximately 6% more of the oligodendroglial population expressing both NG2 and O4, which is typical of the transient stage of differentiation. Furthermore, whilst there were very few cells within control cultures which exclusively expressed the OPC marker NG2, preliminary data suggested that there were 8 times as many of these cells in CTGF astrocyte pre-treated cultures (Figure 6.5 c). It is unlikely that this increase in OPC numbers, however small, in treated cultures was due to the increased proliferation of OPCs, since oligodendroglial cell counts (excluding those which did not label for either marker to eliminate astrocytes, microglia and neuronal cells) indicated that cell numbers were relatively unchanged in treatment versus control (45 ± 2.17 and 50.1 ± 3.59, respectively).

Thus, this preliminary data suggests that the actions of CTGF in reducing endogenous myelination may be mediated via astrocyte-dependent mechanisms, with a resulting reduction in the differentiation state of oligodendrocytes within our cultures. However, this data needs to be replicated to demonstrate statistical significance before any real conclusions can be drawn.

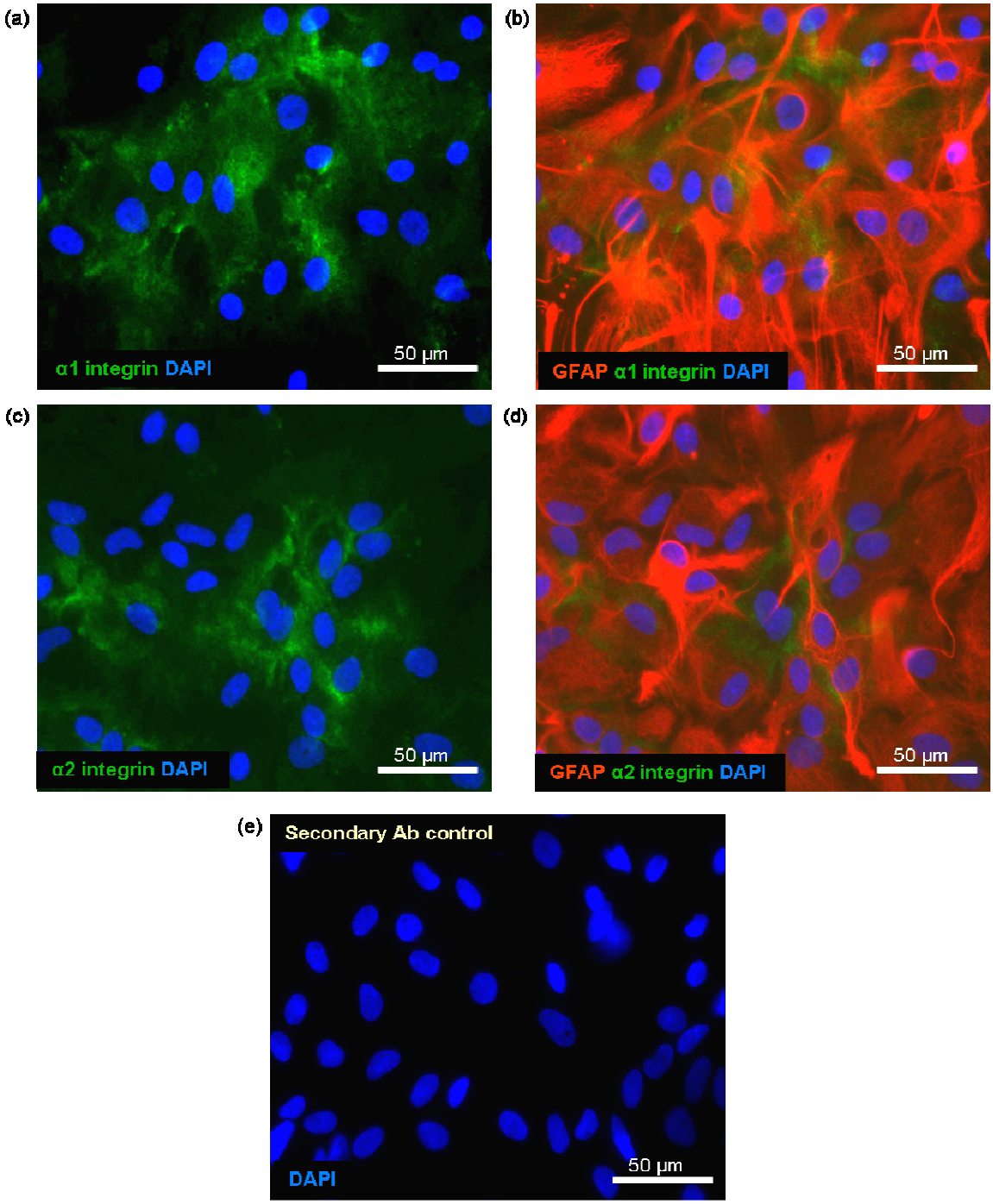


Figure 6.1 *Astrocytes derived from neurospheres expressed both the a1 and a2 integrin receptor sub-units in vitro.* Neurospheres were generated from culturing the dissociated striatum of neonatal rats for approximately 7-10 days. After this period, 10% FBS was used to induce the differentiation of neurospheres into astrocytes, which were cultured to confluency for a further 7 days prior to immunostaining. Immunofluoresence for both the α1 (a) and α2 (c) integrin subunits was evidenced by way of punctuate staining on the cell surface. Isotype controls (e) showing little or no background staining indicated that this staining was unlikely to be an artefact. An antibody against GFAP labelled astrocyte intermediate filaments (b, d). Images were captured with an Olympus BX51 epifluorescence microscope. Scale bar=50 µm.

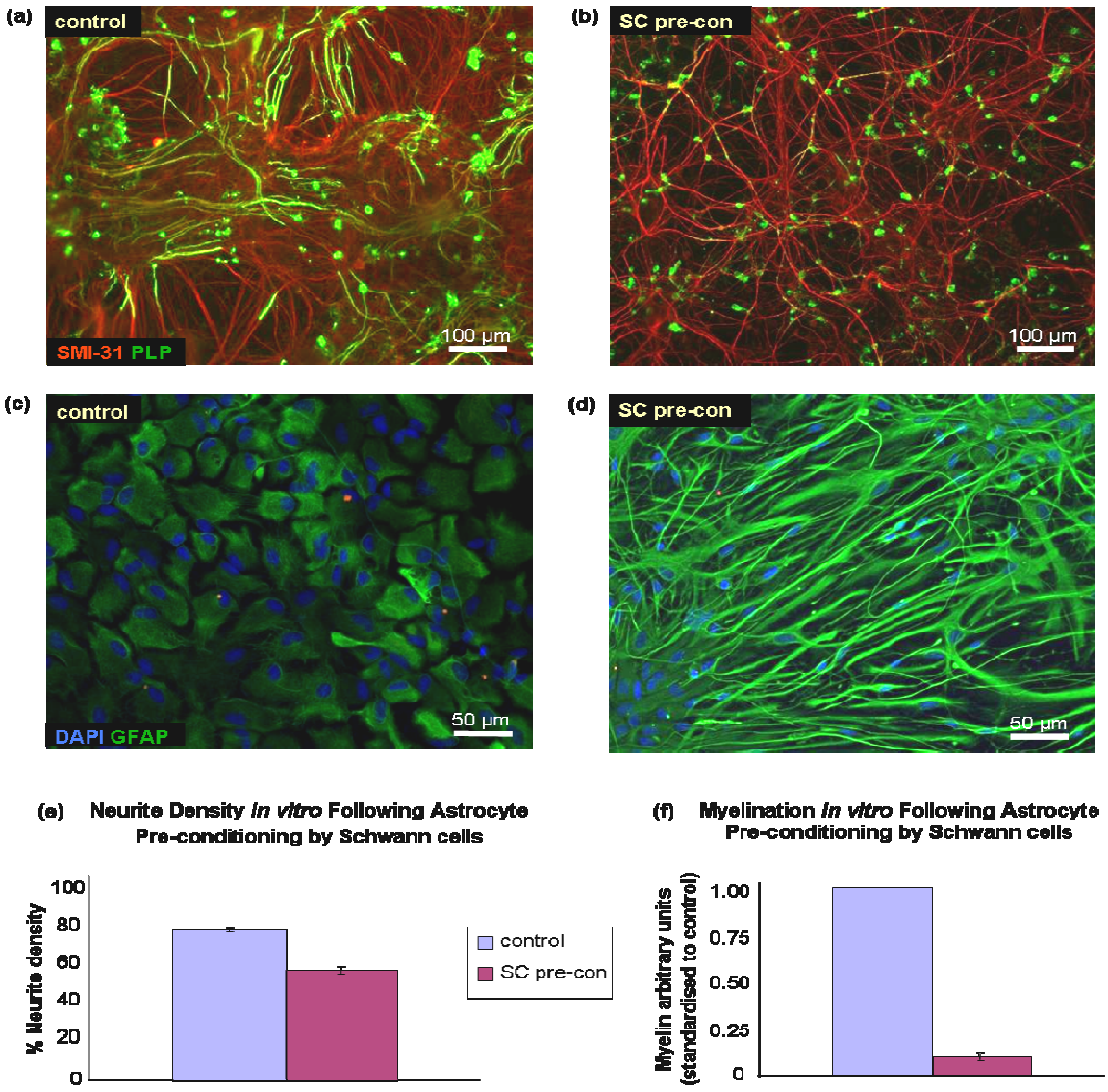


Figure 6.2 – *Pre-conditioning of the astrocyte monolayer with SCs greatly reduced myelination and neurite density, whilst also inducing morphological changes characteristic of reactive hypertrophy in astrocytes.* Confluent monolayers of astrocytes were generated by differentiating neurospheres in 10% FBS. Two confluent coverslips of purified SCs were then placed in a Petri dish and allowed to condition a single astrocyte coverslip for 4 days. Feeding occurred once during this time. The astrocyte monolayer was rinsed in PBS and used in the myelinating cultures as normal. Preliminary results indicated that SC pre-treatment (b, f) greatly reduced the level of myelination compared with controls (a, f). Furthermore, neurite density also appeared to be reduced (e). SC pre-conditioning induced morphological changes typical of gliosis and boundary formation, with an apparent increase in the intensity of GFAP immunoreactivity (c, d) after 4 days of treatment. SMI-31 labelled neurites, an antibody against PLP labelled myelin sheaths and oligodendrocytes, GFAP stained astrocytes and DAPI depicted nuclei. Scale bars=100 µm (a, b) and 50 µm (c, d) Images were captured with an Olympus BX51 epifluorescence microscope. n=2.

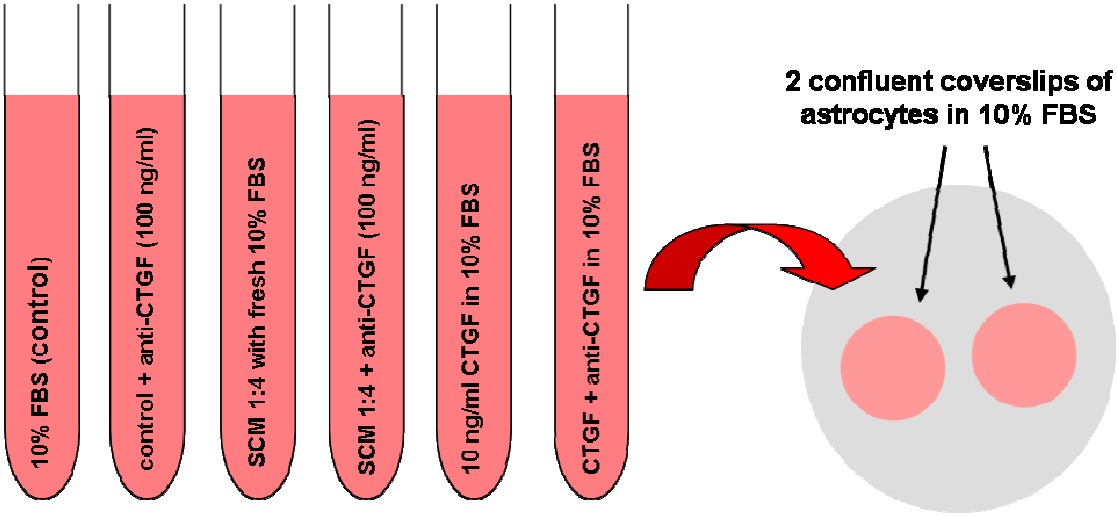


Figure 6.3 - *Pre-treatment of the astrocyte monolayer with SCM or CTGF plus or minus a neutralising antibody to CTGF*. A second methodology of pre-treating astrocytes prior to their use in myelinating cultures was devised whereby CM was taken from SCs and diluted with fresh 10% FBS at a ratio of 1:4 before being added to the cultures every day for 4 days. A neutralising antibody to CTGF (100 ng/ml, neutralises ~10 ng/ml of CTGF) was also added to diluted SCM and then used to treat cultures as above. The 11 kD CTGF peptide was added to 10% FBS, ensuring that the final concentration would be approximately 10 ng/ml in each dish during the course of normal feeding, whereby half the media was removed and replaced each time. Similarly, the neutralising antibody to CTGF was also added to media containing CTGF peptide at a concentration of 100 ng/ml. Controls included feeding with 10% FBS (as per normal astrocyte conditions) and the addition of anti-CTGF to 10% FBS without the addition of SCM or CTGF. After 4 days of treatment, the astrocytes were rinsed briefly in PBS and then used as a monolayer for myelinating cultures, and some were also retained for further analysis.

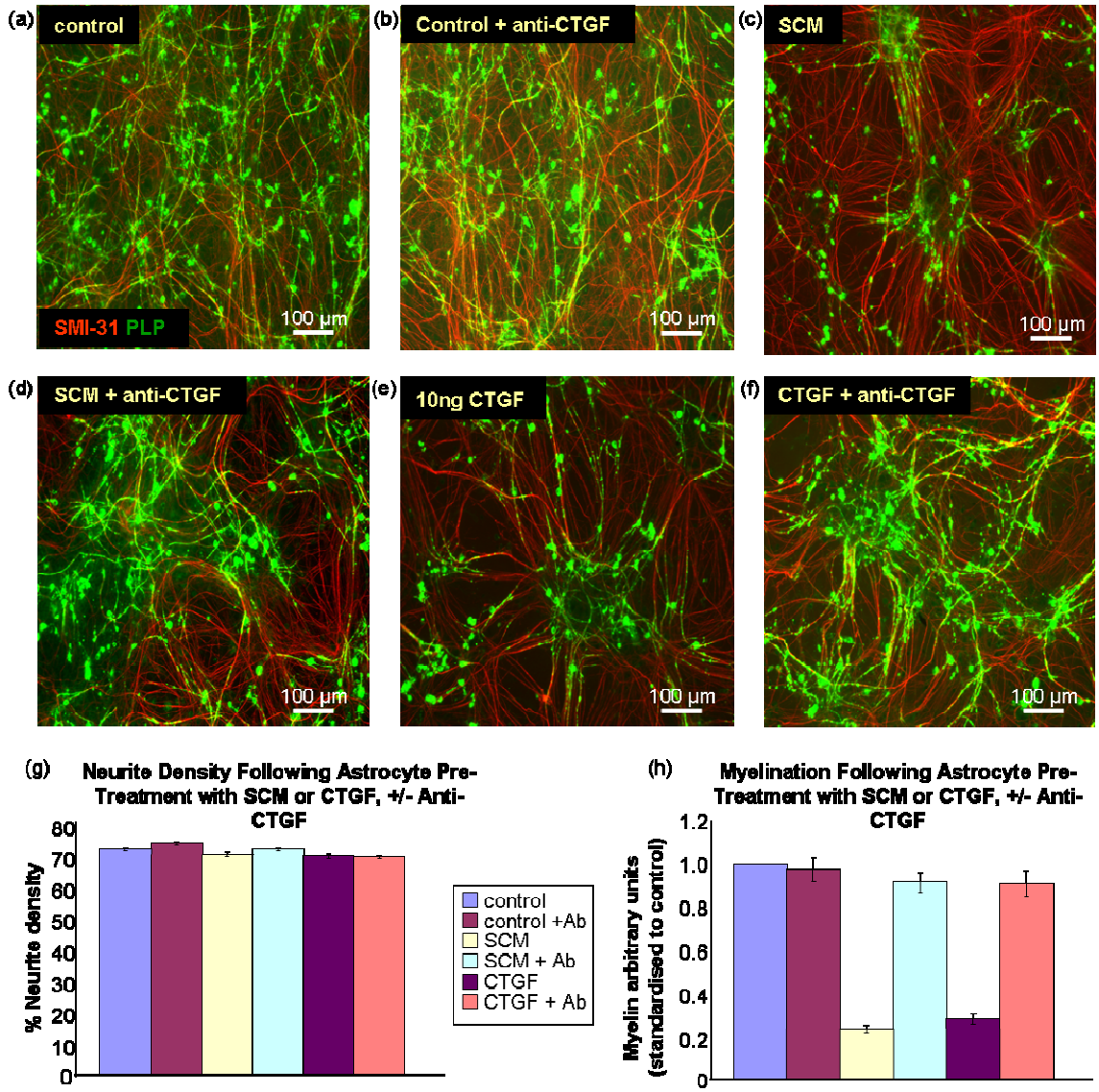


Figure 6.4 - *Pre-treatment of astrocytes with CTGF or SCM greatly reduced myelination compared with controls.* Astrocytes were pre-treated every day for 4 days with 10% FBS mixed with either SCM, CTGF, SCM + neutralising antibody to CTGF or CTGF + neutralising antibody. Additional controls included 10% FBS plus the antibody. Preliminary data suggested that pre-treatment of the astrocyte monolayer with both SCM (c) and CTGF (e) greatly reduced the level of myelination compared with controls (a, h). This effect, however, was reversed by the antibody-mediated neutralisation of CTGF (d, f). Furthermore, the antibody alone did not appear to affect myelination (b). In all conditions, neurite density was unaffected. SMI-31 labelled neurites, and the antibody against PLP labelled myelin sheaths and oligodendrocytes. Scale bars=100 µm. Images were captured with an Olympus BX51 epifluorescence microscope. n=2.

