REMYELINATION STRATEGIES FOLLOWING SPINAL CORD INJURY

by

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B.Sc. (hons.) The University of British Columbia, 2004

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

DOCTOR OF PHILOSOPHY

in

The Faculty of Graduate Studies

(Neuroscience)

THE UNIVERSITY OF BRITISH COLUMBIA
(Vancouver)

August 2012

Abstract

Spinal cord injury (SCI) results in substantial oligodendrocyte death and demyelination. Remyelination is deemed critical because denuded axons not only lack the myelin necessary to achieve normal conduction velocity, but are also at increased risk of degeneration. A more rapid remyelination thus hypothesized to spare more axons from axonal degeneration, ultimately sparing neurological circuitry from the secondary damage that continues in the days and weeks following SCI. In this thesis I undertake two strategies to improve remyelination after SCI.

In Chapter 2, I investigated whether transplantation of murine Platelet derived growth factor (PDGF)-responsive neural precursor cells (PRPs) could differentiate into remyelinating oligodendrocytes and improve functional recovery after SCI. Transplanted PRPs integrated into host tissue, differentiated into extensively branched mature oligodendrocytes that ensheathed multiple axons, and produced mature myelin. Thus, PRP-derived oligodendrocytes were capable of generating mature myelin sheaths on denuded CNS axons. To our surprise, although transplanted PRPs efficiently produced oligodendrocytes in the injured spinal cord, there was no significant increase in the total number of myelinated axons in PRP-transplanted versus media control animals. Likewise there was no improvement in behavioural recovery following transplantation in two separate experiments.

Blocking known inhibitors of oligodendrocyte differentiation or maturation could improve remyelination. Myelin debris is present following SCI and inhibits oligodendrocyte development *in vitro*, and I hypothesized that myelin debris inhibits remyelination after SCI. In Chapter 3, oligodendrocyte precursor cells (OPCs) were grown in culture in the presence of myelin. Using this approach, I found that on myelin there was a robust inhibition of

oligodendroglia maturation, without a corresponding increase in cell death or proliferation. To understand how myelin inhibits maturation, I measured the expression of a number of genes encoding well-characterized transcription factors that negatively regulate oligodendrocyte development. Associated with stalled maturation, I found myelin increases Inhibitor of Differentiation (ID) 2 and 4, which upon overexpression in OPCs is known to stall maturation. Thus, enhanced levels of ID2 and ID4 in oligodendroglia that are in contact with myelin provides a mechanistic understanding as to how myelin inhibits oligodendroglial maturation.

Preface

A version of Chapter 2 has been published[1]. Plemel JR, Chojnacki A, Sparling JS, Liu J, Plunet W, Duncan GJ, Park SE, Weiss S, Tetzlaff W. 2011. Platelet-derived growth factor-responsive neural precursors give rise to myelinating oligodendrocytes after transplantation into the spinal cords of contused rats and dysmyelinated mice. Glia 59(12):1891-910.

In Chapter 2, I conducted or supervised all of the animal work in this study including all the behavioural assessments, histological assessments as well as conducted the electron microscopy. I also conducted the data analysis, wrote the manuscript and helped with experimenatal design. Andrew Chojnacki conducted all of the cell culture work and helped edit the manuscript. Joseph Sparling helped with behavioural assessments and helped edit the manuscript. Jie Liu conducted the surgeries in this study. Ward Plunet helped with animal grouping and blinding. Greg Duncan helped with histological analysis. Sophia Park helped with histological analysis. Sam Weiss supervised the Cell culture and helped with experimenatal design. Wolfram Tetzlaff supervised this project, edited the manuscript, and helped with experimenatal design. This work was conducted with the approval of the Univesity of British Columbia Animal Care Committee. The animal care certificate numbers that were relavent to this work are A10-0017 “Copy of- Anatomical and functional recovery after spinal cord contusion injury” and A06-1529 “Anatomical and functional recovery after spinal cord contusion injury”.

A version of chapter 3 has been submitted for publication[2]. Plemel JR, Manesh, S, Tetzlaff W. 2011. Myelin inhibits oligodendroglial maturation by increasing transciptional inhibitors of the inhibitors of DNA binding family.

In Chapter 3, I designed the experiment. I conducted all of the cell culture and supervised or conducted all of the culture quantification. I also conducted the data analysis and wrote the manuscript. Sohrab Manesh conducted cell culture quantification. Wolfram Tetzlaff supervised this experiment. This work was conducted with the approval of the Univesity of British Columbia Animal Care Committee. The animal care certificate numbers that were relavent to this work are A08-0200 “Myelin inhibition of myelination”.

Footnotes with similar information are presented with each chapter.

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List of Abbreviations

PDGF	-Platelet-derived growth factor
PRP	-PDGF-responsive neural precursors
PDGFR-α	- Platelet-derived growth factor receptor- α
ID	-Inhibitor of differentiation
SCI	-Spinal cord injury
CNS	-Central nervous system
PNS	-Peripheral nervous system
MS	-Multiple sclerosis
OPC	-Oligodendrocyte precursor cell
4-AP	-4-Aminopyridine
TEA	-Tetraethylammonium
ACOX1	-Acyl-coenzyme A oxidase-1
BMP	-Bone morphogenetic protein
CNTF	-Ciliary neurotrophic factor
LIF	-Leukemia inhibitory factor
GFAP	-Glial fibrillary acidic protein
DT	-Diphtheria toxin
PLP	-Proteolipid protein
MBP	-Myelin basic protein

MAG	-Myelin-associated glycoprotein
MOG	-Myelin oligodendrocyte glycoprotein
OMgp	-oligodendrocyte myelin glycoprotein
MAG	-Myelin associated glycoprotein
CNP	-2´, 3´- cyclic nucleotide 3´-phosphodiesterase
NAD	-Nicotinamide adenine dinucleotide
Sirt	- Sirtuin
ROS	-Reactive oxygen species
RGC	-Retinal ganglion cell
Caspr	-Contactin-associated protein
VLCFA	-Very long chain fatty acids
MCT	-Monocarboxylate transporters-1
Shh	-Sonic hedgehog
FGF	-Fibroblast growth factors
bFGF	-Basic fibroblast growth factors
EGF	-Epidermal growth factor
NT-3	-Neurotrophin-3
IGF	-Insulin-like growth factor
HLH	-Helix-loop-helix
bHLH	-Basic-helix-loop-helix
HMG	-High mobility group

Sox	-SRY-related HMG-box
YY1	-Ying Yang 1
MRF	-Myelin-gene regulatory factor
NICD	-Notch intracellular domain
Gpr17	-G protein-coupled receptor 17
HDAC	-Histone deacetylase
DRG	-dorsal root ganglion
mEPSP	-miniature post-synaptic potentials
NMDA	-N-Methyl-D-aspartic acid
NMDAR	- N-Methyl-D-aspartic acid receptor
AMPA	-α-amino-3-hydroxyl-5methyl-4-isoxazolepropionic acid
AMPAR	-α-amino-3-hydroxyl-5methyl-4-isoxazolepropionic acid receptor
NCAM	-Neural cell adhesion molecule
PSA-NCAM	-Polysialylated form of neural cell adhesion molecule
NOGO	-Neurite outgrowth inhibitor
LINGO	- Neurite outgrowth inhibitor receptor-interacting protein
TLR	-Toll-like receptors
EAE	-Autoimmune encephalitis
ATP	-Adenosine-5'-triphosphate
TNF-α	-Tumour necrosis factor- α
IL-1β	-Interleukin-1 β

IL-2	-Interleukin-2
APC	-Adenomatous polyposis coli (also known as CC1)
NG2	-Neuron-glial antigen 2
O1	-Oligodendrocyte marker 1
O4	-Oligodendrocyte marker 4
ESC	-Embryonic stem cells (ESCs),
GFP	-Green fluorescent protein
BBB	-Basso, Beattie, and Bresnahan (BBB) locomotor rating scale
BOS	-Base of support
ANOVA	-Analysis of variance
LSD	-Least significant difference
SEM	-Standard error of the mean
DMEM	-Dulbecco's modified eagle medium
OGM	-Oligodendrocyte growth medium
PCR	-Polymerase chain reaction
RNA	-Ribonucleic acid
DNA	-Deoxyribonucleic acid
LPC	-Lysolecithin
EB	-Ethidium bromide

Acknowledgments

I will ever be indebted to my supervisor and mentor Wolfram Tetzlaff. He took a chance on a misguided student and showed him that in science the horizon extends as far as ones imagination. I will always be thankful to him for allowing me the freedom to find my own path and try to find answers to those questions that interest me most.

I would like to express my gratitude to my supervisory committee members, Dr. Tim O'Connor, Dr. Timothy Murphy and Dr. Matt Ramer and thank them for their patience and guidance. Thank you to Matt Ramer for your passion of science; you are an inspiration to young scientists.

Thank you to the Canadian Institutes of Health Research, Michael Smith Foundation of Health Research and Multiple Sclerosis Society of Canada for the studentships. I would also like to thanks Canadian Institutes of Health Research, Michael Smith Foundation of Health Research, Rick Hansen Man in Motion Foundation, the Canadian Stem Cell Network of Excellence and the Multiple Sclerosis Society of Canada for their financial support to conduct this research.

Through the years I have had an incredible support team in the Tetzlaff lab. Thank you to my partner in crime, Joseph Sparling, for your continued support, creative ideas and conversations at our coffee breaks. I will always be indebted to you for your amazing editorial support throughout our time together. Clarrie Lam, you are a star among lab managers, your technical assistance and help throughout was very much appreciated. Likewise, Jie Liu, your technical brilliance in the surgery room made this work possible. Thank you to my ever-supporting network of graduate students in the lab, past and present, for your encouragement, ideas, criticisms and discussions: Greg Duncan, Peggy Assinck, Ward Plunet, Carmen Chan, Dave Stirling and Brett Hilton. For help with the PRP work, thank you Trevor Scudamore, Casey Shannon and Darren Sutherland for your help with the behaviour and animal care. I would also like to thank Dr Fabio Rossi for providing time on his confocal microscope and to Bahareh Ajami for her support with this equipment and otherwise. I would like to thank Robin Battye and

Quorum Technologies for perhaps the best confocal image of a transplant-derived oligodendrocyte ever taken at a demonstration. With regards to my work culturing OPCs, I would like to give my most sincere thanks to the Barres Lab, especially Jason Dugas and Adiljan Ibrahim for your help. Thank you Lisa Anderson, Jennifer Hinze, Benjamin Richards and Sohrab Manesh for help with quantifying my overwhelming number cell culture experiments.

To my wider graduate student support network, thank you for your continued support. To Angela Scott, Andrew Gaudet, Leanne Ramer, Mark Crawford, Diana Hunter, Jacquelyn Cragg, and Jessica Inskip, thank you for your encouragement and help over the years. Thank you to Chris West for taking the time to read much of this thesis. To my larger ICORD family, thank you Tom Oxland for your enthusiasm and leadership. I would like to thank Lowell McPhail, Cheryl Niamath and Jeremy Green for your guidance and assistance over these many years. As well, to the many outstanding ICORD trainees I have had the opportunity to engage during my time working with ICORD trainee committee, it has been a blast.