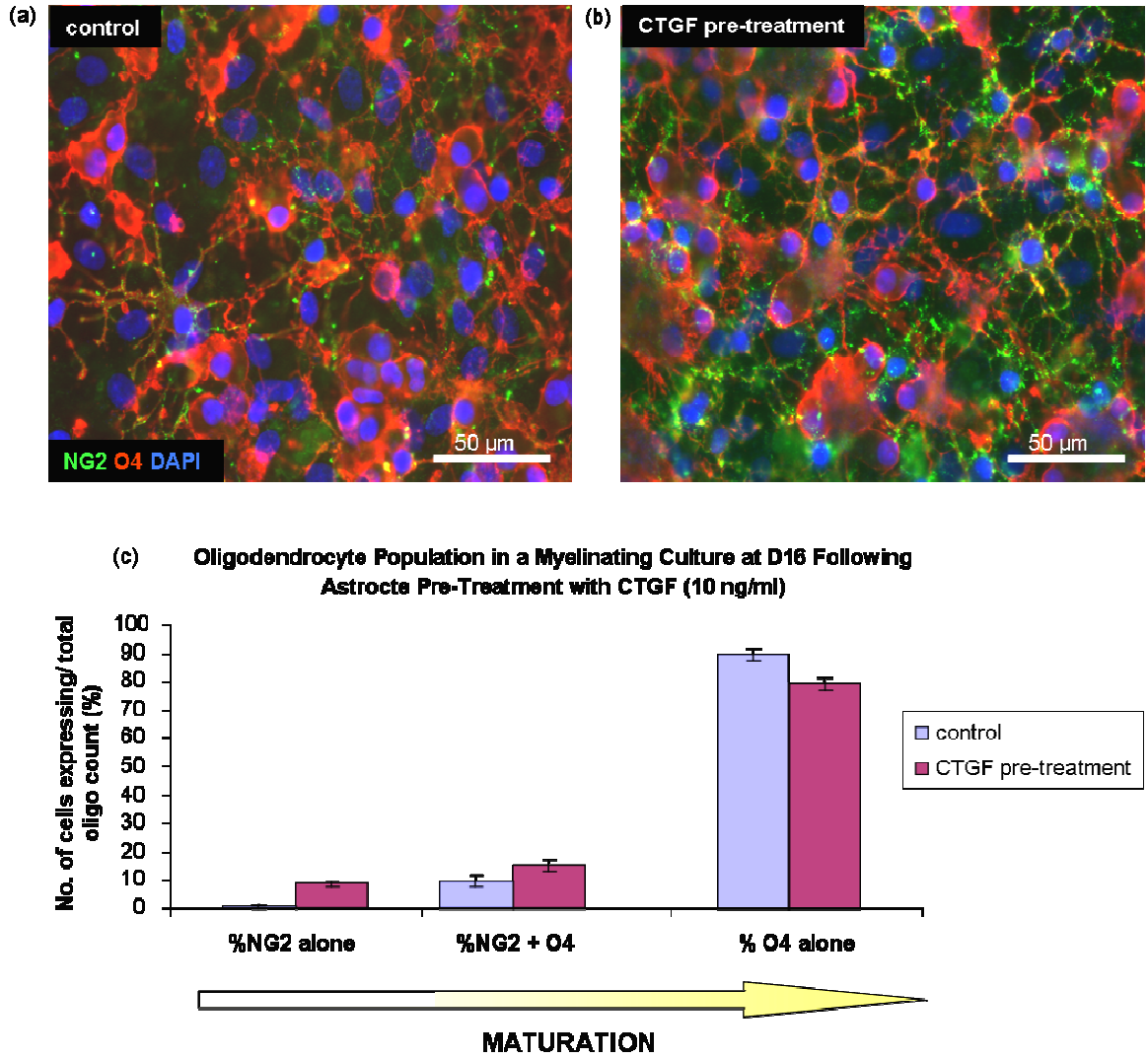


Figure 6.5 - *Pre-treatment of astrocytes with CTGF prior to their use as a monolayer reduced the differentiation of endogenous oligodendroglial cells within the myelinating cultures.* Astrocytes were pre-treated over a 4 day period with 10% FBS containing 10 ng/ml of CTGF (b), before being used as a monolayer in the myelinating culture system. Immunofluorescent labelling was carried out after 16 days *in vitro*. Preliminary data suggested that whilst the majority of oligodendroglial cells expressed O4 alone, there was an apparent 8-fold increase in the NG2 progenitor population along with a slight increase in the number of cells which transiently expressed both markers and a decrease in those exclusively expressing O4, suggesting a reduction in oligodendrocyte differentiation (c). An antibody against NG2 labelled OPCs and early lineage oligodendroglial cells, whilst the O4 antibody recognised both intermediate and mature oligodendrocytes. DAPI labelled nuclei. Scale bars=50 µm. Images were taken using an Olympus BX51 epifluorescence microscope, n=2.

6.3 Preliminary Data Suggested that SCM and CTGF Pre-Treatment Caused Transcriptional Changes in Astrocytes

Following astrocyte pre-treatment with SCM or CTGF or pre-conditioning with SC coverslips, some of the astrocyte monolayers were retained for further analysis. RT-qPCR and Western blot studies (see sections 2.4 and 2.6, respectively) were employed to investigate whether or not astrocytes altered their expression of factors associated with glial scar formation or the inhibition of oligodendrocyte maturation as a consequence of pre-treatment. Two coverslips from each condition were examined for each technique, whilst samples were loaded in triplicate for RT-qPCR studies. Results from RT-qPCR experiments were standardised to control and expressed relative to a housekeeping gene (GAPDH) to give a relative indication of fold change in expression (RQ value), rather than any information directly relating to the *amount* of mRNA in each condition. Quantitative analysis of Western blots was calculated by assessing the optical densitometry of each band from its pixel value in Image J, and this value was expressed relative to the loading control for each condition (GAPDH).

Preliminary results indicated approximately a 3.5 and 4.5 fold change in the expression of both GFAP and BMP-4 respectively, following CTGF pre-treatment, compared with untreated astrocytes (Figure 6.6; n=2). Similarly, SCM appeared to increase expression of both markers by approximately 2.5 - 3 times, compared to controls. The expression of BMP-4 in astrocytes which were pre-treated with CTGF was also up-regulated approximately 2-fold, compared with SCM treated cultures. Smaller increases in TGF ß expression were observed following SCM pre-treatment, and to a lesser extent pre-treatment with CTGF, compared with controls. Repetition of these experiments is needed to provide evidence of statistical significance.

From Western blot analysis, the expression of GFAP did not appear to be greatly altered at the protein level by pre-conditioning with SC coverslips (Figure 6.7; n=2). As an additional control, 3 coverslips of astrocytes were also placed together in a Petri dish to ensure that any changes which may have occurred were due to the presence of SC-derived factors and not related to the physical

constraints of housing 3 coverslips/dish, as opposed to 2 coverslips in our normal experimental procedure. For example, by placing 3 coverslips in a dish, it is possible that there may be a greater demand for nutrients on the cultures and perhaps increased autocrine signalling of astrocyte-secreted factors. However, GFAP levels in the "3 coverslip control" were relatively comparable to those observed in the untreated (2 coverslip) control.

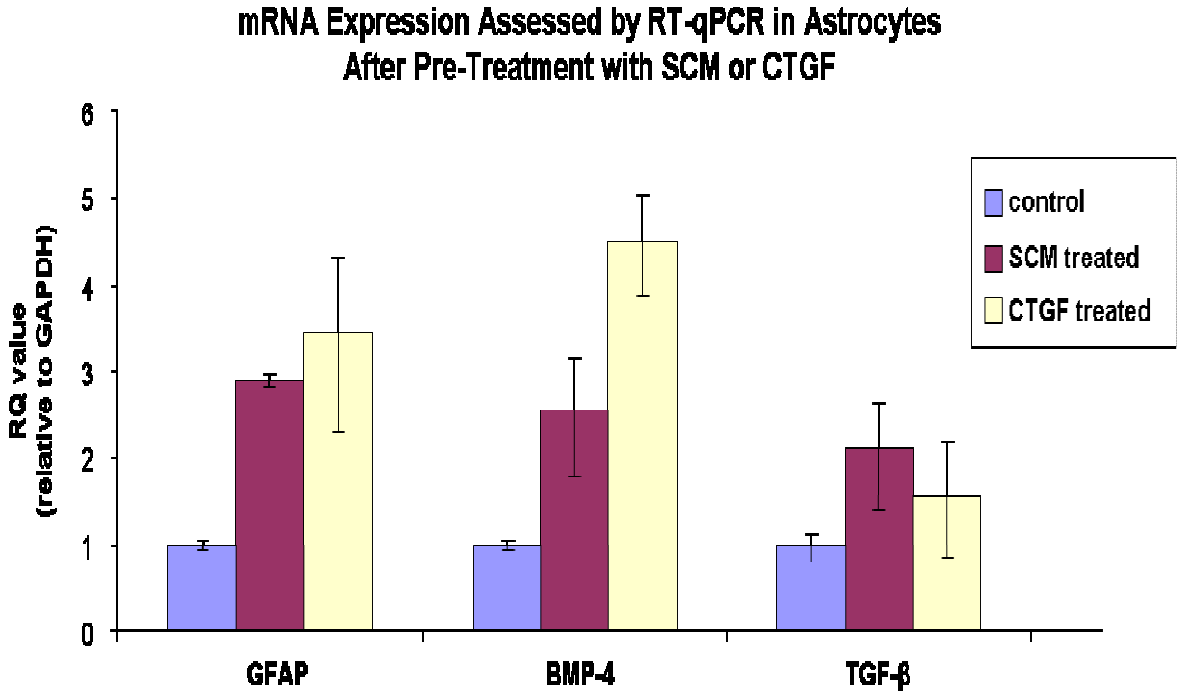
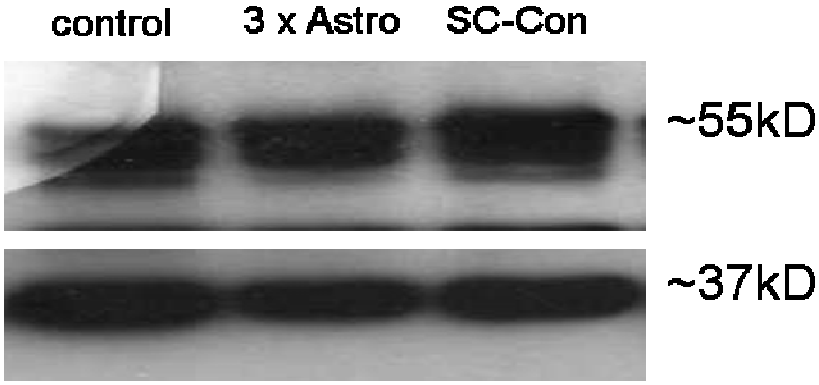


Figure 6.6 - *SCM and CTGF pre-treatment of astrocytes may cause them to alter their expression of mRNA for GFAP, BMP-4 and, to a lesser extent, TGF β.* RNA was extracted from astrocytes which had been pre-treated with CTGF or SCM for 4 days and converted to cDNA using a commercial kit. RT-qPCR was carried out to assess fold changes in mRNA expression (RQ value), which were standardised to control levels and relative to the housekeeping gene, GAPDH. Preliminary results suggested that CTGF pre-treatment caused astrocytes to up-regulate their expression of both GFAP and BMP-4 mRNA compared with untreated controls. Similarly, SCM pre-treatment also induced the up-regulation of GFAP and BMP-4 mRNA versus controls, in the magnitude of 2-3 times. Compared to pre-treatment with SCM, CTGF pre-treatment resulted in a greater increase in BMP-4 mRNA expression. Subtler increases in TGF-β expression were also observed following SCM pre-treatment and to a lesser extent, astrocyte pre-treatment with CTGF compared with controls, though these may not be significant upon repetition. n=2.

(a)



(b)

	CONTROL	3 X ASTRO	SC-CON
GFAP/GAPDH	0.8446638	0.9890852	1.086312

Figure 6.7 - *GFAP protein does not appear to be greatly altered following SC pre-conditioning of the astrocyte*. 2 coverslips of SCs were allowed to condition a single confluent astrocyte coverslip for 4 days. As an additional control, 3 coverslips of astrocytes were also placed together in a Petri dish for 4 days. Quantitative values were made by calculating the optical densitometry (pixel value) for each band and expressing this as a value relative to the loading control/ housekeeping protein for each condition (GAPDH). After conditioning, astrocyte coverslips were rinsed in PBS and lysed for use in Western blot studies. Preliminary results suggested that astrocyte pre-conditioning by SCs did not greatly alter the expression of GFAP. Similarly, GFAP levels remained relatively unchanged in the 3 coverslip control, compared with both SC-pre conditioning and untreated controls. n=2.

6.4 Treatment of Purified OPCs with SCM or CTGF Inhibited their Differentiation by Altering the Expression of Oligodendroglial Markers and Inhibiting Morphological Changes Associated with Maturation

Purified cultures of OPCs were generated by shaking cortical astrocytes to displace the top-dwelling layer of progenitor cells (see also section 2.1.5). Differential attachment was then used to eradicate contaminating microglia (which preferentially attach to tissue culture plastic) and the resulting cell suspension was cultured on glass coverslips in a 24-well plate in Sato media (DMEM-BS modified by Bottenstein & Sato, 1979) supplemented with the mitogens FGF and PDGF to promote self renewal and to maintain the OPCs as progenitors (Noble et al., 1990; Bögler., 2001). After 4-5 days, all media was removed and coverslips were rinsed gently with PBS before treatment began. SCM (collected in Sato/ DMEM-BS) and CTGF (11 kD; 10 ng/ml) were added to Sato media and used to treat cultures every other day for 7 days. Controls included maintaining some coverslips in OPC growth factors (GF) to represent the progenitor population, and feeding others with Sato alone, which induces the normal differentiation of OPCs into mature oligodendrocytes. NG2 and O4 were used as markers to assess the degree of maturation following each treatment.

After 7 days there were significantly more cells following GF treatment, as assessed by counting $DAPI^{+ve}$ nuclei, compared with other treatments, which would be expected given that both FGF and PDGF are known to enhance proliferation and to aid expansion of OPCs (Noble et al., 1988; Wolswijk & Noble, 1992) (Figure 6.8 e; p values= <0.05). There were no significant differences in the cell counts when comparing any other treatments. The % of cells expressing NG2 alone (in the absence of O4) was significantly less in Sato media (Figure 6.8 b), compared with GF treatment (Figure 6.8 a) (p value=0.03) or treatment with SCM (Figure 6.8 c; p value=0.02) or CTGF (Figure 6.8 d; p value=0.03). This sub-set of cells would typically represent an early lineage/progenitor population of oligodendroglial cells and there were virtually no cells matching this description based upon the expression of NG2 alone, as would be expected in Sato media which is used to induce OPC differentiation. There was a slight reduction in the amount of cells solely expressing NG2 in both treatments compared with GF

controls, however these changes were not significant (p values= >0.05.). Equally, there was a significantly greater proportion of cells in the transient stages of differentiation (expressing both NG2 *and* O4) in GF conditions, compared with Sato treatment (p value=0.02); or following treatment with SCM or CTGF compared to Sato conditions (p values=0.02). Again, there were slightly less cells expressing both markers following treatment with SCM or CTGF compared with GF conditions, although this was not significant (p values= >0.05). Approximately 80% of the cells which had been treated in Sato alone solely expressed O4 (in the absence of NG2), and this value was significantly greater than the % of cells expressing O4 alone in GF cultures (p value= <0.01), and in SCM (p value= <0.01) or CTGF treated cultures (p value=0.01), thus suggesting the induction of a less differentiated phenotype following treatment. However, there was a significantly greater proportion of cells labelling with the O4 antibody in the absence of NG2 following treatment with SCM (p value=0.03) or CTGF (p value=0.04) compared with GF controls. When comparing SCM treatment with CTGF treatment, there were no significant differences across all comparisons.

Taken together, these results suggest that whilst the majority of OPCs grown in Sato media lose their expression of NG2 to label with the O4 antibody after 7 days *in vitro*, the cells grown in GF are less differentiated, being fairly evenly spit between a typical $NG2^{+ve}$ progenitor population and a sub-set of cells in the transient stages of differentiation (expressing NG2 and the O4 antibody). Treatment with SCM or CTGF, however, resulted in significant shifts in marker expression suggesting that although these cells were more differentiated than those grown in GF, they were significantly less differentiated than OPCs which were grown in Sato media alone.

When carrying out these experiments, it was also noted that whilst cells from each condition could express the same marker, their morphology was often markedly different. This is unsurprising giving that markers such as the O4 antibody can be expressed in the intermediate stages of oligodendrocyte differentiation and then persist throughout maturation, with O4 antibody labelling frequently being observed in the myelin sheath. Therefore, a system of quantifying differentiation based upon characterising morphology was also used

to re-analyse images as a more sensitive means of assessing oligodendrocyte maturation. Adapted from the methodology published by Huang et al., (2011) to also include a descriptive term for progenitor cells, this system involved characterising cells as pre-simple, simple, complex and membrane forming to describe the level of branching and myelin formation in each cell (Figure 6.9 a). Values were given as % morphology of the total oligodendroglial population.

Quantification using this system confirmed that the majority of cells in GF conditions retained a pre-simple morphology with little obvious branching, typical of a progenitor cells (Bögler et al., 1990). This value was significantly higher than the % of cells displaying this morphology following incubation in Sato media (p value= <0.01) and SCM or CTGF treatment (p values=0.03). However, there was also a higher proportion of cells with this morphology following SCM or CTGF treatment compared with Sato media alone (p values= <0.01). Furthermore, the % of cells with a simple morphology (mostly primary branching) was significantly higher in Sato media (p value=0.04) and SCM or CTGF treatment (p values=0.03, 0.02) compared with GF conditions. There was no significant difference in the % of cells displaying this morphology in either treatment (CTGF or SCM) versus Sato media conditions (p values= >0.05). Virtually none of the cells grown in GF exhibited a complex (highly branched) morphology, and this value was highly significantly less than in Sato media (p value= <0.01) and in SCM or CTGF-treated cultures (p values=0.02 and 0.04, respectively). In addition, there were also significantly fewer cells with complex morphologies after both the aforementioned treatments (SCM or CTGF) compared with Sato media (p values=0.02). Finally, whilst there wasn't a great deal of myelin membrane formation for any conditions, there was significantly more when OPCs were grown in Sato media compared to GF conditions or SCM and CTGF treatment. There were no significant differences when comparing myelin membrane formation in treated cultures (SCM/ CTGF) versus GF conditions (p values= >0.05).

This data indicated that SCM or CTGF treated OPCs were more differentiated than those grown in GF, but significantly less so than OPCs grown in Sato media. Interestingly, however, these differences appeared to be significant in the very

early stages of morphological maturation as well as in the formation of complex branching and maturation, whilst intermediate stages of differentiation did not seem to be significantly altered, based upon morphological characterisation.

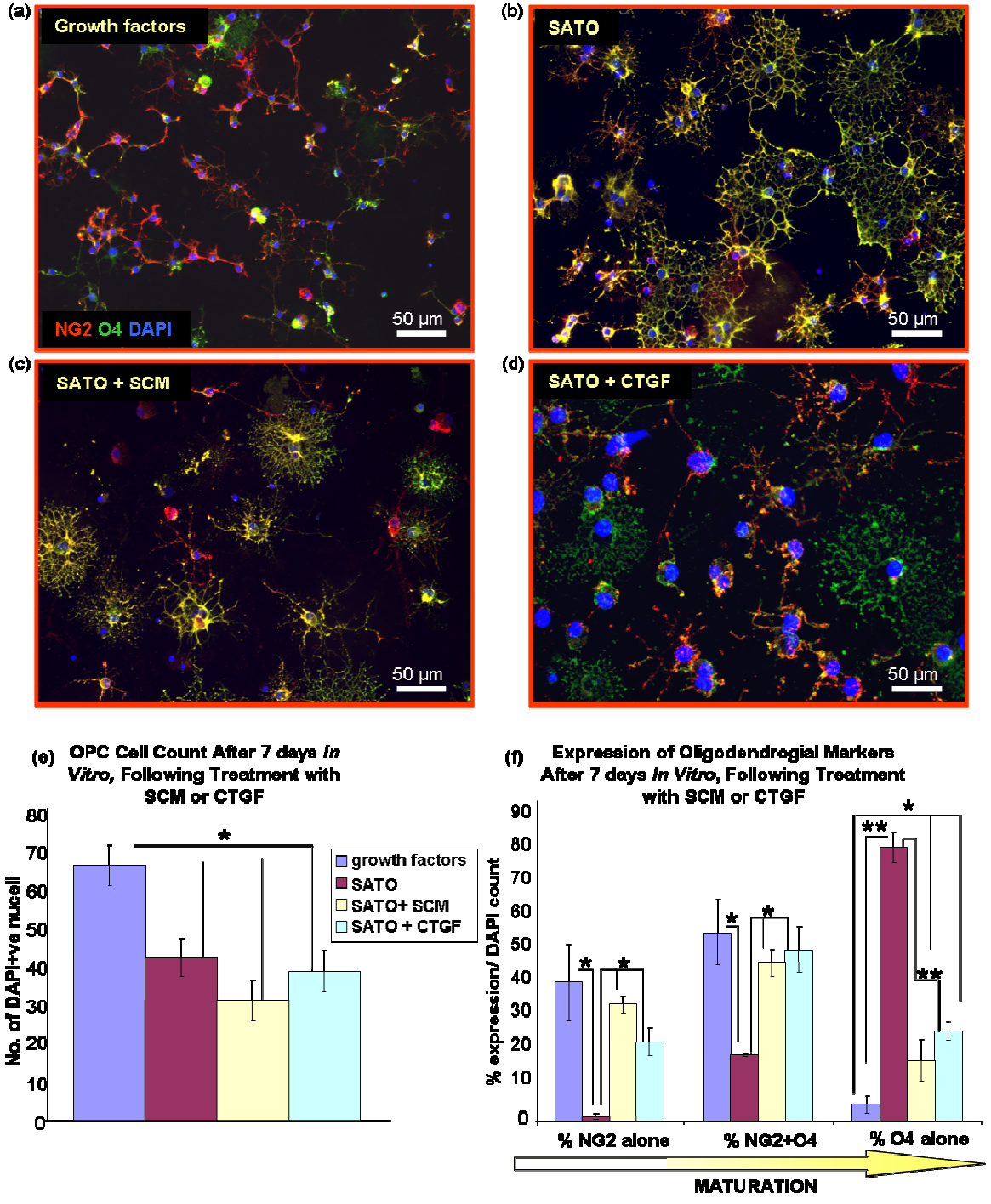
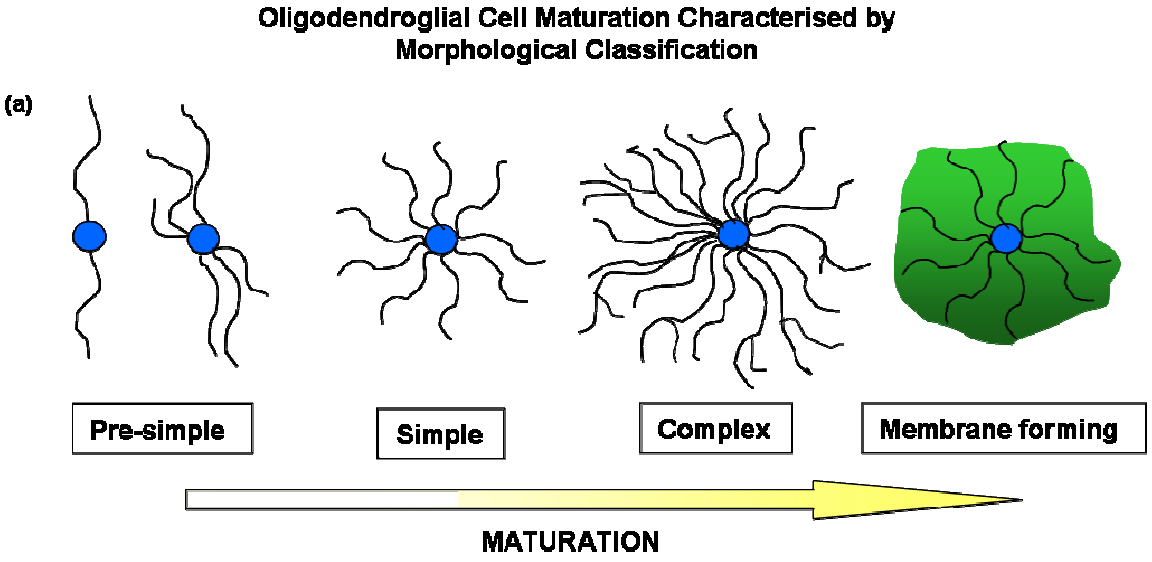


Figure 6.8 – *The expression of OPC differentiation markers is significantly reduced following treatment with SCM or CTGF.* OPCs were shaken from cortical astrocytes and contaminating microglia were eliminated via differential attachment to tissue culture plastic. Cells were maintained in Sato media supplemented with FGF and PDGF for approximately 5 days to allow for expansion. After this period, some were kept in growth factors (GF) (a) to retain their progenitor phenotype, whilst others were placed in Sato media without further supplementation (b) to encourage their differentiation into oligodendrocytes. Treatments included the addition of SCM (c) or CTGF (d) (10 ng/ml) to Sato media. Cells were fed/ treated every other day for 7 days before immunofluorescent labelling. Cells counts were considerably higher in proliferative GF media compared with all other conditions, as would be expected (e). There were no obvious differences in the cell counts when comparing SCM or CTGF treated cultures with those grown in Sato media. Treatment with SCM, CTGF or GF significantly increased the proportion of cells which expressed NG2 in the absence of expression of the O4 antibody (f). However, although there were slightly less cells in this category following treatment with SCM or CTGF compared with GF, this reduction was not significant. Similarly, the number of cells expressing both markers simultaneously was significantly greater in GF conditions and in SCM or CTGF treated cultures, compared with Sato conditions. Whilst there appeared to be a slight decrease in the % of cells expressing NG2 and the O4 antibody after treatment, this reduction was not significant. Approximately 80% of oligodendroglial cells grown in Sato media expressed the O4 antibody without NG2 immunolabelling and this value was significantly higher than that observed in GF conditions, or with SCM or CTGF treatment. The % cell expressing the O4 antibody (in the absence of NG2 expression) was also significantly greater after SCM or CTGF treatment versus GF conditions. There were no significant differences in expression when comparing SCM treatment with CTGF treatment. Antibodies against NG2 labelled early lineage/ progenitor cells whilst the O4 antibody labelled intermediate to mature oligodendrocytes. Scale bars=50 μm An Olympus BX51 epifluorescence microscope was used to capture all images. ***** = p values < 0.05; ****** = p values < 0.01. n=3.


Oligodendroglial Cell Maturation Characterised by
Morphological Classification
(a)
Pre-simple
Simple
Complex
Membrane forming
MATURATION

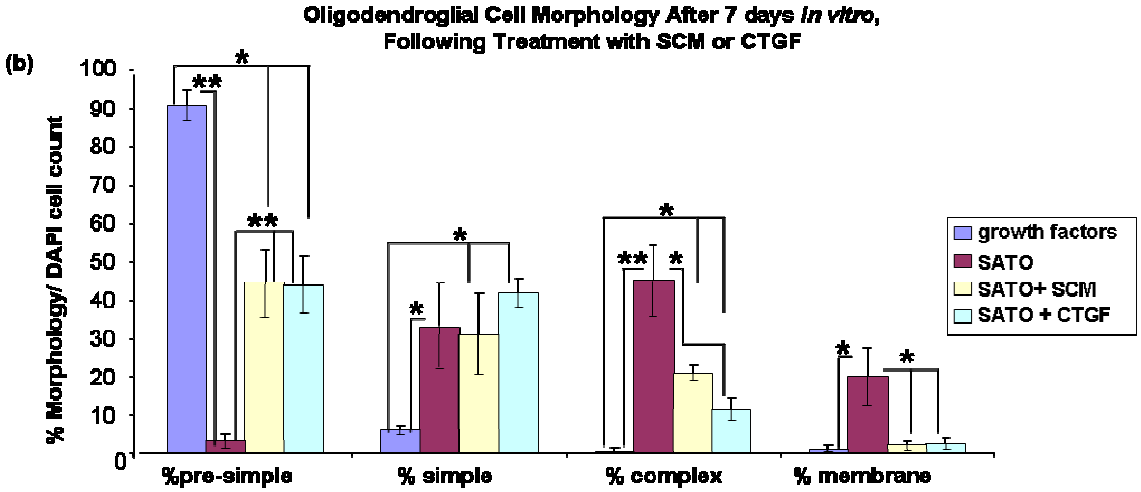

Oligodendroglial Cell Morphology After 7 days *In vitro*,
Following Treatment with SCM or CTGF
(b)
% Morphology/ DAPI cell count
100
90
80
70
60
50
40
30
20
10
0
%pre-simple
% simple
% complex
% membrane
growth factors
SATO
SATO+ SCM
SATO + CTGF

Figure 6.9 – *OPCs treated with SCM or CTGF were significantly less differentiated than those cultured in Sato media, based upon morphological classification.* OPCs were shaken from cortical astrocytes and contaminating microglia eliminated via differential attachment. Cells were maintained in Sato media supplemented with FGF and PDGF for approximately 5 days to aid their expansion. After this period, some were kept in growth factors (GF) to retain their progenitor phenotype, whilst others were placed in Sato media without further supplementation to encourage their differentiation into oligodendrocytes. Treatments included the addition of SCM or CTGF (10 ng/ml) to Sato media. Cells were fed/ treated every other day for 7 days before immunofluorescent labelling. Quantification was carried out by assessing cells according to their morphology (a) as a % over the total oligodendroglial cell count. "Pre-simple" described cells which were either bipolar or exhibited very little branching of their processes. The term "simple" was assigned to cells where there was evidence of moderate primary branching and the cells were no longer polarised. "Complex" described cells with extensive primary and secondary branching, whilst "membrane" was used to characterise cells which formed flat sheets of myelin membrane and were considered to be terminally differentiated. Results showed that there were significantly more pre-simple cells in GF cultures and SCM or CTGF cultures compared with Sato media conditions (b). However, the % of pre-simple cells was also greater in GF cultures versus SCM or CTGF treatment. GF treatment gave rise to significantly fewer cells of simple morphology compared to all other treatments, whilst there were no differences in the occurrence of this classification when comparing Sato cultures to those which had been treated with SCM or CTGF. Very few cells which were cultured in GF displayed a complex morphology and this value was significantly less than that observed in Sato media. Whilst there was a significantly greater proportion of complex cells detected following SCM or CTGF treatment compared with GF conditions, there was a significantly lower % of these cells when comparing these treatments to Sato media controls. Membrane formation was significantly reduced in all conditions compared with Sato media cultures. For all comparisons, there were no significant differences when comparing SCM treatment to CTGF treatment. Antibodies against NG2 labelled early lineage/ progenitor cells whilst the O4 antibody labelled intermediate to mature oligodendrocytes. An Olympus BX51 epifluorescence microscope was used to capture all images. ***** = p values < 0.05; ****** = p values < 0.01. n=3.

6.5 Discussion

Data from this chapter has demonstrated that CTGF may exert its inhibitory effects on endogenous myelination via both direct effects on the differentiation of oligodendrocytes and via indirect mechanisms, since preliminary data has suggested that modulation of the astrocyte monolayer by SCM/ CTGF also greatly inhibited myelination and reduced oligodendrocyte differentiation in the myelinating cultures. Furthermore, initial results suggest that astrocyte pre-treatment with SCM or CTGF may induce transcriptional changes in factors which mediate aspects of glial cell behaviour.

Whilst neurite density was reduced in cultures where the astrocyte monolayer had been pre-conditioned in a Petri dish for 4 days with 2 confluent coverslips of SCs (Figure 6.2), this may have been due to deterioration of the health of the astrocyte due to the depletion of vital nutrients being utilised by 3 coverslips as opposed to the normal 2 used in controls. Equally, SCs are known to induce astrocyte reactivity and it has been demonstrated that in our culture system, the phenotype of the astrocyte is vitally important in supporting adequate survival and myelination of the cultures (Nash et al., 2011). Thus, the resulting poor neurite density may have been due to the induced reactive astrocyte phenotype being less supportive for the culture, as opposed to the actions of SC-secreted factors on the neuronal population since SCs have been well characterised for their expression of factors, such as laminin and NGF, which are known to support neurite outgrowth.

To assess this observation further, astrocytes were also pre-treated with SCM or CTGF (11 kD, 10 ng/ml) plus or minus a neutralising antibody to CTGF (Figure 6.3). In this paradigm, SCM was diluted in 10% FBS, therefore SC-secreted factors were likely to have been at lower concentrations than in the previous experiments, where 2 coverslips of SCs were continually secreting into the shared astrocyte media for 4 days. Preliminary results from these experiments indicated that even though neurite density was not reduced, myelination was still greatly inhibited following SCM or CTGF pre-treatment of the astrocyte

monolayer, suggesting a direct inhibition of myelination. Interestingly, there was no obvious difference in myelination with SCM pre-treatment versus pre-treatment with CTGF. In addition, antibody-mediated neutralisation of CTGF restored myelination in both conditions, suggesting that CTGF may be the sole or dominant factor in mediating this astrocyte-dependent inhibition of endogenous myelination (Figure 6.4). Analysis of the myelinating cultures after 16 days *in vitro* suggested that there were slight changes in the differentiation state of endogenous oligodendrocytes following CTGF pre-treatment of the astrocyte monolayer, in that there appeared to be a greater % of NG2 labelled progenitors in these cultures and a reduction in the number of cells which labelled with the O4 antibody compared with untreated controls (Figure 6.5). However, since this experiment has only been carried out on 2 biological replicates, it needs to be repeated to ensure the statistical significance of our preliminary findings

RT-qPCR analysis of pre-treated astrocytes prior to their use in the myelinating cultures suggested that there may be an up-regulation in the mRNA expression of BMP-4 and GFAP in treated astrocytes compared with their untreated counterparts (Figure 6.6). As discussed in the introduction to this chapter, up-regulation of GFAP is associated with reactive astrogliosis, commonly associated with the glial scar *in vivo*. BMP 4 on the other hand has been shown to inhibit oligodendrocyte differentiation (as observed in astrocyte pre-treated myelinating cultures), whilst enhancing OPC proliferation. Therefore, it may be worthwhile treating the myelinating cultures (following astrocyte monolayer pre-treatment) with BrdU and then double-labelling with antibodies against NG2 and BrdU to assess proliferation of the endogenous oligodendroglial population. There also appeared to be a greater increase in BMP-4 mRNA expression following CTGF pre-treatment when compared to its expression in SCM pre-treated astrocytes. This could indicate that the SCM concentration of CTGF was less than the 10 ng/ml of exogenous CTGF peptide which was used to treat astrocytes. However, changes in message may not necessarily have related to significant changes at the protein level given that both treatments inhibited myelination comparably. It was hypothesised that since TGF-ß, a growth factor associated with glial scar formation, is known to activate CTGF expression that paracrine signalling of CTGF to the astrocytes may have caused them to up or down-regulate their expression of TGF-ß as part of a positive/negative regulatory loop. However,

there appeared to be only slight increases in TGF-ß expression following treatment.

Further clarification of these results at the protein level, perhaps using an ELISA kit, would be required to prove that pre-treatment of astrocytes with SCM or CTGF caused them to up-regulate their expression of BMP-4 protein, in particular. Furthermore, to demonstrate definitively that this factor mediates the astrocyte-dependent inhibition of oligodendrocytes induced by CTGF, noggin (the antagonist of BMP-4) could be added to astrocyte monolayers during pre-treatment and prior to their use in the myelinating cultures. If myelination was restored following this treatment then this would strongly suggest that BMP-4 up-regulation in astrocytes was highly involved in mediating the CTGF-induced inhibition of CNS myelination. It is also possible that several factors could be up or down-regulated in response to CTGF/ SCM treatment and some of these may also relate to the control of myelination, therefore a full microarray analysis of pre-treated versus untreated astrocytes could also provide useful information about important transcriptional changes.

Western blot analysis (Figure 6.7) of SC-conditioned astrocytes suggested that there was little difference at the protein level for GFAP expression compared with untreated controls. However, Figure 6.1 suggested clear morphological differences in astrocytes which had been treated versus those which hadn't. Furthermore, though it requires formal quantification, immunoreactivity for GFAP appeared to be brighter in treated versus untreated astrocytes. However, it could also be argued that changes in morphology can cause certain markers to appear brighter as the protein becomes more concentrated in the redefined cytoplasm (Figure 6.1 c, d).

Although the findings from this chapter are preliminary, they certainly suggest that the astrocyte may be partly responsible for the CTGF mediated inhibition of myelination and that astrocyte pre-treatment may also cause transcriptional changes in important regulatory factors of myelination. These findings are novel in that Stritt et al., (2009) concluded that since they did not observe any losses

in the number of GFAP $^{+ve}$ cells, astrocytes were unaffected by CTGF and that the oligodendrocytes were the primary targets for this growth factor.

Furthermore, Schwab et al., (2000, 2001) demonstrated using human brain tissue from infarct/ trauma patients and rat brains post stab injury, that CTGF was acutely up-regulated in a sub-set of reactive astrocytes, particularly in and around laminin $^{+ve}$ structures, suggesting a possible role for CTGF in modulating the BBB. From this study, the acute expression of CTGF and its up-regulation in reactive astrocytes, coupled with the knowledge that CTGF is known to play a role in regulating components of the ECM, could suggest that CTGF may also be involved in glial scar formation *in vivo*. Furthermore, peri-lesional astrocytes and astrocytes in areas devoid of obvious neuropathology expressed little or no CTGF (Schwab et al., (2000, 2001). In rat models of SCI, similar up-regulation of CTGF was observed in reactive astrocytes and also in invading fibroblasts and endothelial cells at the lesion site (Conrad et al., 2005). Patients with ALS also showed an increased expression of CTGF in reactive astrocytes, whilst little or no glial CTGF expression was detected in healthy patients (Spliet et al., 2003). It is therefore not novel that CTGF may induce astrocyte reactivity; however, our findings that SCs are a source of CTGF may be relevant when considering their interactions with astrocytes *in vivo,* particularly in a SC transplant scenario. It could be hypothesised, given our findings and the work of others, that the induction of boundary formation and astrogliosis induced by SCs could be at least partially attributable to the SC expression of CTGF. Thus, as well as potentially inhibiting oligodendrocyte myelination, SCs could also exacerbate scar formation via their secretion of CTGF.

Results from the treatment of OPCs with SCM or CTGF indicated that both treatments significantly altered their differentiation into oligodendrocytes as assessed by their expression of NG2 and the O4 antibody, as well as their morphological characteristics (Figure 6.8/ 6.9), when compared to Sato conditions. Whilst there was evidence that a small proportion of SCM/ CTGF treated cells were able to differentiate into “complex” oligodendrocytes, suggesting their maturation, they were significantly hindered from doing so, compared with cultures which were grown in Sato media. This data indicated

that CTGF also inhibited oligodendrocyte differentiation via mechanisms which were astrocyte non-dependent *in vitro*.