To my parents, thank you for your unyielding support, your strength and intellect and your determination, thank you Mom and Dad for teaching me the importance of hard work. To my Aunt Cindy, thank you for your ongoing love and support. Chris, Amy, Avalon, Charlotte and Xavier, thank you for your love and advocacy. And last, but not least, thank you Lesley Soril from the bottom of my heart, you are my biggest fan. I cannot ever thank you enough for your unyielding patience, ongoing encouragement and unwavering support- I could not have done this without you. Having you with me through these years has made the high points even brighter and the low points hardly noticeable.

Dedication

To my family: past, present and future.

Chapter 1

General introduction

1.1 Introduction overview

Damage to oligodendrocytes and the ensuing loss of myelin (or demyelination) is a common occurrence in numerous disease conditions, including spinal cord injury (SCI), multiple sclerosis (MS), and stroke. The replacement of myelin, or remyelination, is robust following focal demyelination in animal models, but it can fail in human disease conditions such as MS. Remyelination, in many respects, recapitulates developmental myelination. Thus, understanding development of myelin is important to promote remyelination in the mature central nervous system (CNS). The absence or abnormal production of myelin is also seen in a heterogeneous group of inherited disorders called leukodystrophies that usually occur in infants and often lead to premature death. These conditions have contributed to our understanding of both myelin development and axon support by oligodendrocytes. While much of the discussion in this thesis surrounds both the demyelination and remyelination mechanisms following SCI, there are, in fact, parallels between these aforementioned inherited conditions that can inform our understanding of the mechanisms underlying white matter damage.

Attempts to improve remyelination after SCI commonly involve two strategies: cell transplantation or stimulation of endogenous remyelination. The former as a treatment for SCI will be the focus of Chapter 2. Transplantation of neural precursors/progenitors has been widely successful in promoting improved functional recovery after SCI. However, the optimal cell has yet to be determined. Embryonic stem cells are a source of neural precursors/progenitors and expand relatively easily, but have the potential to produce embryonic tumours when transplanted (Reubinoff et al. 2000; Thomson et al. 1998). Another source of neural precursors/progenitors is

induced pluripotent stem cells, which are differentiated cells that are stimulated to become pluripotent. Like embryonic stem cells, induced pluripotent stem cells are likely to produce embryonic tumours when transplanted. By contrast, Fetal or adult-derived neural precursors/progenitors do not produce tumours when transplanted. In Chapter 2, I transplanted fetal platelet-derived growth factor (PDGF)-responsive neural precursor cells (PRPs), which are multipotential cells capable of self-renewal *in vitro* (Chojnacki and Weiss 2004). **I hypothesized that transplanted PRPs after SCI will remyelinate denuded axons and improve remyelination as well as functional recovery.** I demonstrated that transplanted PRPs integrate with host tissue and differentiate into remyelinating oligodendrocytes. To my surprise, PRP transplantation was not associated with an increase in the number of myelinated axons at the lesion epicenter, nor was it associated with improvement in functional recovery in two separate transplantation experiments.

The second strategy promotes myelin repair by enhancing the endogenous remyelination response. By coaxing endogenous cells to remyelinate more rapidly and efficiently it would be possible to avoid the logistical and perhaps even ethical considerations surrounding cell transplantation. An important rate-determining step in spontaneous remyelination is the presence of factors that block remyelination. These factors could also inhibit the remyelination by transplanted cells. Targeting these remyelination inhibitors might provide a better strategy to improve remyelination. Myelin is one putative remyelination inhibitor that is present following SCI.. Therefore, **I hypothesized that myelin inhibits remyelination after SCI.** In Chapter 3, I studied how myelin inhibits oligodendrocyte precursor cells (OPCs) in culture. I developed a

myelin spot assay and demonstrated that myelin inhibits maturation of oligodendrocyte lineage cells. This was characterized by decreased expression of immature and mature oligodendrocyte markers, impaired production of myelin gene products, and reduced morphological complexity. Myelin gene products are regulated by transcription factors, thus, I measured the expression of transcription factors known to regulate oligodendrocyte differentiation. I found that contact with myelin increased Inhibitor of Differentiation (ID) family members 2 and 4. Since the overexpression of either ID2 or ID4 in OPCs is sufficient to arrest their development (Kondo and Raff 2000; Wang et al. 2001), elevated levels of ID2 and ID4 due to contact with myelin is sufficient to block oligodendrocyte lineage cell maturation. Upregulation of ID2 and ID4 provides a mechanistic explanation as to how myelin blocks this maturation.

In the remainder of this introductory chapter I will first describe myelin, its importance in axonal support, and its development. I then discuss the cellular processes of endogenous remyelination, and the functional consequences of demyelination following SCI. The overall objective of this thesis is to understand and manipulate exogenous and endogenous remyelination in the context of SCI.

1.2 Myelin and its role in axon support: why remyelination matters.

1.2.1 What is myelin?

Through the use of metal stains and light microscopy, Del Rio-Hortega (Bunge 1968; Del Rio-Hortega 1921) was the first to give a complete morphological description of the oligodendrocyte, describing them as process-bearing cells that are found in rows along axons. Due to their position along axons, their appearance at the time of myelination, and their direct relation to myelinated fibres Del Rio-Hortega (1922) and Penfield (1924) concluded that oligodendrocytes were the myelinating cell of the CNS. At the time, this observation was quite contentious and only later with the use of electron microscopy was direct evidence found that lent credence to the concept that CNS myelin was derived from oligodendrocytes (Bunge et al. 1962; Hirano et al. 1966; Kruger and Maxwell 1966; Metuzals 1963; Peters 1964). Based on evidence from light microscopy and electron microscopy, emerged the fundamental concept that a single oligodendrocyte extends numerous processes: each one capable of producing an individual myelin segment (Bunge 1968). The cell bodies and processes of oligodendrocytes can now be imaged together using modern microscopes with fluorescently labelled markers (as demonstrated in Chapter 2); this work demonstrates with exquisite clarity the ability of an oligodendrocyte to myelinate multiple axons (Butt and Ransom 1989). The ability to myelinate multiple axons is an important and fundamental characteristic of oligodendrocytes.

Myelin's primary function is to increase action potential conduction velocity along axons (Harris and Attwell 2012). This increased conduction velocity provides an obvious evolutionary

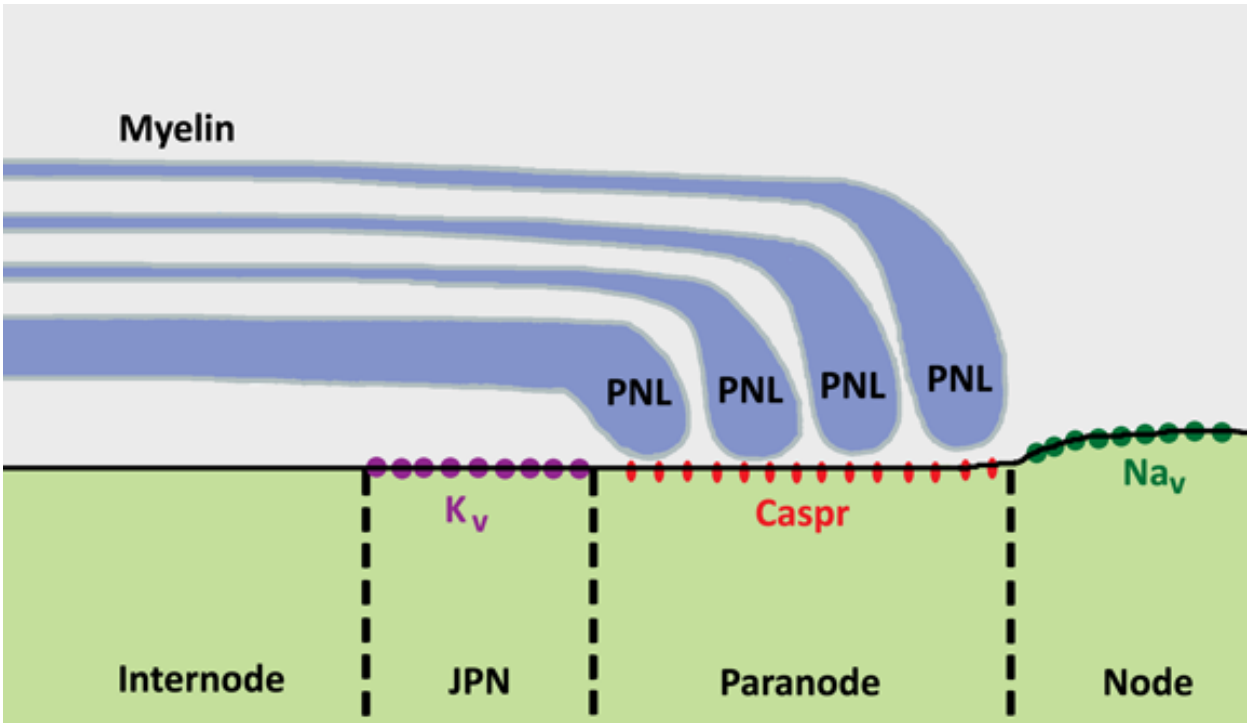
advantage in making a quick escape response or a fast attack move. Thus, it is not surprising that myelin has arisen independently several times in the course of evolution (Hartline and Colman 2007). In myelinated axons, the electrical current enters exclusively at the node of Ranvier. At the adjacent internode, myelin facilitates an increase in the resistance and a decrease in the capacitance of the axonal membrane. This ultimately results in a drop in current conductance through internodal regions. With less axonal membrane exposed, myelin reduces the area into which the current must flow. Myelin also attenuates current loss along the internode, allowing current to travel greater distances. The decreased capacitance along the internode allows faster charging of the internodal membrane. As a result, the presence of myelin along the axon gives rise to a drastic increase in conduction velocity, which is linearly related to the axonal diameter. This contrasts with conduction velocity in unmyelinated fibres, which is related to the square root of the axonal diameter (Moore et al. 1978).

The presence of myelin requires a specific distribution of channels throughout the axolemma (Figure 1.1). For instance, in myelinated axons Na+ channels are located almost exclusively within the axonal membrane of the nodes of Ranvier (Ritchie and Rogart 1977; Waxman 1977; Waxman 2006). This distribution allows the entry of inward Na^+ current almost entirely at the nodes of Ranvier. In contrast, K^+ channels in myelinated fibres are largely restricted to the juxtaparanode (Figure 1.1). In an unmyelinated axon, the outward K^+ current repolarizes the axonal membrane, as demonstrated by the prolonged action potentials that occur when K^+ channels are blocked with 4-Aminopyridine (4-AP) or tetraethylammonuim (TEA) (Malenka et al. 1981; Preston et al. 1983). As fibres become myelinated in the CNS they lose

their sensitivity to 4-AP, and thus prolongation of the action potentials becomes substantially diminished (Gordon et al. 1988; Kocsis and Waxman 1980). In myelinated fibres, voltage clamp experiments demonstrate that K+ channels are normally absent from Ranvier nodes (Chiu and Ritchie 1980; Chiu and Ritchie 1981; Chiu et al. 1979). Taken together, myelination alters the arrangement of voltage-sensitive K+ channels into regions along the axon that are covered by the myelin sheath. However, if myelin sheaths are disrupted, the K+ channels become unmasked. The K+ channels $K_v1.1$ and $K_v1.2$ of the shaker family of delayed rectifier K+ channels (Rhodes et al. 1997; Wang et al. 1993) are 4-AP sensitive (Mathie et al. 1998) and located in the juxtaparanode underneath the myelin sheath. The role of these juxtaparanodal K+ channels is not only thought to maintain the resting potential of the axon, but also to stop re-entrant excitation and prevent aberrant excitation (Chiu and Ritchie 1981; Chiu and Ritchie 1984; Vabnick et al. 1999; Zhou et al. 1998).

Much less is known about Ca^{2+} channels along the axolemma. In the optic nerve, P/Q-type Ca^{2+} channels cluster in the axolemma during myelin development at early nodes of Ranvier (Alix et al. 2008). P/Q-type Ca^{2+} channels are necessary for the development of Ranvier nodes, but their role and location in mature myelinated axons is unclear. There is also evidence that L-type Ca^{2+} channels reside in the axolemma of myelinated axons (Ouardouz et al. 2003). Ca^{2+} entry through L-type channels is thought to activate ryanodine receptors, which then allow Ca^{2+} entry from intracellular stores. L-type Ca^{2+} channels contribute to axonal Ca^{2+} levels during ischemia (Stirling and Stys 2010), but their role during development and non-pathological conditions is also unclear.

Figure 1.1 Characterization of axonal structures adjacent to nodes of Ranvier. The axon is organised into discrete regions: the internode, the juxtaparanode, the paranode and the node of Ranvier. These regions of the axolemma express characteristic markers voltage-gated potassium channels (K_v) occupy the juxtaparanode, whereas contactin Associated Protein (Caspr) is located in the paranode. The node of Ranvier is characterized by expression of voltage-gated sodium channels (Na_v). The paranodal loops (PNL) at the outer edge of the myelin sheath axonal structures at the paranode axolemma. This figure was adapted from Perlin and Talbot (2007).



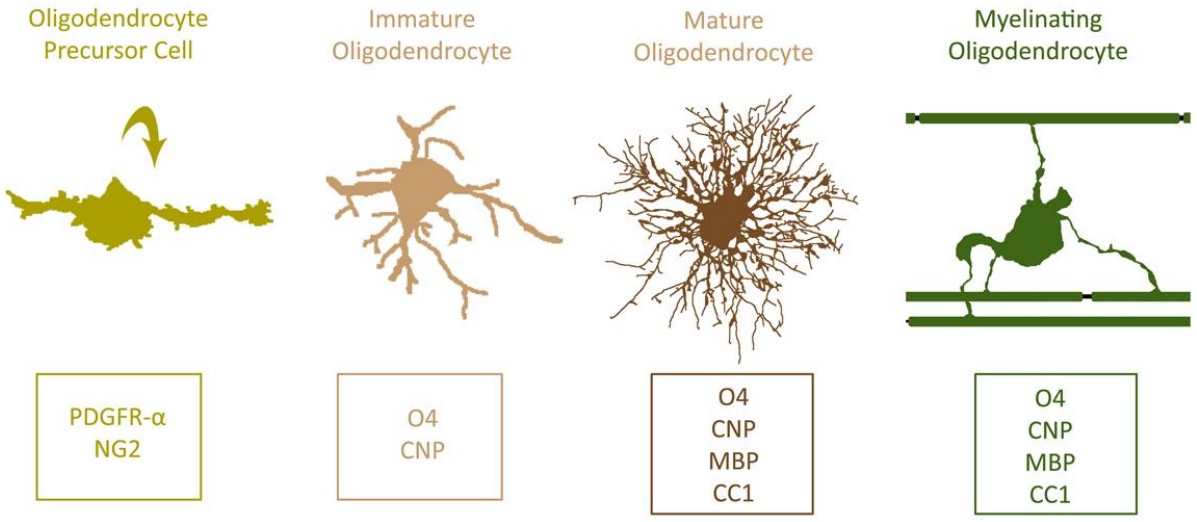
1.2.2 Important concepts in oligodendrocyte development

Oligodendrocytes are postmitotic cells that develop from the specification, migration and proliferation of OPCs. Our understanding of oligodendrocytes is due in large part to the abundance of lineage-specific markers which have been used to describe the stages of oligodendrocyte development: OPC, immature oligodendrocyte, mature oligodendrocyte and myelinating oligodendrocyte (for markers in this thesis see Figure 1.2). To understand oligodendrocyte development, some basic principles and definitions must be introduced. When using common strategies to culture OPCs from postnatal animals (Barres et al. 1992; Chen et al. 2007; Dugas et al. 2006; McCarthy and de Vellis 1980), OPCs are largely fate-restricted to the cells of the oligodendrocyte lineage. OPCs are also fate restricted *in vivo* as indicated by 'fate mapping' of postnatal animals using the OPC-specific platelet-derived growth factor (PDGFR)-α promoter, which almost exclusively labels oligodendrocyte lineage cells without labelling astrocytes (Kang et al. 2010; Rivers et al. 2008). Still, *in vitro* OPCs can differentiate into type 2 astrocytes, characterized by their thin glial fibrillary acidic protein (GFAP)-positive processes. This occurs when OPCs are exposed to certain factors such as fetal calf serum (Lillien and Raff 1990; Raff et al. 1983), morphogenetic proteins (e.g. bone morphogenetic proteins [BMPs]) (Kondo and Raff 2004; Mabie et al. 1997; Wang et al. 2011), ciliary neurotrophic factor (CNTF) (Hughes et al. 1988; Lillien et al. 1990), leukemia inhibitory factor (LIF) or oncostatin-M (Gard et al. 1995). While the ability of OPCs to make astrocytes under pathological conditions *in vivo* is still debated (Fulton et al. 1992; Kang et al. 2010; Richardson et al. 2011; Rivers et al. 2008; Skoff 1990; Tripathi et al. 2010; Zawadzka et al. 2010), *in vitro* it is undeniable that OPCs can

produce multiple cell types depending on culture conditions. Within this thesis, the down-regulation of OPC markers or the end of cell division will be referred to hereafter as differentiation. By definition, differentiation is the process whereby a less specialized cell becomes more specialized. The term maturation will be used to distinguish the stages of oligodendrocyte development that occur after OPCs stop proliferating and lose OPC-specific antigens, such as increased morphological complexity, or increased production of myelin proteins. *In vivo,* it is often more difficult to distinguish maturation and differentiation as there is a paucity of immature oligodendrocyte markers and a stable OPC density after early development. Thus *in vivo,* an increase in the number of oligodendrocytes will be referred to as oligodendrocyte differentiation.

OPCs are maintained as OPCs, at least in culture, by mitogens that drive their proliferation (Tang et al. 2001; Tang et al. 2000). Under optimized culture conditions, and in the presence of mitogens, OPCs proliferation can be extended for over a year due to an apparent lack of cellular senescence (Tang et al. 2001; Tang et al. 2000). Upon mitogen removal, OPCs stop dividing and differentiate into oligodendrocytes; this occurs spontaneous *in vitro*, even in the absence of differentiating cues or axons (Barres and Raff 1999).

Figure 1.2 Oligodendrocyte lineage cell characterization. OPCs, characterized by the expression of PDGFR-α and NG2, can self-renew when in the presence of mitogens PDGF *in vitro* and *in vivo*. In culture, the transition to an immature oligodendrocyte is associated with the down-regulation of OPC markers as well as a decreased capacity to proliferate. Immature oligodendrocytes express the markers O4 and CNP. As oligodendroglial cells mature, they express additional myelin proteins such as MBP or late stage markers such as CC1 (also known as APC). In the presence of axons, oligodendrocytes can ensheathe and wrap multiple axons, ultimately producing compact myelin. This figure was adapted from de Castro and Bribian (2005).



1.2.3 Myelin provides trophic support to axons

Historically, myelin was primarily seen as a means to increase the speed of axonal conduction (see above). However, more recently oligodendrocytes and the myelin they produce have been found to play a role in the trophic support of axons. Axons extend great distances from their cell bodies, often in excess of a metre. This length, and the time required to obtain cell body-derived resources pose a great challenge to the normal metabolic function of axons. This is not a trivial impediment. For example, if fast axonal transport of mitochondria occurs at approximately 50mm/day in mammals, it would require 20 days for a mitochondrion to travel from the cell body to the distal end of the axon (Grafstein and Forman 1980). With the metabolic enzymes that undergo slow axonal transport at a rate of approximately 0.2-6mm/day in mammals (Brady and Lasek 1981; Oblinger et al. 1988), it would require 166 days (at 6mm/day) to travel from the cell body to the distal end of the axon. Some have hypothesized that oligodendrocytes support axons to accommodate their length (Nave 2010a; Nave 2010b; Nave and Trapp 2008). Oligodendrocytic support of axons is important in discussions of white matter damage, because demyelination results in a loss of this support, providing one explanation as to why persistently demyelinated axons are more prone to axonal degeneration (Irvine and Blakemore 2008). If axons are persistently demyelinated and unsupported by oligodendrocytes, then a more rapid remyelination is predicted to spare more axons from the secondary damage that continues in the days and weeks following SCI. Increased axonal sparing from a more rapid remyelination may be one mechanism underlying the correlation between remyelination and functional recovery after SCI.

1.2.3.1 Loss of oligodendrocyte results in robust axonal degeneration

Loss of oligodendrocytes, if excessive, results in axonal degeneration, and this fact provides compelling evidence that oligodendrocytes support axons. For instance, oligodendrocytes can be selectively ablated in transgenic mice. Oligodendrocyte-specific ablation can be induced by the injection of diphtheria toxin (DT) into transgenic mice that express the DT receptor specifically in oligodendrocyte (Buch et al. 2005; Ghosh et al. 2011). Alternatively, in a second transgenic mice line, oligodendrocyte-specific production of the death-inducing A-subunit of DT can be induced by systemic administration of the estrogen receptors agonist tamoxifen (Pohl et al. 2011; Traka et al. 2010). In both transgenic lines, oligodendrocyte death causes demyelination, astrogliosis, microgliosis, dramatic tissue vacuolation and robust axonal damage (Pohl et al. 2011; Traka et al. 2010). Surprisingly, damage to oligodendrocytes does not increase the permeability of the blood-brain barrier (Pohl et al. 2011) or result in significant T-cell or B-cell infiltration (Locatelli et al. 2012; Pohl et al. 2011; Traka et al. 2010). Thus, damage to axons is independent of the adaptive immune system and likely due to the loss of oligodendrocyte support (Locatelli et al. 2012; Pohl et al. 2011).