Since the expression of oligodendroglial markers was reduced but not completely inhibited by treatment, but morphology was significantly altered by OPC treatment with SCM or CTGF, it could be hypothesised that CTGF directly inhibits oligodendrocyte process extension in the absence of astrocytes. Uhm and colleagues (1998) demonstrated that the treatment of OPCs with an agonist to PKC enhanced their process extension, whilst also inducing their up-regulation of the ECM modulator, matrix metalloproteinase-9 (MMP9) on an astrocyte substrate. Furthermore, i*n vivo* studies demonstrated that MMP9 expression peaked with the onset of myelination, suggesting its role in process extension and myelination (Oh et al., 1999; Larsen et al., 2006). These reports could suggest an interesting link to the data demonstrated in this chapter, in that CTGF also regulates components of the ECM and could therefore play a role in oligodendrocyte process extension via similar mechanisms to those previously reported. Also of note is the finding that MMP9 regulates process extension on an *astrocyte* substrate, given that oligodendrocytes have been shown to remyelinate *in vivo* in astrocyte rich areas (as discussed in the introduction to this chapter). To correlate our findings with those of Uhm and to elucidate the mechanisms governing the CTGF-mediated inhibition of oligodendrocyte maturation and process extension, OPCs could be treated with CTGF and then analysed using RT-qPCR for their expression of MMP9. A down-regulation of MMP9 mRNA (and further confirmation at the protein level) following SCM or CTGF treatment could suggest that CTGF inhibits endogenous CNS myelination by inhibiting MMP9-mediated oligodendrocyte process extension.

In summary, data from this chapter has indicated that astrocytes are likely to play a role in the CTGF-mediated inhibition of endogenous myelination, possibly via the up-regulation of BMP-4 which reduces oligodendrocyte maturation. However, CTGF also works via astrocyte-independent mechanisms to significantly inhibit oligodendrocyte differentiation, and these mechanisms could perhaps involve hindering process extension through interactions with the ECM. Whilst these two mechanisms appear to be exclusive, within the myelinating cultures,

as it would be *in vivo*, it is possible that a combination of both of these mechanisms is at play in inhibiting oligodendrocyte maturation. Taken together, results from this chapter have provided further evidence to suggest that the transplantation of differentiated SCs without suitable modification may not be the most functionally beneficial following SCI due to the undesirable effects of mature SCs on endogenous CNS glia.

Results Chapter 7:

Developing the Optimum Design *In Vitro* for a Cell-Seeded Biodegradable Scaffold to Promote CNS Repair

7.1 Introduction

The data reported throughout this thesis, in combination with what is known in the literature, has indicated that whilst SCs are able to promote neurite outgrowth following transplantation into the damaged CNS, their efficacy may be limited given that they form boundaries with astrocytes (Lakatos et al., 2000) and inhibit CNS myelination *in vitro* via the secretion of CTGF (Chapter 5). Therefore, OECs may be the more favourable candidate for transplant-mediated repair of the injured CNS, as they naturally co-exist with astrocytes in the olfactory bulbs and, as this thesis has shown, enhance endogenous oligodendrocyte myelination *in vitro*. However, previous transplantation studies have demonstrated that whilst OECs can enhance neurite outgrowth and restore functional recovery to some extent, regenerating neurites rarely exit the glial scar and their re-growth and orientation is often random and disorganised (Barnett & Riddell., 2007; Toft et al., 2007; Fitch et al., 2008). For this reason the concept of using a biodegradable scaffold to bridge the lesion was conceived. By introducing topographical guidance cues such as grooves and ridges, it was postulated that neurite outgrowth could be directed in order to maximise the reorganisation and reformation of functional neuronal connections (Flynn et al., 2003; Stokols et al., 2004; Patist et al., 2004; Nomura et al., 2006). Nonetheless, spinal cord injuries are complex and multi-faceted, and competent repair is limited due to the presence of both physical *and* molecular barriers to regeneration. Therefore, a combined therapeutic approach using a biodegradable scaffold seeded with OECs could be a promising strategy for maximising functional recovery following damage to the CNS, such as in spinal cord trauma.

Although the focus of this thesis was on CNS repair, bioengineering strategies can be used to combat a host of medical and scientific problems, such as the design of competent drug delivery strategies using nanoparticles (Harmia et al., 1986; Li et al., 1986; Verdun et al., 1990; Feng et al., 2009 Chen et al., 2012; Madan et al., 2012); and in the manufacturing of medical implants for bone repair (Narang et al., 1975; Rubin & Marshall 1975; Fan et al., 2008; Lovald et al, 2009; Pilliar et al., 2012). Certain biomaterials may be deemed optimal for a particular problem, based upon their biochemical and mechanical properties; such as

degradation time, durability and ease of manipulation/ modification. Thus, selecting the most appropriate biomaterial is vitally important in order to maximise the effectiveness of a given bioengineering strategy.

One example of a commonly used biomaterial is polycarbonate, which is a type of plastic easily altered by heat, known as a thermoplastic. It is known for its strength and high transparency to visible light, as well as its ability to bend at room temperature without becoming brittle and breaking, thus making it an attractive biomaterial. Polycarbonate has been used in the development of implantable biodegradable neuronal recording devices, which degrade after approximately 220 days *in vivo* (Lewitus et al., 2011). Furthermore, polycarbonate-urethane has also been demonstrated for its use in bone implants where it effectively cushion joints and minimizes bone degradation caused by shearing (Elsner et al., 2010; Zur et al., 2011; St John & Gupta 2012).

Poly(methyl) methacrylate (PMMA) is another transparent thermoplastic, which is often used as a more economical alternative to polycarbonate. It has previously been demonstrated to be biocompatible within the human body and was formally used in the manufacturing of contact lenses (Millidot et al., 1979; Mandell et al., 1982) prior to its replacement with a more gas-permeable alternative. It has also been used in the bioengineering of dental implants (Leigh 1975; Klawitter et al., 1977; Peterson et al., 1979), as well as for the effective delivery of antibiotics to combat orthopedic infections (Shipley et al., 1981; Grieben 1981; Vécsei & Barquet 1981). Scaffolds bioengineered from polystyrene (another thermoplastic) have also been used to induce different aspects of cell behavior, such as differentiation and proliferation in rat mesenchymal stem cells *in vitro* (Wang et al., 2012). Although little is currently known about their role in promoting CNS repair, it may be worth investigating the aforementioned biomaterials for use in this context, given their current application in a range of bioengineering paradigms and the ease at which they can be modified by heat.

Poly-L-lactic acid (PLLA), which is produced by polymerizing lactic acid from natural sources, is also an attractive candidate for use in bioengineering

strategies given its ability to degrade within a period of 2 years, leaving behind non-hazardous lactic acid. As with the previously described polymers, PLLA is used extensively in implants to aid bone/ cartilage formation (Chang et al., 2007; Izal et al., 2012; Schofer et al., 2012). However, PLLA has also been demonstrated to enhance neurite outgrowth and Schwann cell migration following implantation into the site of peripheral nerve injury (Ngo et al., 2003; Cai et al., 2005), as well as to facilitate neurite alignment and the infiltration of host cells in the transected thoracic spinal cord in rats (Wang et al., 2009).

In addition, poly-ε-caprolactone (PCL) has been recognised for its potential application within the field of biomedical engineering, perhaps due to its low melting point of 60 °C, as well as its moderate degradation time of approximately 1 year by physiological hydrolysis of its ester bonds. In addition, previous work from the Barnett lab demonstrated that a 2D PCL scaffold embossed with a serious of micro grooves and ridges ranging from 12.5–25 μm wide promoted the alignment of neurites *in vitro*, as well as supporting myelination within the myelinating culture system described in this thesis (Sørenson et al., 2007, 2008). However, over time the alignment of these cultures decreased as the topographical cues were lost with increased cell density, thus posing a potential problem for their use in long-term repair strategies. Subsequent studies have also demonstrated the use of PCL as an appropriate substrate for enhancing the directed outgrowth of regenerating neurites in both the peripheral and central nervous system *in vivo* (Hwang et al., 2011; Neal et al., 2011; de Luca et al., 2012), as well as in enhancing the differentiation of neuronal cells *in vitro* (Nisbet et al., 2008).

Furthermore, the 2D PCL scaffolds used by Sørenson and colleagues can be developed into a 3D "Swiss-roll" structure (Figure 7.1) containing a series of pores, as well as a micro-pattern (Seunarine et al., 2008). The pores aid the diffusion of nutrients throughout the core of the scaffold, as would be required for its effective use *in vivo*, whilst the pillars retain separation between the layers of the PCL, thus maintaining the integrity of its structure. It is hoped that the modification of the scaffold in this way may ultimately lead to the development of an implantable device which could be used as part of a

combinatorial therapeutic approach for the treatment of SCI, by enhancing neurite outgrowth and orientation, as well as maximising cell survival. However, prior to the integration of such an intricate scaffold to the complex CNS environment, further investigation as to the effects of topography on distinct neuronal/ glial populations needs to be investigated *in vitro*. Furthermore, to ensure the optimal scaffold design, it may be worth investigating other commonly used biomaterials for their ability to support CNS behaviour relating to functional repair, such as myelination.

In summary, whilst OEC-mediated cell transplants have demonstrated their potential in promoting neurite outgrowth following SCI, combining this kind of therapy with the implantation of an optimised micropatterned scaffold may maximise functional recovery by aiding the effective orientation of regenerating fibres *in vivo*, thus combating the physical *and* molecular impediments to repair.

7.1.1 Aims

The main aim of this chapter was to examine the potential of a biodegradable micro-patterned PCL scaffold to support the growth and survival of glial and neuronal cells. This was carried out by:

(i) Demonstrating the ability of PCL to support a range of glial cells, including OECs, which would ideally be used in a cell-seeded scaffold strategy *in vivo*

(ii) Assessing whether or not increasing the depth of the topography could promote a more prolonged cellular alignment as cell density increased.

(iii) Determining if PCL was the optimum substrate for use in this context by comparing it to a range of other biomaterials, which were assessed for their ability to support myelination *in vitro* using the myelinating culture system

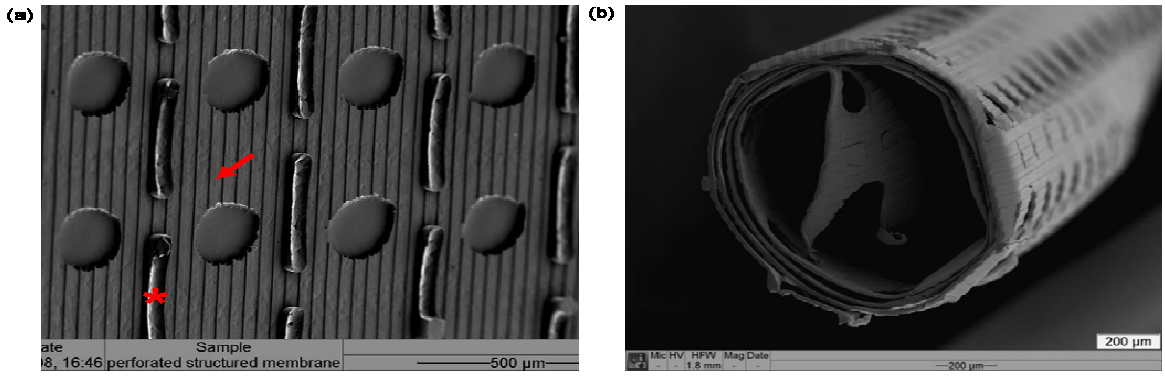


Figure 7.1- *3D "Swiss roll" structure of a PCL scaffold*. PCL can be embossed with a micro-pattern of grooves (red arrow) with defined dimensions to enhance cellular alignment (a). To produce a model which may perhaps be more suitable for use in vivo, pillars (red asterisk) can also be introduced to this scaffold to allow it to maintain its structural integrity when rolled into a 3D structure, which should support cell growth and alignment throughout each layer (b). By incorporating pores into the design of the scaffold, nutrients should be able to diffuse freely throughout its structure, thus improving cell survival. Scanning electron microscopy was used to capture these images, which were provided by Toby Lammel.

7.2 Cell Attachment Was Improved by Plasma Treating Poly-L-Lysine Coated PCL Scaffolds

Initial experiments culturing primary cells on poly-L-lysine (PLL)-coated PCL scaffolds demonstrated poor attachment and survivability, which was thought to be due to the hydrophobicity of the scaffold surface. Plasma, which is a state of matter similar to gas, contains a proportion of particles which are charged ions. Thus, plasma-treatment/ plasma etching can be used to change the surface chemistry of a given substrate to make it more or less permissive for attachment by altering its charge (Yang et al., 2002; Wan et al., 2004; Zhao et al., 2006). To investigate whether or not plasma-treatment could improve cell attachment in our cultures, PCL scaffolds were prepared as previously described (2.7.1) and treated with plasma after fabrication (Harrick Plasma Cleaner; Harrick Plasma, USA), using a Hi setting for 5 min (740V DC, 40 mA DC, 29.6W). PCL scaffolds were immediately placed into ethanol to ensure their sterile transfer into a tissue culture hood, where they were coated in PLL as previously described (see section 2.1). Neurosphere-derived astrocytes were seeded onto the PCL scaffolds in 10% FBS at a density of 50,000 cells/ 100 µl. After 3 days, the DNA dye DAPI was used to identify nuclei under a fluorescent microscope in order to formally quantify the number of cells remaining in plasma-treated PCL scaffolds versus those which were untreated.

Treatment of the PCL scaffolds with plasma prior to PLL-coating significantly increased the number of cells which were present after 3 days *in vitro,* by approximately 5 times (Figure 7.2) (n=3; p value= <0.01). Thus, for all subsequent fabrications, scaffolds were plasma treated as above prior to their use.

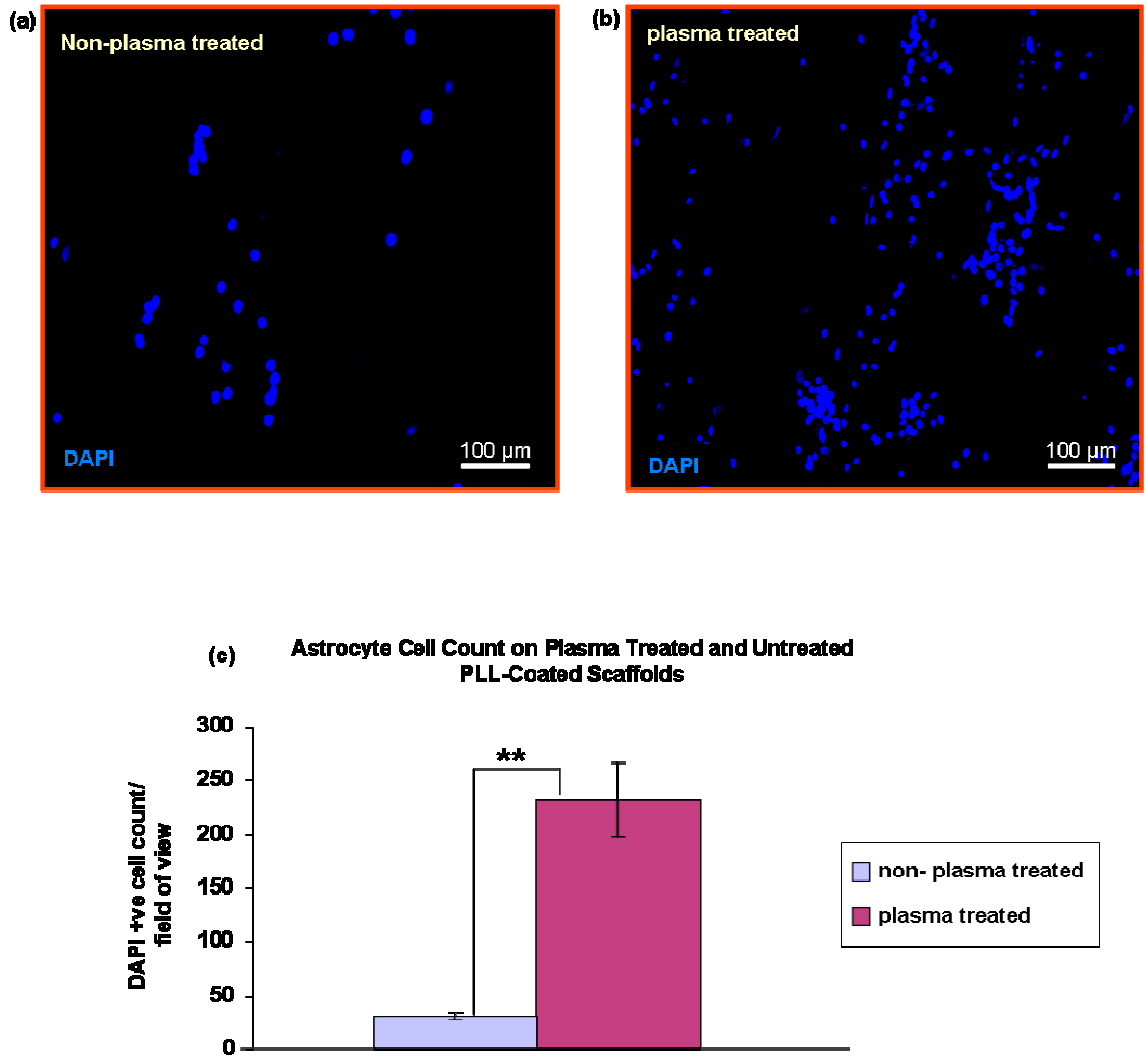


Figure 7.2 – *Plasma treatment of PCL scaffolds prior to cell-seeding significantly increased cell attachment*. PCL scaffolds were treated in a plasma cleaner for 5 min at a high setting to alter their surface chemistry, before being coated with 13.3 µg/ml of PLL. Neurosphere derived astrocytes were then seeded at a density of 50,000 cells/ 100 µl in 10 % FBS. After 3 days in vitro, DAPI labelling of nuclei revealed that there were approximately 5 times more cells remaining on PCL scaffolds which had been plasma treated (b), compared to those which were untreated (a). Images were taken using an Olympus BX51 epifluorescence microscope. Scale bars=100 µm. ** = p values <0.01.

7.3 Glial Cells Followed the Orientation of the Micropattern by "Aligning" with Grooves

Ns-Astrocytes were plated onto high molecular weight PCL scaffolds (h.MW PCL; see section 2.7.2) at a density of 50,000 cells/ 100 µl and cultured for 7 days in 10% FBS, prior to immunolabelling with antibodies to GFAP. Each scaffold contained defined areas of groove widths, including 5, 25 and 50 µm, at a constant groove depth of 5 µm (Figure 7.3). Similarly, myelinating cultures were plated directly onto scaffolds and cultured for 7 days in DM, although this was done in the absence of the usual astrocytes monolayer in order to accurately assess the response of endogenous oligodendrocyte and neurites to topography.

After 7 days, astrocytes displayed a typical protoplasmic morphology with little or no orientation of their processes when cultured on PCL without grooves (no topography) (Figure 7.4 a). However, on groove widths of 25 µm or less, astrocytes appeared to reside within the grooves, extending their processes in the direction of the topography giving them an "aligned" appearance. However, as groove widths increased to 50 µm, this alignment was lost, with astrocytes cultured under these conditions displaying characteristics similar to those cultured on areas of no topography (Figure 7.4 b-d).