1.2.3.2 Loss of certain myelin proteins results in axonal degeneration prior to demyelination

Studies on mice deficient in Proteolipid protein (PLP) —a protein found in CNS myelin (Griffiths et al. 1998) —provide a strong link between oligodendrocytes and axonal support. PLP-deficient mice develop myelin normally, but after 2-3 months begin developing axonal

swellings that are normally associated with axonal degeneration. These axonal swellings also contain phosphorylated neurofilaments, which are a marker of axonal degeneration (Petzold 2005). PLP-deficient mice also develop ataxia at 2-3 months of age and die prematurely (Griffiths et al. 1998). In mice that are deficient in myelin basic protein (MBP), another CNS and peripheral nervous system (PNS) myelin protein, there is no compact myelin in the CNS and yet there is very little axonal degeneration in these mice at 2-3 months of age. This suggests that the loss of compact myelin *per se* is not detrimental to axons during this period. When PLP-null mice are bred with MBP-deficient mice, the PLP-null and MBP-deficient offspring are phenotypically similar to PLP-null mice and contain extensive axonal swellings, but without compact myelin (Griffiths et al. 1998). Therefore, the loss of PLP is associated with an oligodendrocyte-specific defect that results in axonal degeneration independent of compact myelin. It is not entirely clear how PLP is linked to the support of axons. Proteomic analysis of myelin in PLP-deficient mice has revealed several proteins that are found at lower levels within PLP-deficient myelin, indicating that PLP might have an important transport role within myelin (Werner et al. 2007). One protein almost entirely absent in the myelin of PLP-null mice is the nicotinamide adenine dinucleotide (NAD+)-dependent deacetylase sirtuin 2 (Sirt2) (Werner et al. 2007). Sirt2 has been demonstrated to play a protective role in a *Drosophila* model of Parkinson disease (Outeiro et al. 2007) and can deacetylase the transcription factor FOXO3, reducing cellular levels of reactive oxygen species (ROS) (Wang et al. 2007). Thus, loss of Sirt2 from myelin in PLP-null mice might account for the axonal degeneration, but this requires further testing *in vivo*.

Similar to PLP loss, the deficiency of the enzyme 2´, 3´- cyclic nucleotide 3´-phosphodiesterase (CNP)—a protein found in the early development of oligodendrocytes and maintained in myelinating oligodendrocytes (Yu et al. 1994)—results in axonal swelling, phosphorylation of neurofilaments, and substantial axonal loss (Lappe-Siefke et al. 2003). Like PLP, loss of CNP is not associated with a gross defect in myelin development. Small axonal swellings are often surrounded by a seemingly normal myelin sheath, indicating that axonal degeneration precedes any myelin loss. Axonal damage in CNP-deficient mice correlates with motor defects, ultimately resulting in premature death in these mice (Lappe-Siefke et al. 2003). The mechanistic link between these effects of CNP loss and axonal degeneration is unclear. CNP participates in the regulation of Ca^{+} levels in the mitochondria as well as the regulation of mitochondria permeability transition (Azarashvili et al. 2009), which is an increase in mitochondria permeability that can result in mitochondrial swelling and cell death. Alternatively, CNP loss has been associated with impaired paranodal integrity that might contribute to axonal degeneration (Rasband et al. 2005). Loss of paranodal integrity can be toxic to axons as the disruption of one structural paranodal protein—contactin-associated protein (Caspr)—is associated with abnormal paranodes and ataxia (Bhat et al. 2001).

Another oligodendrocyte protein that is involved in axonal support is myelin associated glycoprotein (MAG). MAG is an oligodendrocyte protein on the surface of mature myelin; it directly contacts the axonal surface. Myelination can occur in the absence of MAG and is generally normal in MAG-deficient mice (Bartsch et al. 1997; Marcus et al. 2002). In the absence of MAG (Nguyen et al. 2009; Pan et al. 2005; Sheikh et al. 1999) or its axonal receptors

GD1a and GT1b (Collins et al. 1997a; Collins et al. 1997b; Yang et al. 1996) there is axonal degeneration in the CNS. Axonal contact with MAG-expressing cells in culture protected axons from undergoing axonal degeneration (Nguyen et al. 2009), suggesting that MAG can activate a protective signalling pathway within axons. The combination of MAG deficiency and PLP deficiency causes very minor myelin defects, but produces an earlier onset of axonal degeneration than the loss of either PLP or MAG alone (Uschkureit et al. 2000), arguing that these proteins act via independent mechanisms. The downstream signalling initiated by axonal contact with MAG might provide insight as to how MAG protects axons from degeneration.

1.2.2.3 Disruption of oligodendrocyte peroxisomes produces inflammation and axonal degeneration

Oligodendrocyte peroxisomes are another link between oligodendrocytes and axonal support. Peroxisomes are ubiquitous eukaryotic organelles that are important for the detoxification of reactive oxygen species (ROS) and the β-oxidation of very long chain fatty acids (VLCFA) (Kassmann et al. 2011; Titorenko and Terlecky 2011; Wolf et al. 2010). To study the role of peroxisomes in oligodendrocytes, Kassmann and colleagues removed the enzyme peroxin-5 (2007)—an enzyme that is needed to import cytosolic enzymes into peroxisomes (Gould and Collins 2002; Wolf et al. 2010)—in oligodendrocyte lineage cells. Peroxisomes are numerous in oligodendrocytes, but when their function is impaired by the absence of peroxin-5, there is no major defect in myelin development (Kassmann et al. 2007). However, the loss of peroxin-5 in oligodendrocyte lineage cells results in a progressive ataxia that is associated with axonal degeneration and demyelination. The finding that axonal damage

precedes demyelination indicates that oligodendrocyte peroxisomes have a protective role for axons. Peroxisomal defects are also associated with activated microglia, astrogliosis, and inflammation, including a T-cell response that is predominantly of the cytotoxic CD8+ subset. The inflammation arising in mice with oligodendrocyte peroxisomal defects is intriguing because it suggests that oligodendrocytes might regulate inflammation. Mechanistically, such oligodendrocytic regulation of inflammation may occur through the cell's degradation of lipid products, including prostaglandins and leukotrienes, which are broken down in peroxisomes (Diczfalusy 1994; Funk 2001; Mayatepek and Tiepelmann 1996). Without functional peroxisomes, an accumulation of lipid by-products might trigger inflammation. Since prostaglandins and leukotriene levels are often elevated during CNS pathology, oligodendrocyte-peroxisomes are unexplored regulators of neuroinflammation

Ongoing regulation of gene expression in oligodendrocytes is necessary for peroxisomal function and axonal integrity. Blocking gene expression regulation by microRNA in maturing oligodendrocytes impairs β-oxidation and axonal degeneration (Shin et al. 2009). MicroRNAs are small non-coding RNAs that serve as post-transcriptional regulators of roughly 30% of genes (Bushati and Cohen 2007). Conditional removal of the enzyme Dicer blocks the majority of microRNA production (Yang and Lai 2011). Dicer-deficient OPCs fail to differentiate normally and to produce myelin during development (Dugas et al. 2010; Zhao et al. 2010). However, removing Dicer in differentiated oligodendrocytes in 2-week-old mice does not alter developmental myelination, but instead is associated with demyelination, oligodendrocyte apoptosis, astrogliosis, microgliosis, and a reduced lifespan (Shin et al. 2009). The loss of Dicer

is associated with acyl-coenzyme A oxidase-1 (ACOX1) reduction, a rate-limiting enzyme in the peroxisomal β-oxidation pathway (Fan et al. 1996), as well as an overall decrease in β-oxidation. This suggests that impairing microRNA synthesis contributes to diminished peroxisomal function. Mice with Dicer-deficient oligodendrocytes also have elevated oxidative damage and elevated levels of proteins associated with stress and redox homeostasis, including proteins that detoxify ROS, such as catalase and peroxiredoxin. Therefore, the ongoing gene expression regulation by microRNAs in oligodendrocytes is important for the continued function of peroxisomes.

1.2.3.4 Potential role of oligodendrocytes in energy homeostasis

The long distance that metabolic enzymes must travel from neuronal cell bodies to the distal ends of axonal processes has led researchers to hypothesize that oligodendrocytes provide metabolic support such as glycolysis products to long axons (Nave 2010a; Nave 2010b; Nave and Trapp 2008). Axons are thought to receive glucose from astrocytes that contact at Ranvier nodes (Butt et al. 1994a; Butt et al. 1994b), but oligodendrocytes ensheathe large portions of the axons and contact a much greater surface area of the axon than do astrocytes. Although it is theoretically possible to provide sufficient energy for oxidative phosphorylation in the optic nerve by providing glucose solely through the nodes of Ranvier (Harris and Attwell 2012), additional axonal metabolic support by oligodendrocytes might be necessary during peak performance or during periods of low glucose or oxygen deprivation. Lactate is a potential source of energy for neurons and can replace glucose. It is hypothesized to be important for axonal function *in situ* (Brown et al. 2001), although its importance *in vivo* is debated (Allen et

al. 2005; Chih and Roberts Jr 2003). In the CNS, lactate and can be produced by glycogenolysis in astrocytes (Dringen et al. 1993a; Dringen et al. 1993b). Because glycogen storage occurs predominantly within astrocytes (Cataldo and Broadwell 1986), these cells are thought to be a major source of lactate. Although it is assumed that lactate is transported from astrocyte to axon, it is possible that oligodendrocytes are responsible for lactate transport into axons. In culture, oligodendrocytes take up lactate to a greater extent than do neurons (Sanchez-Abarca et al. 2001) through the transporter monocarboxylate transporters-1 (MCT-1) (Rinholm et al. 2011). This lactate uptake can support oligodendrocyte development, rescuing the impaired myelin development that occurs in low glucose environments (Rinholm et al. 2011). Although there is no direct evidence that lactate from oligodendrocytes can be transported to axons, indirect evidence for lactate transport from oligodendrocyte is suggested by the location of the axonal lactate transporter MCT-2, which is found throughout the internode adjacent to the myelin sheath. Mice with oligodendrocytes that are deficient in MCT transporters will be required to determine whether lactate transport from oligodendrocyte to axon occurs *in vivo*.