When culturing myelinating cultures on the scaffolds in the absence of the astrocytes monolayer, there was little or no evidence of neuronal survival. However, O4 $^{+ve}$ branched oligodendrocytes were present after 7 days, suggesting their ability to survive and differentiation to some extent on the PCL scaffolds (Figure 7.4 e-h). Furthermore, oligodendrocytes also displayed some alignment of their processes in the direction of the micro grooves, though this decreased as the width of the grooves increased to 50 µm.

Purified OECs (see section 2.1.2) were also seeded onto PCL scaffolds at a density of 50,000 cells/ 100 µl and cultured in their optimum growth media (OMM/ACM 10%) for 7 days. In this manner, investigation into their potential for use in a cell-seeded scaffold designed to promote CNS repair could be carried

out. After 7 days, OECs were still present on the scaffolds (Figure 7.5). Although OECs are a polarised cell, they were randomly orientated in the absence of topography (Figure 7.5 a, e), as they would be in culture. However, in response to microgrooves, OECs became greatly aligned with the direction of the topography, particularly at groove widths of 25 µm or less (Figure 7.5 b, c).

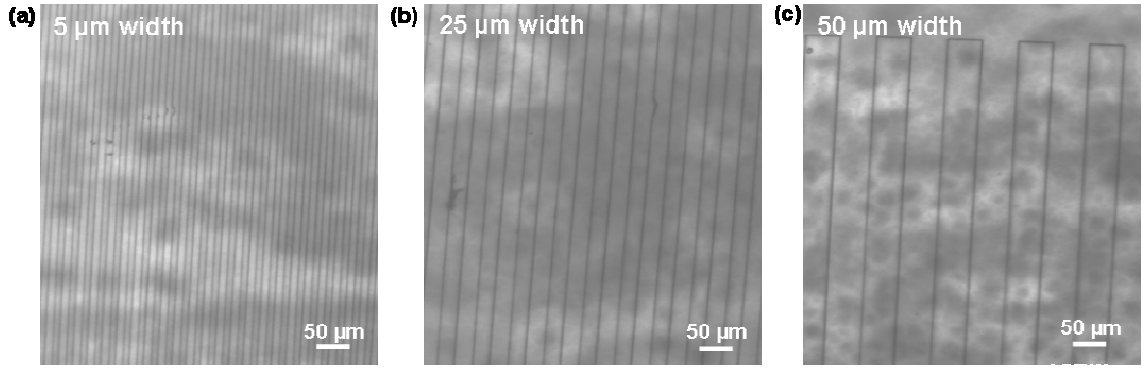


Figure 7.3 – *PCL scaffolds were hot-embossed with 3 defined areas of a micro-pattern of grooves and ridges to promote cellular alignment*. PCL scaffolds were hot embossed using a quartz template containing areas of topography with groove ridges of 5 µm, 25 µm and 50 µm at a constant depth of 5 µm. An Olympus microscope was used to take phase/ contrast images of the scaffolds. Scale bars=50 µm.

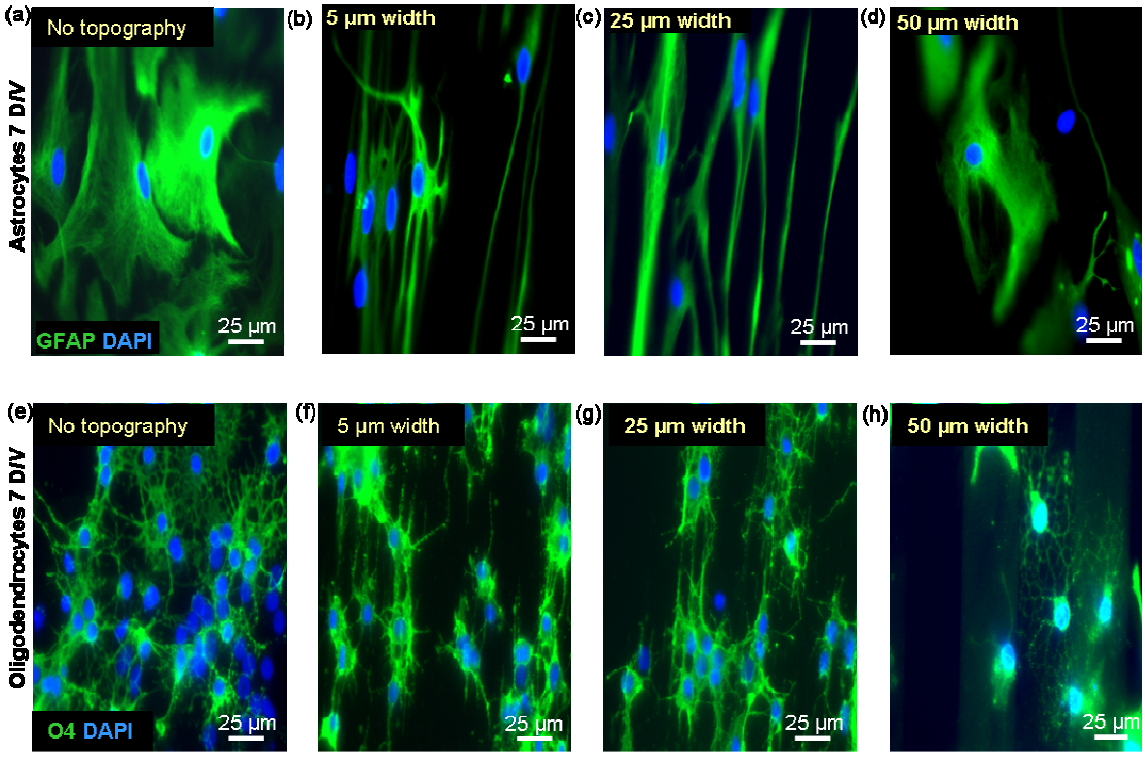


Figure 7.4 – *Astrocytes and oligodendrocytes aligned preferentially with groove widths of 25 µm or less.* Ns-Astrocytes were seeded onto micro-patterned scaffolds at a density of 50,000 cells/ 100 µl and cultured in 10% FBS for 7 days (a-d). Mixed embryonic spinal cord cells were also seeded onto scaffolds at 150,000 cells/ 100 µl in the absence of the typical astrocyte monolayer (e-h). Astrocytes appeared to be confined within the grooves and were observed extending their processed in the direction of the topography when the width of the groove was 25 µm or less (b, c), giving them a more "aligned" appearance than those grown in the absence of topography (a). This alignment was lost when astrocytes were cultured on areas of groove widths of 50 µm (d). Whilst culturing embryonic spinal cord cells in the absence of an astrocyte monolayer resulted in little or no axons remaining after 7 days, branched oligodendrocytes were still present at this time (e-h). Similarly to astrocytes, oligodendrocytes also appeared to extend and align their processes in the direction of the topography with a preference for groove widths of 25 µm or less. Groove depths were consistent for all conditions at 5 µm. An antibody to GFAP and the O4 antibody labelled astrocytes and oligodendrocytes, respectively, whilst DAPI labelled nuclei. Images were captured using an Olympus BX51 epifluorescence microscope. Scale bar=25 µm. n=2.

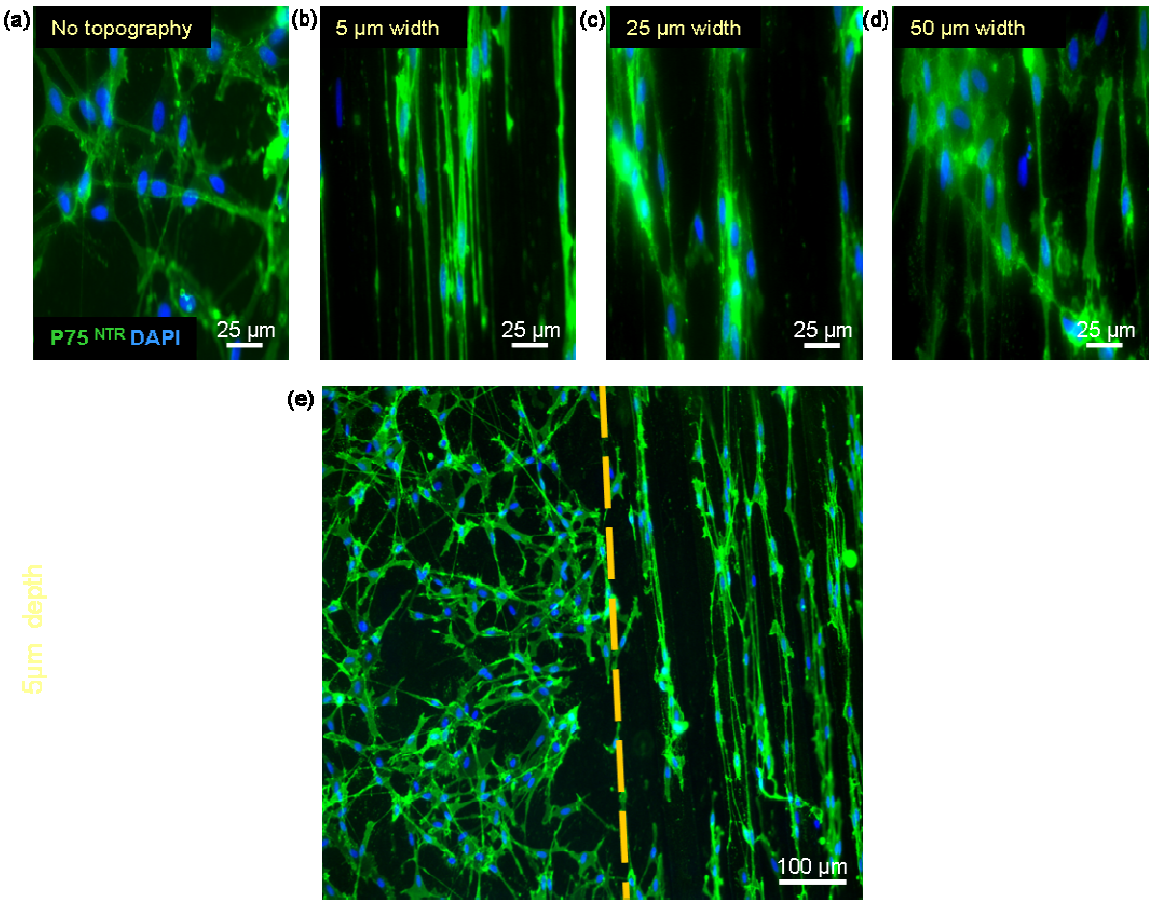


Figure 7.5 - *OECs align with microtopography*. Purified OECs were seeded onto micro-patterned PCL scaffolds at a density of 50,000 cells/ 100 µl and cultured in optimal growth media (OMM/ACM 10%) for 7 days. OECs appeared randomly orientated in the absence of topography, as is shown in panel (a) and panel (e), which illustrates the interface between an area of microgrooves and no topography (represented by broken yellow line). In areas embossed with a micropattern (b-d), OECs appeared to be confined within the grooves and were observed extending their processes in the direction of the topography, though this alignment was less apparent when the width of the grooves exceeded 25 µm (d). Groove depths were consistent for all conditions at 5 µm. An antibody to p75NTR labelled OECs, whilst DAPI labelled nuclei. Images were captured using an Olympus BX51 epifluorescence microscope. Scale bar=25 µm (a-d), and 100 µm (e). n=2.

7.4 Increasing Groove Depth Improved Alignment as Cell Density Increased

Previous work by Sørenson et al., (2007) demonstrated the ability of PCL scaffolds to support both the alignment of neurites and the subsequent myelination of axons within the myelinating culture system. However, their findings also demonstrated that as the density of the culture increased, the degree of alignment decreased. To try to optimise the design of the PCL scaffolds to best support their use as part of a long-term therapy to promote neurite outgrowth and functional repair, the depth of the groove was increased from 5 µm to 10 µm. As groove widths of 25 µm were shown to be optimal for alignment, both within this thesis and by Sørenson and colleagues, all other groove widths were excluded for this experiment. Ns-Astrocytes were seeded onto micro-patterned PCL scaffolds at densities of both 50,000 and 100,000 cells/ 100 µl and cultured in 10% FBS for 14 days. A previously published scoring system of 1-5 was used to describe the degree of alignment (Sørenson et al., 2007), and scoring was carried out by 4 "blinded" assessors to produce average alignment scores for each condition. 15 images per condition were analysed for each biological replicate (n=2).

Whilst alignment scores decreased with increased cell density in spite of an increased groove depth (Figure 7.6), scores remained at least 1 point higher in conditions where astrocytes were grown on 10 µm deep grooves, versus growth on a micropattern with a depth of 5 µm at a comparable cell density. The highest alignment score was observed when approximately 50,000 cells were seeded onto PCL scaffolds with a groove depth of 10 µm (Figure 7.6 b), which resulted in an average score of 4, indicating that 60-80% of the astrocytes were described as being aligned in the direction of the micropattern. Conversely, the lowest alignment score obtained (asides from "no topography") was 1.5 when 100,000 astrocytes were plated onto scaffolds with a groove depths of 5 µm, indicating that only approximately 20-30% of the astrocytes were considered to be aligned. At the same cell density, the average alignment score was 3.5 when astrocytes were grown on scaffolds with 10 µm deep grooves, suggesting that approximately 60-70 % of the cells were classified as aligned (n=2).

To determine the effects of groove depth on improving neurite alignment, myelinating cultures were also cultured onto an astrocytes monolayer and grown on PCL scaffolds containing a micropattern of grooves with a constant width of 25 μm and a depth of 10 μm for 26 days. Whilst preliminary observations indicated that increasing groove depth resulted in enhanced neurite alignment at the end-stages of culture when compared directly to cultures grown in the absence of topography (Figure 7.7), the overall reproducibility of the myelinating cultures was poor on h.MW PCL scaffolds, thus preventing formal quantification of this data.

In summary, whilst PCL appeared to support the growth and alignment of OECs, astrocytes and oligodendrocytes, myelination within neuronal cultures was variable. Furthermore, although an increase in groove depth did maintain alignment to a certain extent as cell density increased, deeper grooves may need to be considered for use in vivo to promote the long-term alignment of neurites required to support competent regeneration.

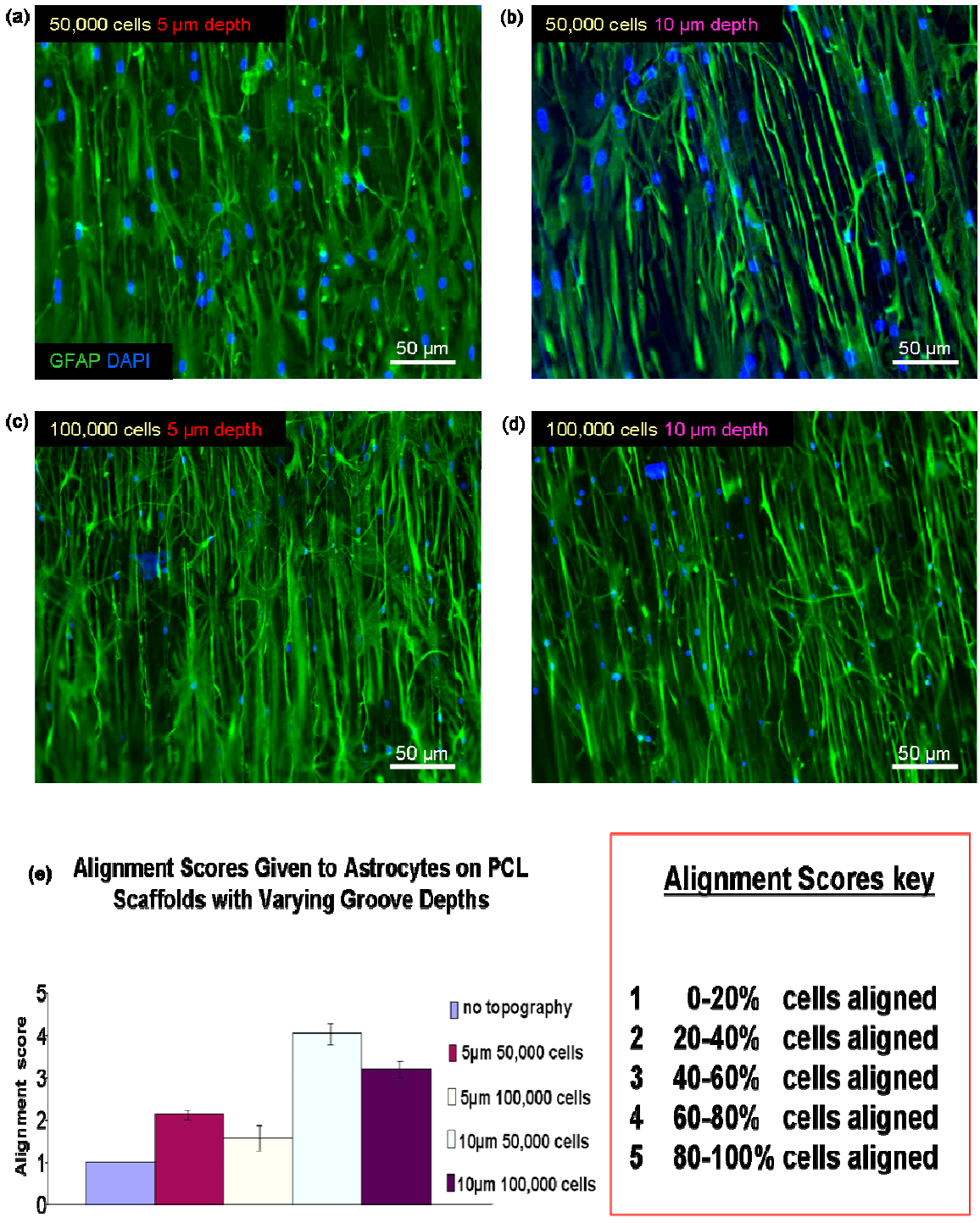


Figure 7.6 – *Increasing the depth of the groove in micro-patterned PCL scaffolds improved alignment scores as cell density increased.* Ns-Astrocytes were plated onto patterned PCL scaffolds with grooves at a constant width of 25 µm and a depth of either 5 µm (a,c) or 10 µm (b,d) and cultured in 10 % FBS for 14 days. The degree of cellular alignment was assessed on a scale of 1-5 by 4 independent and blinded assessors, based upon a previously published scoring system and average scores for each condition were given. Alignment scores decreased with an increase in cell density for both groove depths, however by increasing the groove depth to 10 µm scores remained on average at least a point higher when compared to the same cell density on shallower grooves (e). An antibody to GFAP labelled astrocytes, whilst DAPI labelled nuclei. Images were taken using an Olympus BX51 epifluorescence microscope. n=2. Scale bar=50 µm.

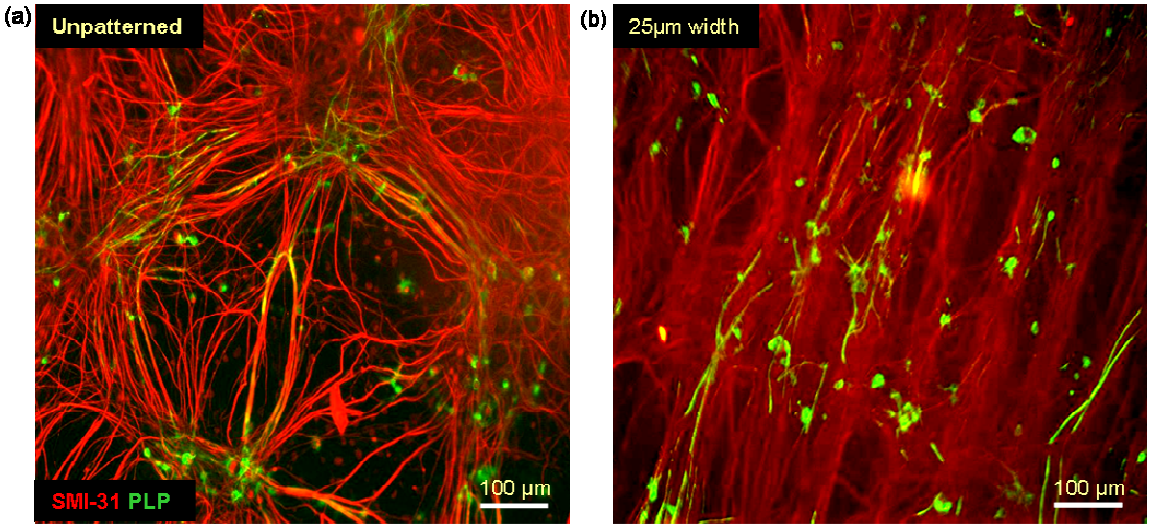


Figure 7.7 - *Neurites were aligned on groove depths of 10 µm after 26 days in culture.* Myelinating cultures on an astrocytes monolayer were cultured on PCL scaffolds containing either no topography (a), or a serious of grooves and ridges with a constant width of 25 µm and a depth of 10 µm for 26 days. Immunolabelling and fluorescent microscopy were carried out after this period. Neurite alignment appeared to be greater on micropatterned scaffolds compared with unpatterned scaffolds (a), with bundles of fibres orientating in the direction of the topography (b). Small amounts of myelin were also present, ensheathed around axons in the direction of the micropattern (b). SMI-31 labelled phoshorylated filament in neurites and an antibody against PLP labelled oligodendrocytes and myelin sheaths. Images were taken using an Olympus BX51 epifluorescence microscope. Scale bar=100 µm.

7.5 Inconsistent Myelination May be due to Induced Astrocyte Reactivity, as Opposed to Toxicity of PCL

As previous experiments for this thesis have demonstrated, myelination in cultures grown on h.MW PCL scaffolds was generally inconsistent and irreproducible, thus making formal quantification difficult. To assess if these anomalies were due to the possibility that PCL could be leaching something which was detrimental to one or all components of the culture system over time, sterile PCL beads were placed in a Petri dish containing the 2 glass coverslips with myelinating cultures grown on an astrocyte monolayer and left to "condition" the culture for 26 days. Feeding was carried out every other day by removing half the media and replacing with fresh DM as normal.

Immunofluorescent labelling of neurites and myelin sheaths revealed that the addition of PCL beads into the microenvironment did not significantly alter the level of myelination observed after the usual 26 day culture period (Figure 7.8; n=3, p value= >0.05) (data also described in Donoghue et al., 2013). These results suggest that the low levels of myelination observed in our cultures on PCL scaffolds were unlikely to be due to toxic chemicals leaching from the PCL substrate itself.

As this thesis and multiple other publications have demonstrated extensively, modulation of the astrocyte either via paracrine/ autocrine signalling or in response to environmental cues, such as changes to the ECM, can greatly alter their ability to support myelination (Levison et al., 2000; Albrecht et al., 2002; Nash et al., 2011). Thus, it was hypothesised that astrocytes grown on a h.MW PCL scaffold may be in a more reactive state compared with their counterparts, which were cultured on glass coverslips. To assess this, Ns-astrocytes were seeded at a density of 100,000 cells/ 100 µl onto PCL scaffolds with groove widths of 25 µm, at a constant depth of either 5 µm or 10 µm, as well as onto scaffolds with no topography. As an additional control, comparisons were also made to astrocytes grown on glass coverslips. After 7 days, some cultures were lysed for Western blot analysis, whilst others were treated with BrdU prior to immunofluorescent labelling to assess proliferation. As with previous

experiments, results from Western blot studies were quantified by working out the optical density/ pixel value for each band and expressing this as a ratio over the density of the loading control (GAPDH).

GFAP expression was significantly up-regulated when comparing astrocytes grown on micropatterned PCL substrates (Figure 7.9) to those grown on glass coverslips (a condition which typically supports myelination) (n=3; p values= <0.05). Whilst PCL without topography demonstrated a slight trend for an increase in GFAP expression compared with coverslip controls, this value was non-significant (p values= >0.05). Similarly, the differences between GFAP expression in astrocytes grown on a micropattern versus those grown on unpatterned PCL was also non-significant (p values= >0.05).

Whilst treatment with BrdU followed by labelling with an antibody to detect its presence in proliferating cells indicated a trend for an increase in the % of proliferating astrocytes which were grown on glass coverslips (Figure 7.10 a) and PCL scaffolds without a topography (Figure 7.10 b), compared to those grown on micropatterned PCL (Figure 7.10 c,d), this trend was not significant (n=3; p values= >0.05). In general, the rate of proliferation given as a % of proliferating cells over the total GFAP $^{+ve}$ population was less than 13% for all conditions.

Thus, this data indicates that whilst h.MW PCL does not seem to have a negative affect on myelination by leaching soluble factors, it may be inducing some characteristics of astrocyte reactivity, such as increased GFAP expression, though hyperproliferation was not evident in these cultures. Altering the phenotype of the astrocytes in this way could greatly reduce their ability to support myelination in our system.

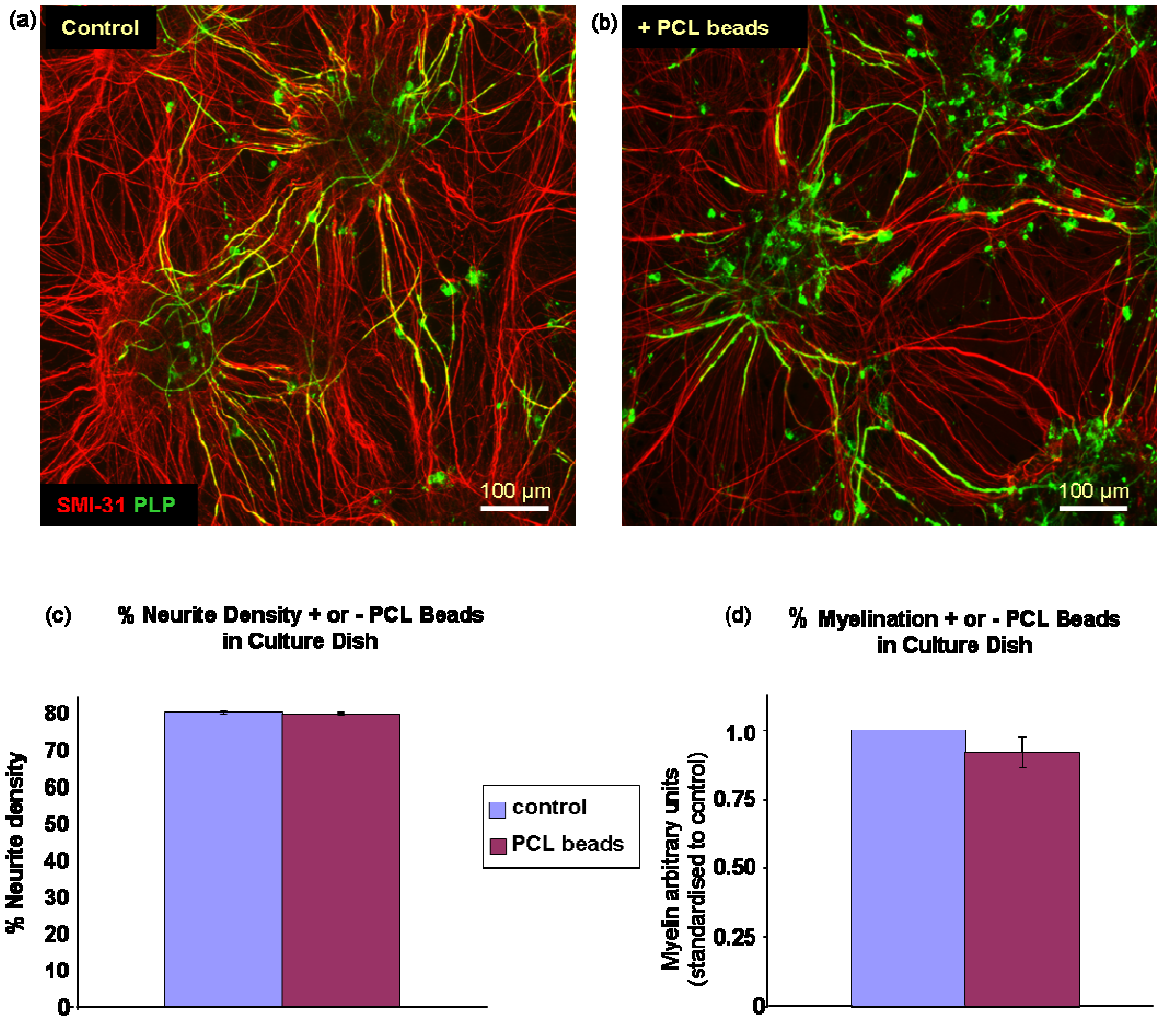
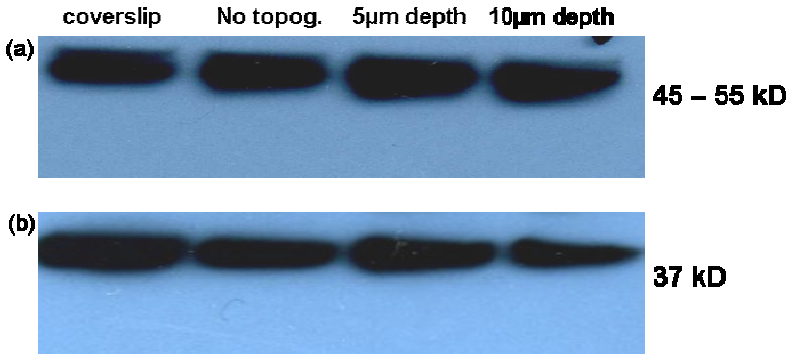


Figure 7.8 – *Conditioning of cultures grown on glass coverslips with PCL beads did not affect myelination*. Myelinating cultures on an astrocytes monolayer were grown on glass coverslips for 26 days as normal prior to immunolabelling. From day 1, PCL beads were added to the Petri dish and allowed to "condition" the culture media. The addition of PCL to the culture environment (b) did not affect myelination (d) or neurite density (c).compared to untreated controls (a). SMI-31 labelled neurites, whilst an antibody to PLP labelled myelin sheaths and oligodendrocytes. p values= >0.05. Scale bar=100 µm. n=3.



(c)

	control	No topography	5µm depth	10µm depth
Ratio of GFAP compared to GAPDH	0.58	0.72	0.80 *	1.01 *

Figure 7.9 – *GFAP expression was significantly increased when astrocytes were cultured on micropatterned scaffolds, compared to those on glass coverslips.* Ns-astrocytes were seeded onto PCL scaffolds with and without topography at a density of 100,000 cells/ 100 µl. Cells were cultured for 7 days in 10 % FBS prior to lysing for Western blot analysis. Comparisons were made to astrocytes which were grown under the same conditions on glass coverslips. Quantification was given as the optical density of each band normalised to a GAPDH loading control (b). GFAP expression was significantly up-regulated on scaffolds with grooves of both 5 and 10 µm depth compared to those on glass coverslips (a, c). Whilst there appeared to be an increase in GFAP expression in unpatterned PCL compared with coverslip controls, this value was not significant. Similarly, the differences between all PCL conditions were also non-significant. n=3. * = p values <0.05.

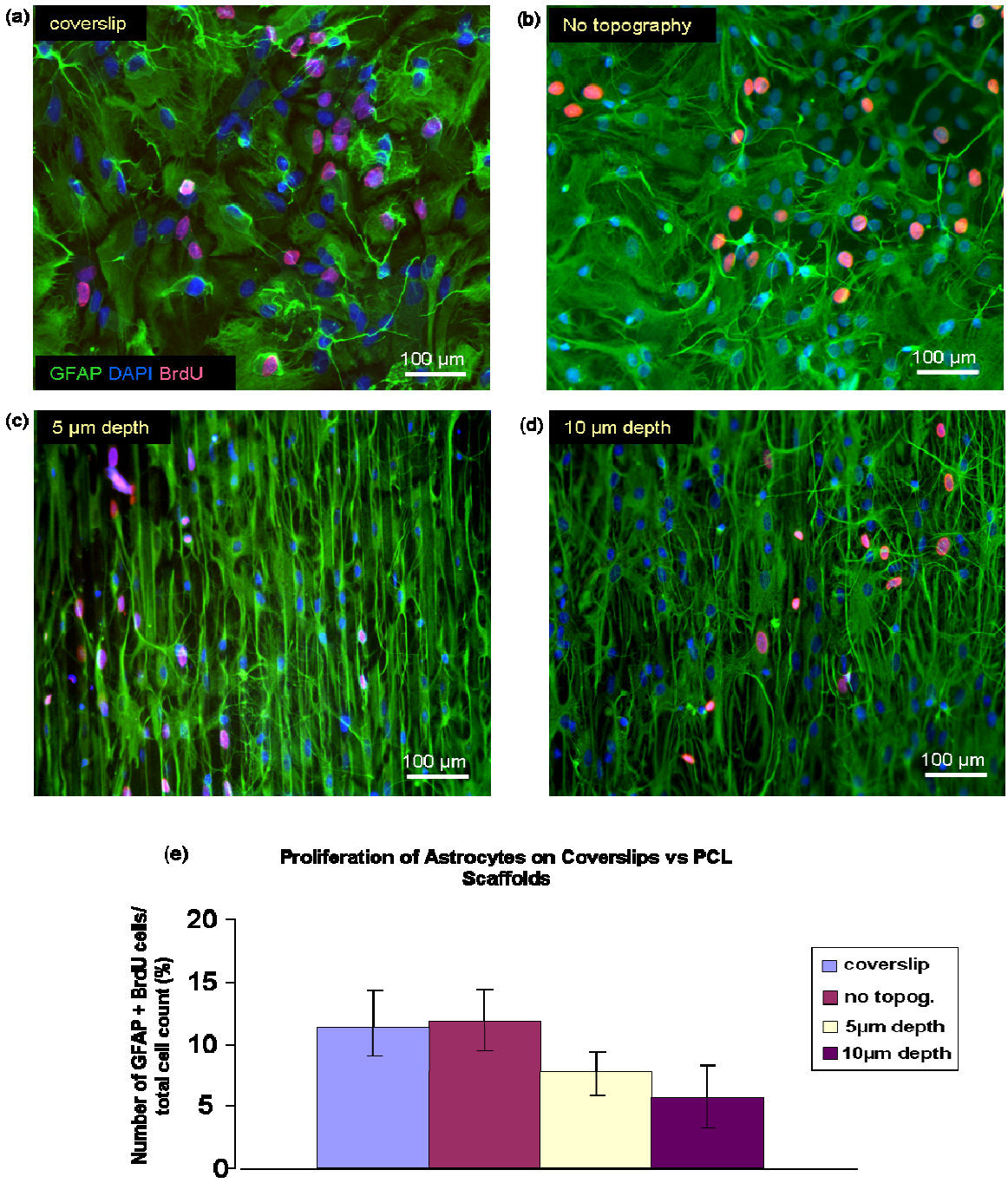


Figure 7.10 - *The rate of proliferation was not significantly altered in astrocytes grown on PCL substrates.* Astrocytes were seeded onto either unpatterned PCL scaffolds or those with groove widths of 25 µm and a depth of either 5 or 10 µm, at a density of 100,000 cells/ 100 µl. Comparisons were made to astrocytes grown on glass coverslips under the same conditions. After 6 days, cultures were treated with BrdU for approximately 16 hrs prior to immunolabelling. The rate of proliferation was given as the number of BrdU+ve cells as a % of the total GFAP+ve population. Whilst there appeared to be a trend for increased proliferation in cultures grown on glass coverslips and in the absence of topography (a,b) compared to the rate of proliferation on micropatterned surfaces (c,d), these changes were not significant (e). For all conditions, approximately 13% or less of the total astrocyte population were undergoing proliferation at the point of immunolabelling. An antibody against GFAP labelled astrocytes, whilst an antibody to detect BrdU labelled proliferating cells. DAPI depicted nuclei. p values= >0.05. Scale bars=100 µm. n=3.

7.6 A Lower Molecular Weight PCL was the Optimal Substrate for Supporting Myelination when Compared to a Range of Other Biomaterials

Since myelination did not appear to be supported by the h.MW PCL used in previous experiments, a range of other biomaterials were investigated for their ability to support a complex CNS system enabling competent myelination.Throughout this thesis, scaffolds were fabricated using PCL with a molecular weight of 90,000 (Sigma Aldrich, UK). However, a lower molecular weight PCL substrate (MW 45,000; Sigma Aldrich, UK) was also investigated, along with polycarbonate, polystyrene, poly(methyl)methacrylate (PMMA) and poly-L-lactic acid (PLLA). To fully assess the effects of the substrate, topography was excluded from these experiments, thus unpatterned membranes of each biomaterial were spun onto glass coverslips to make their handling easier (section 2.7.2 for more details). Astrocyte monolayers were plated down as usual, followed by the seeding of the mixed embryonic spinal cord cells on top. Immunolabelling and fluorescent microscopy were carried out after 26 days in culture. Dr Peter Donoghue fabricated the scaffolds used in these experiments, whilst I performed the cell culture and analysis. The following results are published in Donoghue et al., (2013).

PMMA, PLLA, polycarbonate and polystyrene supported relatively low and comparable levels of myelination, which was significantly less than control coverslips. Whist myelination was greatest on glass coverslips, low molecular weight PCL (l.MW PCL) produced a level of myelination which was not significantly different to this control (Figure 7.11) (n=3; p values= >0.05). Similarly, the level of myelination observed in cultures grown on a l.MW PCL substrate was significantly higher than that achieved on all other substrates (n=3; p values= <0.05). Although it has not been included in this thesis, Donoghue et al., (2012) also directly compared l.MW PCL to h.MW PCL to demonstrate that myelination on the latter substrate was significantly lower (n=3; p values= <0.05). Neurite density was not significantly altered in any condition.

Taken together these findings demonstrate that l.MW PCL may be the most appropriate biodegradable substrate for aiding functional recovery after SCI, given its ability to support neurite outgrowth and the complex cellular mechanisms, which lead to myelination. Furthermore, the data indicates that factors such as the molecular weight of a polymer may be a vitally important consideration when optimising bioengineering strategies.