Oligodendrocytes and astrocytes are connected by gap junctions, making oligodendrocytes convenient conduits for astrocyte-derived metabolites that can be secreted into the periaxonal space and received by axons (Nualart-Marti et al. 2012). Gap junctions are composed of members of the connexin family of transmembrane protein, with two pairs of compatible connexins coupled to form each gap junction (Willecke et al. 2002). The oligodendrocyte-specific connexins 32 and 47 are thought to couple with astrocyte connexins 30 and 43 to mediate gap junction coupling between oligodendrocyte and astrocytes (Altevogt and

Paul 2004; Dermietzel et al. 1989; Menichella et al. 2003; Nagy et al. 2001; Odermatt et al. 2003; Orthmann-Murphy et al. 2007; Rash et al. 2001). Many of these connexins are also involved in oligodendrocyte-oligodendrocyte coupling (O-O) or astrocyte- astrocyte coupling (A-A), creating a glial network. This gap junction network is important in homeostasis as the loss of oligodendrocyte connexin 32 (Sargiannidou et al. 2009) or connexin 47 (Menichella et al. 2003; Odermatt et al. 2003) produces both myelin defects and reduced O-A coupling (Maglione et al. 2010). The loss of both oligodendrocyte connexins results in a more profound phenotype with axonal loss, oligodendrocyte death, myelin vacuolation and early mortality of connexin-deficient mice (Menichella et al. 2003; Menichella et al. 2006; Odermatt et al. 2003). Gap junctions are thought to be important in extracellular potassium buffering arising from axonal activity (Menichella et al. 2006; Neusch et al. 2001), but might also facilitate metabolite movement between astrocytes and oligodendrocytes. Whether this metabolic coupling occurs *in vivo* is still unclear.

The potential mechanisms of axonal support by oligodendrocytes are clearly numerous. The oligodendrocytic regulation of oxidative stress and β-oxidation of VLCFAs is likely important *in vivo* and might regulate inflammation during pathology (Kassmann et al. 2007; Kassmann and Nave 2008; Shin et al. 2009). Oligodendrocytes are an intermediate in a glial network, receiving metabolites from astrocytes or indirectly from blood vessels and potentially releasing them onto the axonal membrane (Nave 2010a; Nave 2010b; Nave and Trapp 2008; Rinholm et al. 2011). The disruption of such a network during pathology is predicted to decrease the energy available to an axon and would likely exasperate axonal degeneration. The loss of

certain myelin proteins, such as PLP and MAG, results in axonal degeneration in the absence of compact myelin, whereas the absence of compact myelin results in little if any axonal degeneration, suggesting that additional mechanisms of oligodendrocyte support of axons exist that are not dependent on mature myelin (Griffiths et al. 1998; Nguyen et al. 2009; Uschkureit et al. 2000). Understanding how oligodendrocytes support axons is central to the fields of axonal degeneration and myelin repair as it provides a mechanistic understanding for how demyelination might be linked to axonal degeneration (Bjartmar et al. 2000; Ganter et al. 1999; Lovas et al. 2000; Trapp and Stys 2009). In this context, remyelination is hypothesized to increase axonal sparing. Remyelination has many direct parallels to developmental myelination and a thorough understanding of remyelination requires an understanding of myelin development.

1.3 Myelin and its development: what are the lessons for repair

Bearing in mind that many aspects of remyelination are a recapitulation of development, I review here the major stages of oligodendrocyte development: specification, proliferation/ migration, differentiation and myelination. SCI occurs primarily in late adolescence, adulthood, and old age, making it important to recognize the persistence of adult OPCs that are responsible for remyelination. I will also briefly review adult OPCs.

1.3.1 Oligodendrocyte specification and transcriptional control

During development, the spinal cord is stratified into genetically separate regions segmented along the dorsal-ventral neuraxis. This pattern is largely the result of secreted molecules such as Sonic Hedgehog (Shh), Wnts, BMPs and Fibroblast growth factors (FGF) that provide positional information to neuroepithelium progenitor cells (Briscoe and Novitch 2008; Ulloa and Marti 2010) and is reflected by a combinatorial code of transcription factors that initiate spinal cord oligodendrocyte development (Briscoe et al. 2000; Jessell 2000; Richardson et al. 2006; Rowitch and Kriegstein 2010; Wilson and Maden 2005). Initially, the secreted morphogen Shh acts in a concentration-dependent manner to induce cellular differentiation, originating in the notochord and later in the floorplate in the most ventral aspect of the neural tube (Marti et al. 1995a; Marti et al. 1995b; Roelink et al. 1995). Shh is hypothesized to induce cellular differentiation different cell types by activating or repressing a subset of homeodomain and basic-helix-loop-helix (bHLH) transcription factors in a concentration-dependent manner (Briscoe et al. 2000). Then, through cross-regulatory interactions by transcription factors (reciprocal inhibition), the dorsal-ventral boundaries become more defined. Finally, the expression of particular combinations of specific transcription factors defines the ultimate identity of the cell. At first, the majority of OPCs arise from a unique progenitor domain of the ventral spinal cord referred to as the pMN domain (Rowitch and Kriegstein 2010; Rowitch et al. 2002). Days later, a second wave of OPCs originate in the dorsal spinal cord (Cai et al. 2005; Fogarty et al. 2005; Vallstedt et al. 2005).

The specification of dorsally-derived OPCs is different from that of OPCs arising from the pMN domain in that the former do not require Shh signalling (Cai et al. 2005; Chandran et al. 2003; Fogarty et al. 2005). In the spinal cord, approximately 80% of oligodendrocytes originate from the ventral pMN domain, whereas the remainder of oligodendrocytes originate from dorsal domains (Tripathi et al. 2011). Although from separate origins, both ventral and dorsal populations have largely similar electrical properties as well as a similar range and number of internodes, indicating that despite their differences in origin they possess remarkably similar properties (Tripathi et al. 2011). In the forebrain, waves of oligodendrocyte specification occur along the ventral-to-dorsal axis, and if one population is removed, the others can compensate for the loss (Kessaris et al. 2006).

1.3.1.1 Specification by a combination of transcription factors

A combination of transcription factors is required for oligodendrocyte specification. The basic-helix-loop-helix (bHLH) transcription factors Olig1 and Olig2 are both expressed during oligodendrocyte development (Lu et al. 2000; Wu et al. 2006; Zhou et al. 2000). In the absence of Olig1 and 2 there is a complete failure in the brain and spinal cord to produce OPCs as well as oligodendrocytes (Zhou and Anderson 2002). In the brain, Olig1 can partially compensate for Olig2 as some oligodendrogenesis does occur, but in the spinal cord Olig2 deficiency results in the complete absence of OPCs (Lu et al. 2002; Park et al. 2002; Takebayashi et al. 2002). In the absence of Olig1, OPC production is normal, but there is impairment in oligodendrocyte maturation (Lu et al. 2002; Xin et al. 2005), suggesting that Olig2 plays a more central role in

oligodendrocyte specification. Olig1 is sufficient to specify oligodendrocytes as transfection of Olig1 in cortical progenitor cultures induces oligodendrogenesis (Lu et al. 2000)

Multiple transcription factors are required for oligodendrocyte specification. The bHLH transcription factors Olig1 and Olig2 require binding partners to specify oligodendrocytes. This provides an explanation as to why Olig2 electroporation into the spinal cord produces precocious OPCs only in specific regions surrounding the pMN domain (Zhou et al. 2001). Olig2 can form homodimers, or heterodimers with other bHLH transcription factors such as Olig1 or Mash1 (Li et al. 2011; Samanta and Kessler 2004). Olig2 can also form heterodimers with other non-bHLH transcription factors such as Sox10, nkx2.2 and zfp488 (Li et al. 2007; Sun et al. 2003; Wang et al. 2006; Wissmuller et al. 2006). The Olig2 partners that regulate oligodendrocyte specification are currently unclear. Co-electroporation of nkx2.2 and Olig2 promotes extensive early production of OPC, suggesting nkx2.2 and Olig2 might partner during oligodendrocyte specification (Zhou et al. 2001). However, nkx2.2 deficiency does not alter OPC production and is thus not essential for this process (Qi et al. 2001). Similarly, there is little or no decrease in OPC production in the absence of Olig1 (Lu et al. 2002) and mash1-deficient spinal cords have reduced OPC production that resolves with time (Sugimori et al. 2008). It is possible that there is redundancy in Olig2 partners that mediate oligodendrocyte specification with these or other undefined binding partners.

1.3.2 Oligodendrocyte production

Once specification has occurred, widespread OPC proliferation and migration is needed to populate the brain and spinal cord. Migration is thought to be the result of multiple soluble and membrane-bound chemotactic cues such as semaphorins, ephrins and netrins that attract or repel OPCs (Cohen 2005; Jarjour and Kennedy 2004). However, live imaging of OPCs during migration *in vivo* indicates that it is likely to be more complex (Kirby et al. 2006). Migrating OPCs guide themselves with multiple large, highly-branched filopodium-like processes. The net movement of OPCs is away from their site of origin yet they frequently and unexpectedly change directions and migrate in a more stochastic manner *in vivo*, stopping apparently upon contact with an axon (Kirby et al. 2006). Once an OPC has stopped, it dynamically sends and retracts processes constantly. Contact with adjacent OPCs results in a retraction of its process, indicating that OPCs occupy mutually exclusive domains. Just as they exhibit an elevated capacity to migrate and proliferate in order to compensate for the depleted pool of OPCs during development (Kessaris et al. 2006), spared OPCs demonstrate a remarkable ability to proliferate and migrate to replace cells destroyed by laser microsurgery (Kirby et al. 2006). OPCs must therefore be responsive not only to environmental chemotactic cues, but may also be responsive to each other. The ability of OPCs to proliferate in response to damage is conserved in adults (Horky et al. 2006; Lytle et al. 2009; McTigue et al. 2001; Zai and Wrathall 2005)

1.3.2.1 Combination of mitogens control OPC proliferation

Astrocytes increase the proliferation of OPCs in culture due to the secretion of PDGF (Noble et al. 1988; Raff et al. 1988; Richardson et al. 1988). In the absence of PDGF-A during development, there is a robust decrease in the number of OPCs that does not correct itself, resulting in hypomyelination (Fruttiger et al. 1999). There is a similar robust decrease in the number of proliferating OPCs when retinal ganglion cell (RGC) activity is blocked during optic nerve development, which can be rescued by the presence of exogenous PDGF (Barres and Raff 1993). This suggests that during optic nerve development, neuronal activity stimulates proliferation and/or survival of OPCs. In culture, OPCs proliferate in response to several other mitogens. For example, basic FGF (bFGF) (Barres et al. 1993; Bogler et al. 1990; Eccleston and Silberberg 1985), neurotrophin-3 (NT-3) (Barres and Raff 1994; Barres et al. 1994; Barres et al. 1993), and insulin growth factor-1 (IGF1) (Barres et al. 1993; McMorris and Dubois-Dalcq 1988) can all promote proliferation, although NT-3 and IGF1 can only induce proliferation with each other or with another OPC mitogen (Barres and Raff 1994). The presence of bFGF is sufficient to induce proliferation and can also increase PDGFR-α expression. The combination of PDGF and bFGF inhibits spontaneous differentiation, decreases the cell cycle time, and reduces the time required for a population of OPCs to double (Bogler et al. 1990; McKinnon et al. 1990; Tang et al. 2000). NT-3 similarly increases the percentage of proliferating cells in culture when in combination with PDGF or insulin (Barres and Raff 1994; Barres et al. 1994; Barres et al. 1993). In development, multiple mitogens are likely required to induce sufficient OPC proliferation, which is supported by the finding that blocking either NT-3 or PDGF-A *in vivo*

robustly reduces OPC proliferation, though the loss of either does not completely block OPC proliferation (Barres and Raff 1993; Barres et al. 1994; Fruttiger et al. 1999).