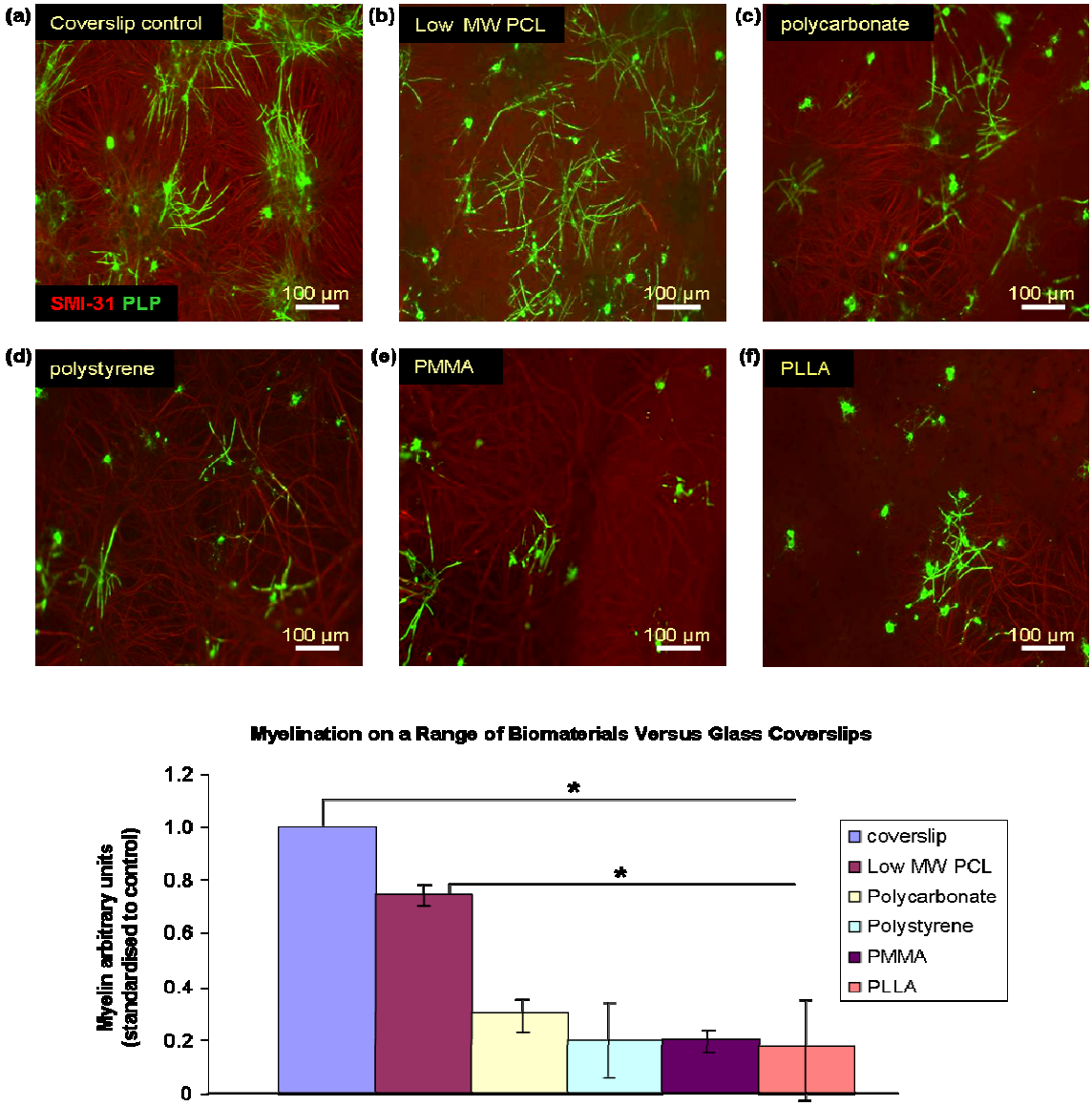


Figure 7.11 -*PCL best supports myelination compared to a range of biomaterials other than glass.* Myelinating cultures on an astrocyte monolayer were seeded onto unpatterned membranes of low MW PCL, polycarbonate, polystyrene, PMMA and PLLA and cultured as normal for 26 days in DM media. Comparisons were made to controls cultured in the same way on glass coverslips (a). The highest level of myelination was observed on glass coverslips, with polycarbonate (c), polystyrene (d), PMMA (e) and PLLA (f) supporting a significantly lower amount of myelination. Asides from coverslip controls, low MW PCL best supported myelination at a level which was significantly higher than the other biomaterials tested. Neurite density was not significantly altered across all conditions. SMI-31 labelled neurites and an antibody against PLP labelled oligodendrocytes and myelin sheaths. Images were taken on an Olympus BX51 epifluorescence microscope. Scale bars=100 µm. n=3. *= p values <0.05. This figure was modified from Donoghue et al., (2013) Tissue Engineering Part A. 19 (3-4) p 497-507.

7.7 Discussion

Data from chapter 7 has indicated that PCL is able to support the growth and survival of astrocytes, oligodendrocytes and OECs. Furthermore, by introducing a micropattern consisting of a serious of grooves and ridges, cell growth could be orientated in the direction of the micropattern, with a preference for grooves with 25 μm width or less (Figure 7.4, 7.5). Previous studies had reported a decrease in cellular alignment as cell density increased, thus masking topographical cues, when the depth of the grooves was consistently 5 μm (Sørenson et al., 2007). However, by increasing groove depth to 10 μm, cellular alignment was prolonged to an extent as cell density increased (Figure 7.6). Whilst initial observations suggested that neurite alignment could also be prolonged after 26 days *in vitro* by increasing the depth of the micropattern, myelination on the whole was poor and not greatly reproducible when cultures were grown on a h. MW PCL substrate (Figure 7.7). This did not appear to be due to toxicity issues relating to PCL, since conditioning of myelinating cultures on glass coverslips with PCL beads did not affect myelination or neurite density (Figure 7.8). However, phenotypic analysis of astrocytes grown on PCL versus glass coverslips demonstrated that GFAP expression was increased under these circumstances, perhaps indicating reactivity (Figure 7.9), although significant hyperproliferation was not evident (Figure 7.10). Introducing a lower molecular weight PCL greatly improved its ability to support myelination at a level which was significantly greater than other biomaterials tested (with the exception of glass coverslips), including the original h.MW PCL (Figure 7.11) (Donoghue et al., 2013).

More detailed analysis is required to assess the effects of longer term culture on the survivability of OECs grown on PCL substrates, in order to further justify their use in the design of a cell-seeded scaffold. Future experiments could involve BrdU treatment and labelling of OECs grown on scaffolds versus coverslip controls to determine their rate of proliferation in response to PCL. Longer cultures of OECs grown on PCL substrates for up to 4 weeks would also be useful in determining the effects of time and increased cell density on their long-term alignment. If the alignment of OECs could be maintained by increasing groove

depth, as it appears to be with astrocytes, it would be interesting to determine if neurites were therefore able to "align" with the orientated OECs as the density of the culture increased, despite the underlying topography being masked by their growth. Given the role of OECs in guiding and ensheathing regenerating olfactory receptor neurons (ORNs) from the periphery *in situ*, it is conceivable that neurite outgrowth could be directed by initially establishing alignment in OECs within the scaffold, thus encouraging neurites into the graft and guiding their exit from the glial scar *in vivo*.

The observation that myelination was poor on h.MW PCL (MW 90,000) appeared to contradict the findings of Sørenson et al., (2007), who demonstrated the onset of myelination on PCL scaffolds after 3 weeks using the same myelinating culture system. Evidence from this thesis has suggested that this may be due to a change in the reactivity status of astrocytes grown on PCL, rendering them less supportive of myelination. However, studies using BrdU to assess proliferation may not have been the most accurate way to determine reactivity based upon hyperproliferation. As cell density increases, proliferation decreases as cells become contact inhibited by the confines of their microenvironment. Indeed, the physical constraints of the microenvironment induced by the presence of grooves and ridges on the PCL substrate may have induced contact inhibition in astrocytes cultured under these conditions, thus reducing their rate of proliferation. Future analysis of this sort could include plating astrocytes at a lower density and carrying out BrdU treatment and subsequent antibody labelling within 3 days of seeding to provide more accurate information.

These anomalies in findings, along with the apparent induced reactivity in astrocytes, could be due to vital differences in the mechanical properties of the PCL used in each study. For example, the PCL used to fabricate scaffolds for use in the work carried out by Sørenson and colleagues had a molecular weight of 65,000 (Sigma Aldrich, UK), as opposed to the 90,000 MW PCL initially used for this thesis. Furthermore, as previously described, l.MW PCL (45,000) appeared to support myelination at a significantly greater level than h.MW PCL (Figure 7.11) (see also Donoghue et al., 2013). This data could indicate the importance of the specific properties of a given biomaterial in relation to their function. Indeed,

the molecular weight of a polymer can relate to its strength, with a higher molecular weight representing a stiffer substrate. Previous studies have reported the importance of substrate stiffness on regulating cellular behaviour, such as proliferation, differentiation and even neurite elongation (Engler et al., 2006; Khatiwala et al., 2007; Jiang et al., 2008). For example, Balgude et al., (2001) demonstrated that the outgrowth of DRGs decreased as the stiffness of the agarose hydrogel on which they were grown on increased. They explained this observation according to the theory of Heidemann & Buxbaum (1994), who used a "tension-pull" model to describe the way in which the growth cones of extending neurites responded to the mechanical properties of their surface to mediate outgrowth.

In order to justify the use of OECs in a combinatorial approach to enhance CNS repair, it is important to confirm the early findings from this thesis relating to the effects of exogenous OECs on oligodendrocyte myelination by repeating these experiments on PCL. Equally, for considering scaffold use *in vivo*, where directed neurite outgrowth is vitally important, a balance must be achieved between designing the optimal topography as well as the correct cellular composition of the scaffold. However, although l.MW PCL lends itself well to supporting the growth and differentiation of endogenous CNS cells leading to myelination, its mechanical properties are such that it loses its structural integrity when "hot-embossed" to introduce a micropattern. These findings are not uncommon given that molecular weight often specifically relates to the transition temperatures of polymers, therefore altering their thermal properties. Thus, l.MW PCL is perhaps currently unsuitable for future studies, whereby the cellular response to topography is an important consideration, until its fabrication can be optimised. Whilst the PCL used by Sørenson et al., (2007) may have been an ideal candidate, it has since been discontinued by its manufacturers (Sigma, UK). However, further work carried out by Dr Peter Donoghue demonstrated that although myelination was poor on h.MW PCL substrates after the usual 26 days in culture, by 46 days the level of myelination was comparable to that observed in control cultures, where there was no obvious demyelination (Donoghue et al., 2013). Furthermore, it was shown that this delay in myelination was due to the secretion of an astrocytic factor(s), which appear to support the findings from this thesis that astrocytes grown on PCL

show characteristics of reactivity, such as an increased expression in GFAP. By identifying this factor it may be possible to optimise the scaffold design in such a way that astrocyte reactivity is not induced or exacerbated by a PCL substrate *in vivo*, thus improving its suitably for enhancing repair in the damaged CNS.

In summary, a scaffold made from PCL incorporating a micropattern of grooves and ridges with an optimal depth may be used to direct cellular outgrowth throughout the glial scar. By eventually incorporating OECs into a porous 3D scaffold design it is hoped that both the physical and molecular barriers to regeneration can be overcome and that the non-permissive scar environment can be modified in such a way to enable cell survival and differentiation, as well as neurite outgrowth, thus maximising functional recovery. However, prior to its effective use *in vivo*, this system must be fully optimised *in vitro* in order to provide a comprehensive understanding with regards to the response of all endogenous glial/ neuronal cells and their responses to PCL and topography.

Chapter 8

Final Discussion

8.1 Summary of Main Findings

8.1.1 OECs and SCs Differ Significantly in Their Effects on Oligodendrocyte Myelination

I have demonstrated using myelinating cultures that the addition of OB-OECs enhanced the level of oligodendrocyte myelination; whilst exogenous SCs significantly reduced endogenous CNS myelination *in vitro*. Furthermore, neither OECs nor SCs appeared to myelinate CNS axons in our culture system, as evidenced by a lack of P0 staining in the myelin sheaths. The mechanisms governing each of these effects appear to be distinct, in that SCs secrete a factor(s) which negatively affects oligodendrocyte myelination in culture, regardless of whether or not the SCs were exposed to paracrine signalling from endogenous CNS glia and neurons. Conversely, OECs enhance endogenous CNS myelination *only* when they are exogenously added to the cultures or when they directly secrete factors into the media shared by the myelinating cultures, thus exposing them to paracrine signalling. When CM was collected from OECs in the absence of endogenous CNS glial/ neuronal influences, it did not affect oligodendrocyte myelination (Chapter 4).

It is unclear from this thesis which factors were involved in mediating the OEC-induced increase in endogenous CNS myelination, although ELISA data demonstrated that purified OECs grown in isolation were a source of the pro-myelinating factor, CNTF, at comparable levels to SCs. It could be hypothesised that the interactions between OECs and endogenous CNS glia and neurons could cause them to up-regulate their expression of CNTF, thus enhancing myelination. Similarly, there may be several other pro-myelinating factors, which could be up or down-regulated in OECs in response to their exposure to endogenous CNS cells. For example, brain-derived neurotrophic factor (BDNF) has been shown to be expressed by OECs *in vitro* (Woodhall et al., 2001; Lipson et al., 2003; Pastrana et al., 2007) and throughout the olfactory system, where it is thought to support neurogenesis (Jones et al., 2007). Furthermore, in cuprizone models of demyelination whereby BDNF had been knocked down, there was a reduction in the $NG2^{+ve}$ progenitor population and deficits in the extent of remyelination in the corpus callosum (VonDran et al., 2011). In the context of SCI, McTigue et al.,

(1998) also demonstrated that the transplantation of fibroblasts engineered to express BDNF into the contused rat spinal cord resulted in a significant increase in the number of regenerating fibres present within the graft, as well as increased MBP expression, suggesting enhanced remyelination. Taken together, this data could partially explain how OECs, as a source of BDNF, are able to promote neurite outgrowth *in vivo* and to enhance oligodendrocyte myelination *in vitro* by aiding the initial expansion of OPCs. This hypothesis could also correlate with my observation that concentrated OCM resulted in an over-confluency of the myelinating cultures, causing them to "slough off"; an effect which could be mediated by the presence of potent mitogens (such as BDNF) in OCM (Chapter 4). An interesting future study could be to directly compare the transcriptomes of OECs grown in isolation with those grown under the influence of CNS cells using a microarray to detect any differences in expression at the gene level, which could later be confirmed using ELISA, Western blotting or immunocytochemistry. Data generated from this kind of investigation could provide novel evidence to support the view that the ability of OECs to promote CNS repair could be modulated by their interactions with the cellular environment at the injury site.

8.1.2 The SC-Induced Inhibition of CNS Myelination is Mediated via the Expression of CTGF

Biological comparisons between purified OECs and SCs suggested that whilst both cell types secreted comparable levels of the pro-myelinating factor, CNTF, SCs expressed significantly higher levels of CTGF mRNA and protein than OECs (Chapter 5). Furthermore, I have demonstrated that the neutralisation of CTGF in SCM restored the level of myelination (Chapter 5). A previous study by Stritt et al., (2009) reported that CTGF negatively regulated oligodendrocyte myelination *in vitro* and *in vivo*; an effect that they postulated to be mediated via the sequestering of insulin, which is known to enhance oligodendrocyte maturation and myelination (Brunner et al., 1989; Ye et al., 2002; Zeger et al., 2007), by CTGF. However, my thesis also provided novel evidence to suggest that CTGF signalling may be more complicated than originally thought, given that the use of a CTGF peptide lacking an IGF binding domain still resulted in a reduction in oligodendrocyte myelination *in vitro* (Chapter 5). Experiments from this thesis on purified OPCs have shown that CTGF can act directly upon

oligodendroglial cells to inhibit their antigenic and morphological maturation *in vitro*. Furthermore, preliminary data has also suggested that astrocytes may be involved in this process, via the secretion of secondary factors which negatively regulate myelination, such as BMP4 (Chapter 6); or potentially via the down-regulation of pro-myelinating factors. As discussed in Chapter 6, BMP4 is known to negatively affect oligodendrocyte differentiation and myelination (Huang et al., 2004; Meulemans et al., 2004; Ara et al., 2008; Sabo et al., 2011) and has also been reported to be up-regulated by reactive astrocytes in a contusion model of SCI (Wang et al., 2011). It has been suggested that BMP4 could exert its effects on myelination by inducing the down-regulation of the transcription factors, Olig 1 and Olig2, which are required for oligodendrocyte differentiation (Lu et al., 2000; Zhou et al., 2000; Cheng et al., 2007). Thus, the possible mechanisms by which CTGF could negatively regulate endogenous CNS myelination are likely to be highly complicated and may involve multiple overlapping pathways, as summarised in Figure 8.1.

Future work could focus on directly comparing the properties of untreated astrocytes, LPS-induced reactive astrocytes and SCM/CTGF-treated astrocytes, perhaps using a microarray or chemokine/ cytokine arrays, in order to identify other possible mediators which may be involved in the SC/CTGF induced inhibition of oligodendrocyte myelination. For example, CXCL10 has been reported to be up-regulated *in vitro* in astrocytes which do not support myelination; whilst neutralisation of this factor restored the level of myelination to control values (Nash et al., 2011). Similarly, CXCL12, which has been shown to stimulate MBP production and to enhance oligodendrogliogenesis *in vitro*, has been demonstrated to be activated by astrocytes in cuprizone models of demyelination in the corpus callosum (Patel et al., 2012). The increased expression of this cytokine was reported to induce OPC proliferation and to aid remyelination in acute lesions. Conversely, remyelinative failure in this particular disease model correlated with the down-regulation of CXCL12 in astrocytes. Therefore, CTGF could induce multiple changes in the secretory profile of astrocytes, which could impact negatively upon oligodendrocyte myelination. However, the preliminary data from this thesis relating to the indirect role of astrocytes in the CTGF inhibition of myelination must initially be repeated in order to demonstrate statistical significance before any further

conclusions can be drawn from these experiments. Thereafter, a neutralising antibody to BMP-4 could be used to treat myelinating cultures where the astrocyte substrate had been pre-conditioned with SCM or CTGF prior to its use as a monolayer. If myelination was restored in these cultures, provided that the addition of the antibody alone to control cultures had no significant effect, this data could suggest that SC-secreted CTGF can reduce the myelinating capability of oligodendrocytes by inducing the up-regulation of BMP-4 in astrocytes.

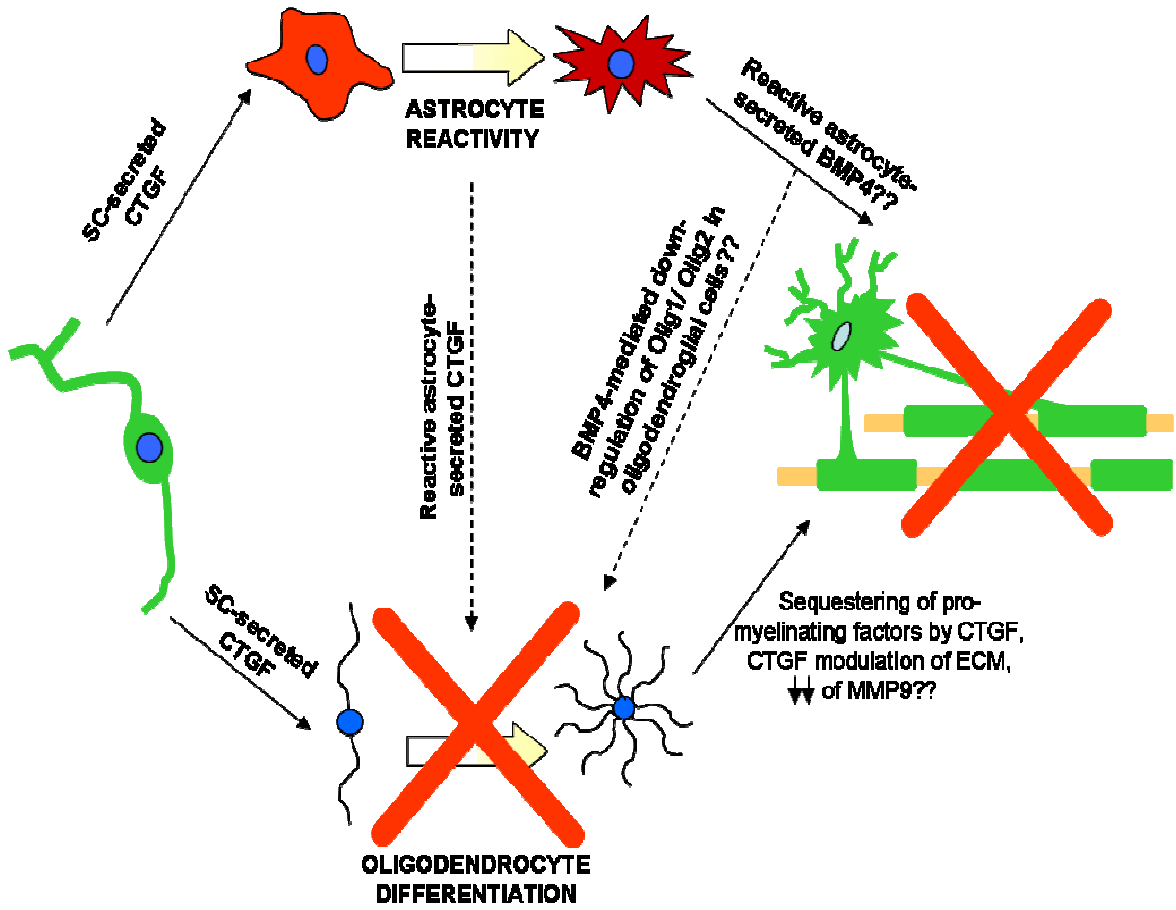


Figure 8.1 - *Schematic of hypothesised signalling mechanisms of CTGF*. Evidence from this thesis has shown that SC conditioned media (SCM), which contains CTGF, and/or CTGF alone significantly reduced the differentiation of purified OPCs in culture. Based upon the literature, this direct inhibition of oligodendrocyte maturation could be due to the sequestering of vital pro-myelinating factors by CTGF, or via the CTGF-induced down-regulation of MMP9 in oligodendroglial cells, which is known to mediate aspects of process extension. SC-secreted CTGF (and other possible factors) could also affect myelination indirectly, by enhancing astrocyte reactivity. Reactive astrocytes could then increase their expression of negative mediators of myelination, such as BMP4, which may induce the down-regulation of vital transcriptional factors in oligodendrocytes, namely Olig1 and Olig2. BMP4 could also negatively affect myelination via unknown mechanisms, which may not necessarily directly block the differentiation of oligodendrocytes. Furthermore, previous studies have shown that reactive astrocytes are also a source of CTGF after injury, which could exacerbate the SC-induced block on oligodendrocyte differentiation by enhancing the bioavailability of CTGF at the lesion site. Due to the highly complex nature of the CTGF molecule, it is possible that CTGF affects myelination through multiple pathways. Furthermore, its signalling mechanisms are also likely to involve several unknown mediators of glial and neuronal cell behaviour, culminating in this reduction in oligodendrocyte myelination.

8.1.3 A Biodegradable Poly-ε-caprolactone (PCL) Scaffold Supports a Complex CNS System of Neurons and Glia

In the interest of providing novel evidence towards the development of a combinatorial repair strategy for the treatment of SCI, data from this thesis has also shown that from a range of biomaterials tested, PCL was the best substrate for supporting myelination *in vitro*, to a level which was comparable to that obtained on glass coverslips (Chapter 7). Furthermore, micro-patterned PCL was shown to support the survival of OECs and astrocytes in culture, and to induce their alignment in response to topography. Given that it has also been shown to be biodegradable and well-tolerated *in vivo* (Woodward et al., 1985; Darney et al., 1989; Kweon et al., 2003; Tay et al., 2007 a,b), this data could suggest the suitability of PCL for fabricating an implantable cell-seeded device, which could enhance the directed outgrowth of neurites, perhaps promoting their successful exit from the graft. Further work would be required to modify this scaffold design for use in long-term cultures in order to overcome the loss of cellular alignment, which occurs with increased cell density. In addition, PCL scaffolds can be fabricated into 3D structures containing pores to aid diffusion throughout its structure; however, this design may need to be modified for *in vivo* use to ensure the successful implantation of such a structure into a more complex injury model, such as a contusion lesion (Seunarine et al., 2006; Sun et al., 2011).

8.2 Correlation of Findings *In Vivo*

Whilst Stritt & colleagues (2009) demonstrated that adenovirus-mediated administration of CTGF into the corpus callosum of mice significantly reduced oligodendrocyte differentiation and myelination, relatively little is known about the expression of CTGF in the CNS and the possible role that it may play in both normal development and disease pathology. Heuer et al., (2003) demonstrated the increased expression of CTGF mRNA associated with neurons in layer VII of the adult mouse cortex, whereas the expression of CTGF was reported to be moderate in the forebrain and in the nucleus of the olfactory tract. Similar expression patterns were observed in embryonic mice from approximately E16. Interestingly, these researchers did not report any findings of CTGF expression in

non-neuronal cells within the CNS. Furthermore, these researchers reported that cerebral trauma did not result in increased CTGF expression, despite the presence of extensive gliosis (Heuer et al., 2003). However, these findings are at odds with a previous report from Spliet et al., (2003), who showed that CTGF was significantly up-regulated by reactive astrocytes in the ventral horn and white matter, and to a lesser extent in motor neurons, in post-mortem tissue derived from patients suffering from amyotrophic lateral sclerosis (ALS). Schwab et al., (2000, 2001) also demonstrated the up-regulation of CTGF associated with reactive astrocytes in human patients after cerebral infarction using immunohistochemistry (as discussed in Chapter 6). Furthermore, in the context of SCI, Conrad et al., (2005) reported the increased expression of CTGF in reactive astrocytes, fibroblasts and endothelial cells associated with blood vessels following a dorsal column lesion in rats. In the aforementioned study, the expression of CTGF was prominent in the acute stages of pathology, suggesting that the ability of CTGF to induce collagen deposition and to modulate the ECM may be fundamental in glial scar formation (Frazier et al., 1996; Grotendorst, 1997; Dammeier et al., 1998; Lasky et al., 1998; Mori et al., 1999; Blom et al., 2001; Stratton et al., 2001).

However, the findings from this thesis that SCs are also a source of CTGF *in vitro* are novel and may provide interesting insights into the mechanisms driving SC-induced astrogliosis. Whilst heparin sulphates, FGF9 and N-cadherin have previously been implicated in SC/astrocyte boundary formation (Fairless et al., 2005; Santos-Silva et al., 2007; Higginson et al., 2012), SC-secreted CTGF could also be an important mediator in this process. Furthermore, by inducing astrocyte reactivity, SCs could actually drive the secretion of CTGF by astrocytes, thus exacerbating the non-permissive environment arising after CNS injury via autocrine and paracrine signaling. This could be demonstrated to some extent using RT-qPCR and ELISA to determine any differences in CTGF expression in untreated astrocytes compared with SC/ CTGF pre-treated astrocytes.

It is more difficult to correlate my findings with relevant *in vivo* data with an emphasis on oligodendrocyte myelination, since the majority of OEC or SC transplantation studies focus on reporting the ability of these exogenously added

cells to myelinate, as apposed to the ways in which the myelinating capability of oligodendrocytes may be affected. As discussed extensively throughout this thesis, early work has suggested that SC remyelination overtakes endogenous remyelination in the CNS in toxin-induced models of demyelination, and that both central and peripherally myelinating glia are predominantly segregated by the presence of astrocytic end-feet (Blakemore, 1975; Blakemore & Patterson., 1975; Sims & Gilmore, 1983).Whilst this in no way proves that SCs are inhibitory to oligodendrocyte myelination *in vivo,* via the secretion of CTGF or other factors, it may suggest an interesting correlation which requires further investigation. However, work carried out in the lab of Prof Edgar Meinl by graduate student Hema Mohan in 2010 reported the up-regulation of CTGF mRNA in remyelinated lesions, active demyelinating lesions and in chronic inactive lesions in tissue obtained from MS patients (http://edoc.ub.uni-muenchen.de/12467/2/Mohan_Hema.pdf). Although these findings were not expanded upon or demonstrated at the protein level, the biggest increase in CTGF expression compared to healthy white matter was observed in chronic lesions, whereby remyelination by oligodendrocytes had ceased. It was unclear from this study which cells were expressing CTGF; however similar up-regulations were also noted in TGF-ß, FGF5 and NT4/5, suggesting that whilst CTGF could potentially play a role in the remyelinative failure of the CNS by endogenous glia, other factors are likely to be involved.

8.3 Implications for Cell Transplant-Mediated Repair of the CNS

Data from this thesis, along with previous observations in the literature, could suggest that the transplantation of SCs into a spinal cord lesion may inhibit the myelinating capacity of oligodendrocytes, in addition to exacerbating glial scar formation. Although transplanted SCs have been shown to myelinate CNS axons in peripheral myelin in injury models (Gilmore 1971; Blakemore, 1975; Felts & Smith, 1992; Honmou et al., 1996; Baron-Van Evercooren et al., 1997; Pearse et al., 2005; Black et al., 2006), it is unclear what the long-term implications would be of ensheathing CNS axons in a myelin coating which has fundamental differences in its composition. Thus, these observations could be interpreted as suggesting that OECs may be a more suitable candidate for cell-mediated repair

strategies, given their ability to support neurite outgrowth (Ramon-Cueto et al., 2000; Lu et al., 2002; Li et al., 2003), to remyelinate CNS axons *in vivo* (Franklin et al., 1996; Barnett et al., 2000; Sasaki et al. 2004; Lankford et al., 2008) and to enhance endogenous myelination *in vitro* (Chapter 4). However, my preliminary findings that the exogenous addition of both OECs *and* SCs resulted in increased endogenous myelination, above the level of controls, could suggest that cell-transplantation therapies combining both cell types could be more effective in the treatment of SCI (Chapter 4). The synergistic effects of OECs and SCs combined in transplantation studies have also been touched upon by other groups, in that OECs are thought to secrete SPARC, which is said to enhance the growth promoting abilities of SCs (Au et al., 2007; Cao et al., 2007; You et al., 2011). However, it has also been shown that CM from SCs causes OECs to form boundaries with astrocytes *in vitro* (Santos-Silva et al., 2007; Higginson et al., 2012). Therefore, this undesirable effect could also be induced *in vivo* following the transplantation of both cell types.

Donor age could also be an important consideration for maximising the effectiveness of SC-mediated transplants, as discussed in section 1.5.2.2. Toma et al., (2001) demonstrated that skin-derived precursor cells (SKPs) could be cultured from human scalp tissue and maintained in culture for up to 50 passages. In addition, these progenitor cells could give rise to neural-crest derivatives, including SCs, though it has been suggested that protocols need to be refined in order to generate sufficient numbers of adult SKPs for transplantation (Biernaskie et al., 2006). Furthermore, the transplantation of SC precursors (SCPs) derived from embryonic rat nerves resulted in their differentiation into myelinating SCs, which displayed enhanced survivability and integration with host astrocytes, compared to transplanted differentiated SCs in EB demyelinating lesions (Woodhoo et al., 2007). In addition, transplanted SCPs were reported to increase neurite outgrowth, as well as reducing glial scar formation and the expression of CSPGs at the lesion site, compared with non-transplanted controls, in contusion and dorsal column crush injury models (Biernaskie et al., 2007; Agudo et al., 2008). Given that they do not appear to induce astrogliosis, it would be interesting to investigate whether or not SCPs differ from SCs in their ability to alter the myelinating potential of oligodendrocytes. If so, then SCPs could also be a very suitable choice of

candidate for autologous cell-mediated repair of the damaged CNS. Similarly, transplanted boundary cap cells show enhanced potential to produce more migratory SCs post-transplantation, thus potentially alleviating the problems associated with poor SC graft/host integration (Zujovic et al., 2007). Induced pluripotent stem cells derived from human fibroblasts are also an interesting candidate for CNS repair given the non-invasive means by which these cells can be generated for autologous transplantation, in addition to their ability to produce CNS derivatives with no undesirable effects post-transplantation (Wang et al., 2013).

8.4 Critical Analysis of Findings

One possible criticism of the body of work carried out for this thesis may be that it was solely carried out *in vitro*. Whilst *in vivo* clarification would be necessary for the formation of clinically relevant conclusions, *in vitro* work can provide valuable information which often translates *in vivo*. In the context of this thesis in particular, the mechanisms of SC/astrocyte boundary formation and reactive gliosis was originally described *in vitro* (Lakatos et al., 2000) before being confirmed *in vivo* (Plant et al., 2001; Lakatos et al., 2003). Similarly, Stritt et al., (2009) firstly demonstrated the ability of CTGF to inhibit oligodendrocyte maturation and myelin formation in culture before validating these findings in the corpus callosum. A further criticism could be that the culture system used throughout this thesis demonstrated the effects of exogenous glia on CNS myelination, as opposed to remyelination. I have discussed in Chapter 1 that remyelination, as occurs to some extent in the adult CNS following disease or injury, is carried out by adult $NG2^{+ve}$ glia, which are distinct from the immature OPCs giving rise to myelinating oligodendrocytes developmentally (ffrench-Constant & Raff, 1986; Wolswijk & Noble, 1989; Keirstead & Blakemore, 1997; Reynolds et al., 1997; Butt et al., 1999; Dawson et al., 2003). Although the precise mechanisms governing remyelination are unclear, adult NG2 glia are thought to respond to similar factors as OPCs, such as FGF, PDGF and IGFs, though they are less mitogenically responsive than neonatal OPCs and divide more slowly (Wolswijk & Noble, 1992; Engel & Wolswijk, 1996; Shi et al., 1998). Thus, it is conceivable that adult NG2 progenitors could respond in a similar

fashion to immature OPCs following exposure to CTGF, though this suggestion is merely speculative and would require appropriate experimental validation.

It could also be critiqued that the focus of this thesis lies predominantly with a single factor, CTGF. Whilst I have shown that the antibody-mediated neutralisation of this factor in SCM reversed the inhibition of oligodendrocyte myelination *in vitro*, it is highly possible that several other factors play a role in modulating this important aspect of SC behaviour. This is particularly evident when considering the data from my CTGF neutralising experiments (Chapter 5), which showed that there was a trend for increased myelination above the level of control when the effects of CTGF were ablated, thus suggesting the presence of less dominant pro-myelinating factors in SCM. For example, SCs have been shown to express IGFs and LIF (Meir et al., 1999; Tofaris et al., 2002; Feng et al., 2010), which are both said to promote oligodendrocyte maturation (Brunner et al., 1989; Mayer et al., 1994; Goodard et al., 1999). As previously discussed, the mechanisms by which SCs induce astrocyte reactivity, thus indirectly reducing myelination, may be multi-factorial (Fairless et al., 2005; Santos-Silva et al., 2007; Higginson et al., 2012).

8.5 Future Perspectives

In addition to those experiments indicated throughout this discussion, there are a number of other studies which could be carried out in order to further this research and to provide evidence of its clinical relevance. Firstly, whilst I have reported the expression of CTGF in SCs in culture, it would be interesting to investigate the presence of CTGF in the sciatic nerve, particularly during Wallerian degeneration and the subsequent repair of peripheral nerves. Also, I have shown that OB-OECs enhance endogenous oligodendrocyte myelination; however it would be worthwhile demonstrating the reproducibility of this phenomenon using LP-OECs, given that LP-OECs are relatively more easily accessible from the dorsal nasal cavity, perhaps making them a more attractive candidate for autologous transplantation. Most importantly, it would be necessary to confirm these findings *in vivo* by investigating the response of endogenous oligodendrocytes to transplanted OECs and SCs in both SCI models

and demyelinating lesions. Homozygous CTGF null mice die perinatally due to skeletal dysplasia (Baguma-Nibasheka & Kaplar, 2008; Doherty et al., 2010). However, in order to prove definitively that SC-secreted CTGF is an important mediator of the inhibition of oligodendrocyte maturation *in vivo*, SCs from transgenic mice whereby CTGF was conditionally knocked down only in SCs could be transplanted into the damaged CNS and the resulting effects on endogenous remyelination and astrogliosis could be studied.

Furthermore, whilst this thesis has provided novel evidence to suggest that the differential expression of CTGF in OECs and SCs correlates with fundamental differences in their behaviour, a more comprehensive analysis should be carried out in order to identify other factors which could be involved in the OEC-mediated enhancement of oligodendrocyte myelination, as well as those which could induce the SC-inhibition of this process. Once these factors had been identified and validated at the protein level, they could be added as exogenous peptides to an appropriate assay, such as the myelinating culture system, to determine their effects on oligodendrocyte behaviour. A study of this kind could also highlight interesting differences in the secretory profile of OECs and SCs, which may regulate important aspects of their behaviour unrelated to their effects on endogenous CNS myelination.

Ultimately, immunohistochemistry of human tissue derived from the lesioned spinal cord or from MS patients, as discussed previously, for the expression of factors like CTGF, could help to build a profile relating to its potential role in pathological processes such as scar formation and remyelinative failure in the CNS. Such evidence could support the view that SCs, as a source of CTGF *in vitro*, may not be the most suitable candidate for the cellular repair of the CNS.

8.6 Closing Remarks

This thesis aimed to provide novel evidence to identify key differences in SC and OEC behaviour, which could be clinically relevant in a transplant scenario. In addition to meeting the aims of this thesis, my findings could also provide a useful contribution to the field of cell-transplantation by highlighting the ways that OECs and SCs differ in their interactions with endogenous CNS glia, and the effects that this could have on endogenous remyelination. Therefore, these observations may be an important consideration for selecting the most appropriate cell candidate to promote the competent long-term functional repair of the damaged spinal cord. In combination with my other findings, I would propose that an OEC-seeded PCL scaffold, as opposed to a scaffold seeded with differentiated SCs, could be more clinically beneficial for the treatment of SCI and less likely to induce negative host responses, such as exacerbated astrogliosis and the inhibition of endogenous oligodendrocyte maturation and myelin formation.

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