1.3.3 Differentiation of oligodendrocytes

Following OPC specification and expansion, several other stages of oligodendrocyte development are required to produce myelin: differentiation, axon ensheathment, axon wrapping, and wrap compaction to produce mature myelin. Oligodendrocyte differentiation occurs spontaneously in culture in the presence of only minimal media requirements, and this event occurs in the absence of axons (Barres and Raff 1999). Oligodendrocyte differentiation is thus hypothesized to be a default state requiring the presence of transcriptional and environmental factors that act to maintain OPCs (Li et al. 2009). According to this model, de-repression of negative regulators during oligodendrocyte development allows oligodendrocyte differentiation. During development, numerous differentiation and myelination inhibitors exist and their downregulation often occurs prior to the onset of myelination. These repressive elements impair the progression of differentiation and maturation, often through the regulation of transcription factors that inhibit oligodendrocyte development. In the absence of transcriptional inhibition, a network of transcription factors is recruited to drive oligodendrocyte-specific gene expression, likely acting in a combinatorial manner (Gokhan et al. 2005). Olig1 and Olig2 bHLH transcription factors are necessary for oligodendrocyte differentiation and maturation. Olig1 knockout mice produce normal OPC numbers, but have impaired oligodendrocyte differentiation (Lu et al. 2002), and Olig1 can upregulate MBP and PLP (Xin et al. 2005). The role of Olig2 during OPC differentiation is often overlooked because the loss of Olig2 almost entirely blocks

OPC specification. However, Olig2 removal from dorsal telencephalon progenitors does not noticeably affect OPC production in the cortex, but markedly impairs OPC differentiation.

1.3.3.1 Transcription factor work in combination to drive myelin gene expression

Numerous other transcription factors that regulate oligodendrocyte development, including the high mobility group transcription factor Sox10 (Stolt et al. 2002). In the absence of Sox10 there is impaired oligodendrocyte differentiation (Stolt et al. 2002). Sox10 is thought to directly control MBP and PLP expression (Stolt et al. 2006). In mice, Sox10 can interact with Olig1 and Olig2, while in zebrafish, Sox10 and Olig1 act synergistically to increase MBP expression *in vivo* (Li et al. 2007; Wissmuller et al. 2006). Sox5 and Sox6 are also located in oligodendroglial cells and they antagonize Sox10 function (Stolt et al. 2006). In addition to bHLH and HMG transcription factor families, zinc-finger transcription factors also regulate oligodendrocyte development. Ying Yang 1 (YY1) is a zinc-finger transcription factor that increases PLP *in vitro* and *in vivo* (Berndt et al. 2001). In the absence of YY1, there is severe inhibition of oligodendrocyte maturation and hypomyelination (He et al. 2007). Myelin-gene Regulatory Factor (MRF), was recently identified as a major regulator of oligodendrocyte maturation (Emery et al. 2009). MRF contains a DNA-binding domain, and it is hypothesized to work as a transcription factor. Identified due to its presence in oligodendrocytes (but not astrocytes and neurons) MRF removal of during development almost completely blocks myelination (Emery et al. 2009). MRF overexpression *in vitro* is sufficient to induce myelin gene expression, but it is still not clear if this is through direct binding to DNA promoter sequences or through indirect regulation.

1.3.3.2 Inhibitors of oligodendrocyte development

Many repressive elements, such as extracellular cues and transcription factors act to inhibit oligodendrocyte differentiation and myelination (Barres and Raff 1999; Baumann and Pham-Dinh 2001; Emery 2010). Extracellular cues might be a signals to OPCs that an axon is to remain unmyelinated or that it is not yet ready to be myelinated. Therefore, oligodendrocyte differentiation requires the removal of extracellular inhibitors. Many extracellular inhibitors act through signalling pathways to regulate transcription factors that inhibit oligodendrocyte differentiation. These inhibitory transcription factors must also be downregulated within OPCs to allow them to differentiate. For example, axonal notch ligands inhibit oligodendrocyte differentiation during development, likely through the Hes transcription factors. The notch receptor family is highly conserved, regulating many aspects of development (Lardelli et al. 1995; Lewis 1998). Notch-deficient animals (Givogri et al. 2002) and conditional Notch removal from OPCs (Genoud et al. 2002) both result in the precocious emergence of immature oligodendrocytes. Notch binding to its ligands Delta, Serrate/Jagged, and Lag-2 result in the proteolytic cleavage and release of its intracellular domain (NICD) and subsequent activation expression of the Hes transcription factors (Martinez Arias et al. 2002). Prior to myelination of the optic nerve, Jagged is expressed in RGCs whose axons project through the optic nerve. Jagged expression begins to decrease prior to the onset of myelination and further continues to decrease as myelination progresses. OPCs express Notch1 and their differentiation is robustly inhibited when cultured in the presence of the notch ligands Delta1 or Jagged1 (Wang et al. 1998). Consistent with the role of Hes transcription factors mediating Notch-dependent

inhibition of OPC differentiation, Hes 5 knockout mice exhibit an upregulation of myelin gene expression and an increase in myelination early in development (Liu et al. 2006a).

Another inhibitor oligodendrocyte development is Wnt3A, which inhibits oligodendroglial maturation (Feigenson et al. 2009; Shimizu et al. 2005). The canonical Wnt signalling pathway acts to stabilize β-catenin from destruction (Clevers 2006). Stabilized β-catenin then interacts with Tcf/LEF transcription factors to regulate gene expression. Expression of a constitutively active β-catenin robustly inhibits oligodendrocyte differentiation (Fancy et al. 2009; Ye et al. 2009). The expression of the TCF/LEF family member Tcf4 (also known as TCF7L2) overlaps highly with oligodendrocyte markers in development (Fancy et al. 2009; Ye et al. 2009), suggesting it is downstream of Wnt signalling in oligodendrocytes. Consistent with the downstream location of Tcf4 in oligodendrocyte Wnt signalling, both Wnt3a and Tcf4 inhibit MBP expression (He et al. 2007; Ye et al. 2009). The Wnt pathway is also negatively regulated by Axin2, which targets β-catenin to be degraded (Jho et al. 2002; Lustig et al. 2002). The deficiency of Axin2 increases β-catenin and impairs oligodendrocyte maturation (Fancy et al. 2011b). Thus, like Notch signalling, Wnt signalling inhibits oligodendrocyte development.

One transcription factor family that negatively regulates oligodendrocyte development is the inhibitors of DNA binding (ID) (Chen et al. 2012). ID transcription factors are helix-loop-helix (HLH) transcription factors that lack DNA binding sites and preferentially dimerize with other bHLH factors to inhibit their function (Benezra et al. 1990; Sun et al. 1991; Yokota 2001). In oligodendrocytes, there is ID1, ID2, ID3, and ID4 expression, but only ID2 and ID4 have a major role in oligodendrocyte development (Kondo and Raff 2000; Samanta and Kessler 2004;

Wang et al. 2001). ID4—and to a lesser extent ID2—but not ID1 or ID3, bind to the bHLH transcription factors Olig1 and Olig2 (Samanta and Kessler 2004). ID dimerization with Olig1 and Olig2 inhibits Olig function and is one proposed mechanism of ID function in oligodendroglia (Samanta and Kessler 2004). Overexpression of either ID2 (Wang et al. 2001) or ID4 (Kondo and Raff 2000) potently blocks oligodendrocyte development. OPCs derived from ID4 and ID2 knockout mice are more prone to spontaneous differentiation *in vitro* (Marin-Husstege et al. 2006; Wang et al. 2001) and ID4 deficient mice show premature myelination *in vivo* (Marin-Husstege et al. 2006). ID4-deficient oligodendrocytes display increased morphological complexity and increased MBP expression, suggesting that ID4 also inhibits maturation (Marin-Husstege et al. 2006). Overexpressing ID4 increases the rate of proliferation of OPCs (Kondo and Raff 2000) and ID2 deficiency decreases OPC proliferation rate (Wang et al. 2001). Hence, ID2 and ID4 block oligodendrocyte differentiation and promote proliferation, in part by antagonizing Olig function.

Several extracellular cues increase ID2 and ID4 expression. For instance, BMP2 and BMP4 increase the production of ID family members in neural precursor cells, which promotes increased astrogenesis and decreased oligodendrogenesis (Samanta and Kessler 2004). Overexpression of either ID2 or ID4 in neural precursor cell cultures is sufficient to drive astrogenesis at the expense of oligodendrogenesis (Samanta and Kessler 2004). Importantly, the BMP-dependent increase in astrogenesis is prevented by ID4 knockdown indicating that ID4 is necessary and sufficient to block oligodendrogenesis; this is likely due to the inhibition of Olig function (Samanta and Kessler 2004). Wnt3a also increases ID2 and ID4 (Ye et al. 2009), with

ID2 suggested as a direct target for β-catenin - TCF transactivation (Memezawa et al. 2007; Rockman et al. 2001). Enhanced ID2 is also observed with increased levels of G protein-coupled receptor 17 (Gpr17) (Chen et al. 2009). Gpr17 is expressed in oligodendrocyte lineage cells, peaks at the onset of myelination and drops precipitously afterwards. If Gpr17 expression is sustained in oligodendrocyte cells, there is profound hypomyelination, indicating that downregulation of Gpr17 is necessary for myelination initiation. Sustained expression of Gpr17 additionally results in nuclear translocation of ID2 and ID4 and is associated with increased Olig1 binding to ID2 and ID4 (Chen et al. 2009).

1.3.3.3 Molecular mechanisms of OPC de-repression

Oligodendrocyte differentiation is repressed by intracellular signalling pathways that ultimately increase the expression of inhibitory transcription factors. Differentiation is thought to proceed when these inhibitory transcription factors are de-repressed. Currently, epigenetic and microRNA-mediated regulation are two known molecular mechanisms of oligodendrocyte de-repression. MicroRNAs are small non-coding RNAs that serve as post-transcriptional regulators. Together with the RISC complex, microRNAs typically repress the expression of critical genes during development (Bushati and Cohen 2007). The majority of microRNAs are produced by the Dicer enzyme, making this protein a useful target in studying the roles of microRNAs during development (Yang and Lai 2011). Dicer removal in OPCs stunts differentiation and enhances OPC proliferation, resulting in delayed and diminished myelination (Dugas et al. 2010; Zhao et al. 2010). MicroRNAs such as miR219, miR338, and miR138 are induced during oligodendrocyte differentiation due to mitogen withdrawal, and the expression of molecules that

mimic miR219, miR338 or miR138 function is sufficient to promote oligodendrocyte differentiation. The addition of miR219 mimics also decreases differentiation inhibitors such as PDGFR-α, sox6, and hes5, which is consistent with the concept that microRNA are acting to de-repress oligodendrocyte development (Dugas et al. 2010; Zhao et al. 2010).

Epigenetic regulation also participates in the oligodendrocyte de-repression. Often, developmental changes in gene expression require chromatin remodelling, which begins with the chromatin compaction. Chromatin compaction impairs gene expression and is stimulated by histone deacetylation (Li et al. 2009; Pazin and Kadonaga 1997). In OPCs, histones become increasingly deacetylated during differentiation; if deacetylation is prevented *in vitro,* oligodendrocyte differentiation does not occur (Marin-Husstege et al. 2002). If valproic acid, a histone deacetylase (HDAC) inhibitor, is delivered during myelin development, there is delayed oligodendrocyte differentiation and hypomyelination (Shen et al. 2005). There are a number of HDACs isoforms, but HDAC1, 2, 3, and 8 are present in oligodendrocytes (Shen et al. 2005). If Hdac1 and Hdac2 are both specifically removed from neuroepithelium precursors, there is nearly complete absence of OPCs and mature oligodendrocytes, indicating that Hdac1 and Hdac2 are necessary for OPC specification (and also potentially differentiation) (Ye et al. 2009). OPC differentiation failure is confirmed by the inability of cultured OPCs that are deficient in Hdac1 and Hdac2 to express an immature oligodendrocyte marker (Ye et al. 2009). HDACs, are thus necessary for oligodendrocyte differentiation through their deacetylation of chromatin, which results in chromatin compaction. In this regard, HDACs are thought to form repressive complexes that block many transcriptional inhibitors of oligodendrocyte development, de-

repressing oligodendrocyte differentiation (He et al. 2007; Li et al. 2009; Shen et al. 2008; Ye et al. 2009). HDAC recruitment to promoter regions can be controlled by transcription factors. For example, HDAC1 binds to the transcription factor YY1 at promoter regions of the transcriptional inhibitors tcf-4 and ID4 (He et al. 2007). Interestingly, HDAC suppresses Wnt signalling, thus linking de-repression to inhibitory signalling cessation (Ye et al. 2009). This is thought to occur by HDAC1 and HDAC2 competing with β-catenin to interact with the Wnt transactivator tcf-4 (Ye et al. 2009).

1.3.4 CNS myelination

OPC differentiation has been an area of intense investigation for decades and much is understood about the transition of an OPC into an immature or mature oligodendrocyte. While this is clearly an important transition for the production of myelin, it is becoming increasingly clear that oligodendrocyte differentiation is not sufficient to produce myelin. This disconnect between myelination and oligodendrocyte differentiation is best demonstrated by early attempts to produce myelinating co-cultures with RGCs (Meyer-Franke et al. 1999). If oligodendrocyte differentiation is sufficient to induce myelination, cultured OPCs in contact with RGCs would automatically produce myelin upon mitogen withdrawal, so long as both neurons and oligodendrocytes are provided with sufficient factors to promote survival. However, this is not the case. Despite prolonged survival, OPCs spontaneously differentiate when cultured with RGC, but do not myelinate (Meyer-Franke et al. 1999). When astrocytes are present in these co-cultures, oligodendrocytes align with and extend MBP+ processes that contact small axon

bundles, but do not ensheathe and wrap axons to produce myelin (Meyer-Franke et al. 1999). This demonstrates that additional elements are required to induce myelination.

Cultured OPCs will myelinate RGC axons that have a substantially increased density due to reaggregation of their cell bodies (Watkins et al. 2008). In OPC cultures plated with reaggregated RGCs, myelination occurs quite early, only 5-6 days *in vitro*. From these culture conditions, Watkins and colleagues (2008) demonstrated that although OPCs are able to myelinate readily, immature (GC+) oligodendrocytes and mature oligodendrocytes have a much-reduced capacity to myelinate RGCs. Therefore, as OPCs differentiate, they lose their intrinsic capacity to initiate myelination. The earliest stage of myelination, axonal ensheathment, also occurs early in oligodendrocyte development prior to maximal MBP expression (Watkins et al. 2008). Surprisingly, the ensheathment of multiple axons by a single oligodendrocyte occurs almost concurrently. Once ensheathment occurs, new segments are rarely added. The low capacity of oligodendrocytes to ensheathe new segments might be important in scenarios where individual myelin segments are lost, such as during remyelination and myelin replacement. Since myelination requires a dense axonal bed and ensheathment of multiple axons occurs synchronously, it is possible that an oligodendrocyte must recognize a certain number of axons prior to the onset of ensheathment. The initiation of ensheathment occurs early in oligodendrocyte development and the capacity to myelinate is diminished as oligodendrocytes develop. Further live imaging studies *in vivo* are likely required to determine if this is an important phenomena or an *in vitro* artifact.

In addition to elucidating the spatial requirements required for myelination, OPC-RGC cultures also demonstrate that initial axonal ensheathment and subsequent axonal wrapping are separate stages, controlled by different mechanisms. Axonal wrapping, but not ensheathment, is promoted by contact with astrocytes (Watkins et al. 2008). Likewise, oligodendrocyte differentiation and axonal ensheathment was increased by treatment with a γ –secretase, but γ –secretase inhibitor treatment does not increase the amount of ensheathed and wrapped axons (Watkins et al. 2008). The protease γ–secretase is required for notch signalling (De Strooper et al. 1999; Dovey et al. 2001). However, increases in axonal ensheathment induced by γ–secretase inhibition is independent of Notch1, indicating that other γ–secretase targets independent of the Notch pathway inhibit ensheathment (Watkins et al. 2008). γ–secretase has at least three dozen substrates (Selkoe and Wolfe 2007). The identity, regulation, and substrate of γ–secretase and its substrate are of great importance in understanding how ensheathment is regulated.

OPC density might also contribute to myelination. During spinal cord development, there is continued OPC proliferation until shortly after birth, when the number of OPCs is reduced at a stage that coincides with the appearance of differentiated oligodendrocytes (Rosenberg et al. 2008). This rapid change in oligodendrocyte differentiation is potentially due to a synchronous transition of OPCs into oligodendrocytes. Lending support to this model, the plating of more OPCs onto a bed of dorsal root ganglion (DRG) neurons results in precocious myelination, which occurs at a critical density of OPCs (Rosenberg et al. 2008). Surprisingly, this onset of myelination was unaffected by plating OPCs on a bed of dead axons that were fixed in paraformaldehyde, demonstrating that myelination can occur independently of axonal signals

(Rosenberg et al. 2008). Ablating OPCs using complement-mediated cell lysis results in delayed myelination, providing additional support for the notion that OPC density may control myelination *in vitro* (Yang et al. 2011). When OPCs are ablated, there is a spare 'pool' remaining, which presumably proliferates to a critical density prior to the initiation of myelination. Taken together, the combination of a high OPC density with a dense axonal bed (live or dead) is sufficient to initiate myelination. It is still unclear, however, which aspect of OPC density is required to initiate myelination. Requiring a certain OPC density to myelinate may have implications for the process of remyelination, as sufficient recruitment of OPCs is likely required before the onset of remyelination can occur.

1.3.4.1 Neuronal activity controls myelination?

In addition to OPC density, there are likely additional mechanisms that control and promote myelination. For example, myelin is remodeled throughout life (Flynn et al. 2003), which is difficult to reconcile with OPC alone expansion controlling myelination. Based on correlative evidence, it is hypothesized that neuronal activity stimulates myelin development in humans (Casey et al. 2000). Myelination improves aspects of information processing as it increases the speed of axonal conduction. Because myelin is potentially regulated by axonal activity though repetitive activation, it is similar to neuroplasticity and might be a novel form of activity-dependent plasticity (Fields 2008). Blocking the activity of optic nerve neurons, which decreases myelination, whereas increases in axonal firing rates boosts myelination (Barres and Raff 1993; Barres and Raff 1999; Demerens et al. 1996). It is likely that several mechanisms exist to couple axonal activity and myelination. Neuronal activity has been shown to upregulate

adhesive molecules (Itoh et al. 1995; Stevens et al. 1998) while also potentially downregulating axonal inhibitors of oligodendrocyte differentiation such as notch ligands or PSA-NCAM. Alternatively, oligodendrocytes can respond to specific molecules that are secreted by axons. Adenosine, for instance, can be released non-synaptically along axons when they are electrically active (Maire et al. 1984). Adenosine promotes OPCs to stop proliferating, even in the presence of mitogens, and promotes increased myelination in a OPC-DRG co-culture (Stevens et al. 2002). Stimulating DRG cell bodies in culture also increases oligodendrocyte myelination (Ishibashi et al. 2006) due to activity-dependent ATP release by axons (also non-synaptically) (Stevens and Fields 2000). ATP acts on astrocytes to promote the release of LIF which, in turn, promotes myelination (Ishibashi et al. 2006). There are lower levels of MBP in LIF-deficient mice (Bugga et al. 1998; Ishibashi et al. 2009), but myelination occurs in the absence of LIF. ATP release also after injury (Huang et al. 2012; Wang et al. 2004) and remyelination is impaired in LIF-deficient mice. Therefore, axonal release of LIF might be more relevant to remyelination (Marriott et al. 2008).

Activity via direct synapses formed between OPCs and axons is another potential mechanism of myelination. These synapses are found in the hippocampus (Bergles et al. 2000) and other grey and white matter regions (Lin and Bergles 2002; Ziskin et al. 2007). At OPC-axonal synapses, glutamate release stimulates miniature excitatory post-synaptic potentials (mEPSP). As OPCs differentiate, these mEPSP are not present in oligodendroglia, suggesting that these synaptic structures are lost during oligodendrocyte development (De Biase et al. 2010; Etxeberria et al. 2010). Given when these synaptic structures appear, many have hypothesized

that they control myelination. This, however is contradicted by the finding that glutamate exposure in OPC cultures inhibits their differentiation (Gallo et al. 1996). However, OPC cultures do not model the synaptic release of glutamate—which would invoke focal activation on OPC cell membranes—compared to diffuse glutamate receptor activation when OPC monocultures are subjected to glutamate. Wake and colleagues inhibited synaptic release in an OPC-DRG co-culture and saw diminished myelination without impaired oligodendrocyte differentiation (2011). Blocking synaptic release also prevented local Ca^{+2} influx within OPC processes, focal activation of Fyn kinase along OPC processes, and local translation of MBP (Wake et al. 2011). Local translation of MBP was blocked by glutamate antagonists (both NMDA receptor and AMPA receptor antagonists), suggesting that synaptic release of glutamate promotes myelination through focal activation of cellular components and increases in local translation of myelin proteins (Wake et al. 2011). Still, genetic removal of the NMDA receptor from OPCs *in vivo* does not impair their ability to produce synapses with glutamatergic axons, or impair the proliferation, differentiation or myelination of oligodendrocyte lineage cells (De Biase et al. 2011). Thus, more work is needed to understand the role of synaptic glutamate release during myelination *in vivo*. OPC synapse with denuded axons after demyelination and might therefore be important for remyelination; like development, these synapses are lost as OPCs differentiate (Etxeberria et al. 2010)

1.3.5 Myelination following myelin development

Even though a significant proportion of OPCs differentiate into oligodendrocytes postnatally, there remains a pool of adult OPCs (Kang et al. 2010; Psachoulia et al. 2009; Rivers

et al. 2008; Rosenberg et al. 2008) that proliferates well into adulthood (Psachoulia et al. 2009; Simon et al. 2011). The dynamics of adult OPCs can be investigated via genetic fate mapping, as it enables the labeling of OPCs and their progeny at different times after birth. OPC genetic fate mapping relies on mice that contain a tamoxifen inducible cre-recombinase in their genome, which is expressed specifically in OPCs. When mice with OPC-specific and tamoxifen inducible cre-recombinase expression are bred with a mice line carrying a cre-recombinase sensitive reporter, tamoxifen administration labels OPCs and their progeny. Whether a population of cells is self-maintained or is replenished by a precursor cell can be determined by measuring the proportion of labelled cells (also known as the recombination frequency) at different times after labelling. If the proportion of labelled cells is constant over long periods in a population of proliferative cells, then the labelled cells are self-maintaining (Hsieh et al. 2007; Meletis et al. 2008). OPC density does not change between postnatal day 45 and 135 despite prominent proliferation in this population of cells (Rivers et al. 2008). The proportion of labelled OPCs at postnatal day 45 (Rivers et al. 2008) or at postnatal day 240 (Psachoulia et al. 2009) is also constant 100 days later, suggesting that OPCs can self-maintain and are not produced by another neural precursor cell. The ability of OPCs to differentiate is not lost, since even OPCs labelled at post natal day 240 continue to produce myelinating oligodendrocytes (Psachoulia et al. 2009). What does change during aging is the pace of the cell cycle, which slows in OPCs during postnatal life (Psachoulia et al. 2009; Simon et al. 2011). These adult OPCs are likely important in myelin replacement/turnover (Lasiene et al. 2009), but might also produce new myelin, as only 30% of axons are myelinated in the corpus callosum of 8-month-old mice (Sturrock 1980).

In states of injury and disease, adult OPCs are the major source of remyelination during injury and disease (see below).

1.4 Remyelination: does it recapitulate development?

Substantial evidence has accumulated suggesting that oligodendrocytes support the function and well-being of axons (see above). As a corollary of this support of axons, rapid remyelination is hypothesized to spare denuded axons from degeneration. Disability in white matter diseases may be directly related to axonal loss (Bulman et al. 1991; Trapp and Nave 2008; Tremlett and Devonshire 2006). For example, axonal loss has been hypothesized to account for the transition from relapsing remitting multiple sclerosis (RRMS) to secondary progressive multiple sclerosis (SPMS), and may also account for the substantial functional decline associated with the latter (De Stefano et al. 2001; Pascual et al. 2007; Petzold et al. 2005; Teunissen et al. 2009; Trapp and Nave 2008; Trapp et al. 1999; Trapp and Stys 2009). Accordingly, treatments focused on axonal protection, including strategies to improve remyelination, could provide an effective means to spare axons in white matter-associated conditions like SCI and MS. I will discuss these topics briefly starting with the immediate consequences of demyelination, the recruitment of adult OPCs, and the differentiation and myelination that ensues.

1.4.1 Consequences of demyelination

To elucidate the importance of remyelination, it is important to consider the immediate consequences of demyelination. Under normal conditions, axonal Na^+ channels are plentiful in

the nodal region located between myelin sheaths and exceedingly rare beneath the sheaths themselves (Ritchie and Rogart 1977; Waxman 1977). Following demyelination, axonal transmission can be blocked due to the paucity of sodium channels on the axolemma of the demyelinated internode and the high number of K+ channels in the adjacent juxtaparanode. Remyelination reinstates the nodal distribution of Na^+ channels and restores axonal transmission, as evidenced by strong correlations between remyelination and electrophysiological functional assessments of axonal conduction in remyelinating injury models (Smith et al. 1979). However, it is possible to achieve axonal conduction without remyelination, even if denuded axons persist through a long region of demyelination (Felts et al. 1997). This might explain the recovery of eye function in MS patients which occurs despite persistent demyelination to all axons within the optic nerve, and is likely a result of the production of additional Na^+ channels (England et al. 1991; Foster et al. 1980; Novakovic et al. 1998) that spread along the length of the demyelinated internodes (Waxman 2006). Remyelination is therefore not the only possible mechanism to restore transmission of an axonal signal following demyelination.

Demyelination leaves axons in a vulnerable state with a greater susceptibility to damage which, over time, may result in axonal degeneration of chronically demyelinated axons. Besides its effects on axonal transmission, the aforementioned increased concentration of voltage–gated Na^+ channels that spread along the demyelinated axon also leads to a dramatic increase in Na^+ influx into the axon during action potential propagation (Waxman 2006). Elimination of the excess Na^+ can come at a steep metabolic expense, as the Na^+/ K+ ATPase (i.e., sodium/potassium pump) is required to maintain the Na^+ electrochemical gradient at the expense

of the cellular metabolite, ATP. Therefore, spreading of voltage–gated Na^+ channels that occurs following demyelination is energetically burdensome to axons and can disrupt the axon's internal energy balance, shifting the ATP/ADP ratio. When ATP levels fall beyond a certain threshold, there is a reversal of the Na^+/ Ca^{2+} exchanger (Stys et al. 1992) and the axon becomes overloaded with Ca^{2+} eliciting a cascade of events such as mitochondrial dysfunction, heightened protease activity, free radical formation, and ultimately, axonal degeneration (Stys 1998; Young 1992). In addition to ATP depletion, other methods of axoplasmic Ca^{2+} loading may contribute to axonal degeneration. For example, axonal Ca^{2+} entry via AMPA receptors can act to stimulate Ca^{2+} release from internal stores through rhyanodine receptors (Ouardouz et al. 2003). IP_3 generated by metabotropic glutamate receptors and kainate receptors can also stimulate Ca^{2+} release via IP_3 receptors on internal stores (Ouardouz et al. 2009a; Ouardouz et al. 2009b; Ouardouz et al. 2003), and Ca^{2+} can enter directly through voltage–gated Ca^{2+} channels (Fern et al. 1995). Ca^{2+} influx is not normally toxic due to the energy-dependent Ca^{2+} buffering system within axons that acts to remove excess Ca^{2+}. However, when ATP is depleted because of changes in Na^+ channel distribution following demyelination, this normal Ca^{2+} buffering fails, and the level of intracellular Ca^{2+} elevates to toxic levels. Simply stated, chronic demyelination leaves axons in an energetically vulnerable state, whereby demyelinated axons experience "virtual hypoxia", increasing the likelihood of axonal loss over time (Stys 1998; Trapp and Stys 2009). This mechanism may explain why neural atrophy continues into the chronic phase of MS, despite the presence of only a few new inflammatory demyelinating lesions developing this phase of the disease (Fisher et al. 2008; Fisniku et al. 2008). Should this be the case,

remyelination may present as an effective therapeutic strategy to protect axons through the restoration of their energetic balance.

1.4.2 Recruitment of adult OPCs

Remyelination is thought to be a robust regenerative response because it occurs spontaneously after demyelination in animal models. The stages of remyelination are very similar to those observed in development. After demyelination, OPCs are recruited to regions of damage before they differentiate and myelinate denuded axons (Franklin 2002; Franklin and ffrench-Constant 2008). Recruitment involves the proliferation of spared OPCs within or adjacent to the area of demyelination. (Levine and Reynolds 1999; Sim et al. 2002; Watanabe et al. 2002). Recruitment might also involve OPC migration. Currently, adult OPCs are thought to be solely responsible for remyelination, but this does not negate the participation of other CNS progenitors that potentially also produce OPCs. The finding that genetically-labelled adult OPCs differentiate into remyelinating oligodendrocytes following focal demyelination is compelling evidence that OPCs are responsible for remyelination (Zawadzka et al. 2010). Similar to the developmental loss of myelinating capacity that occurs as OPCs differentiate (Watkins et al. 2008), differentiated oligodendrocytes have a limited capacity to remyelinate over the course of differentiation. Mature oligodendrocyte transplantation promotes sparse remyelination (Targett et al. 1996). Using an *in vivo* model of demyelination that enabled OPC removal and concomitant sparing of oligodendrocyte cell bodies, Keirstead and Blakmore demonstrated that that remyelination is prevented in the absence of OPCs, despite the presence of mature oligodendrocytes (1997). The mature oligodendrocytes still contacted axons, but were unable to

produce new myelin sheaths (Keirstead and Blakemore 1997). Therefore, OPCs must be recruited after demyelination; failure to recruit adult OPCs is associated with an inability to remyelinate (Blakemore and Patterson 1978; Hinks et al. 2001; Irvine and Blakemore 2008).

1.4.2.1 Remyelination in the CNS by Schwann cells

Despite the similarities, there are marked differences between remyelination and development. For instance, during development, Schwann cells are confined to the PNS. However, following central nervous system injury (Biernaskie et al. 2007; Plemel et al. 2008), demyelination (Hampton et al. 2012; Talbott et al. 2005; Zawadzka et al. 2010), or disease such as MS (Itoyama et al. 1983) Schwann cells can remyelinate the CNS. CNS remyelination by Schwann cells was generally assumed to be the result of migratory Schwann cells from the periphery. However, recent evidence suggests that the majority of CNS Schwann cells arise from resident CNS OPCs (Zawadzka et al. 2010). How an OPC becomes a Schwann cell is not understood, but extracellular cues following injury may promote this transition. Astrocytes likely suppress the OPC-to-Schwann-cell transition, as transplanted OPCs can become Schwann cells in astrocyte-free regions following injury (Talbott et al. 2006; Talbott et al. 2005). The OPC-to-Schwann-cell transition might require BMP because the elevated expressions of the BMP inhibitor noggin, inhibits this transition (Talbott et al. 2006). Much remains to be determined regarding how OPCs become Schwann cells, as well as the functional relevance of this transition.

1.4.2.2 Mitogens drive OPC proliferation during recruitment

As in development, mitogens drive OPC proliferation after demyelination (Murtie et al. 2005; Wolswijk and Noble 1992; Woodruff et al. 2004). The combination of mitogens may be different after demyelination than during development because the immune system secretes many additional inflammatory proteins during remyelination that are known to promote OPC proliferation *in vitro*. PDGF levels and other mitogens` expression levels increase after demyelination (Hinks and Franklin 1999; Hinks and Franklin 2000), and PDGFR-α deficiency impairs OPC proliferation and repopulation following demyelination (Murtie et al. 2005). PDGF regulates the number of precursors during recruitment (Woodruff et al. 2004). Surprisingly, although recruitment is a necessary step for remyelination, increased OPC density due to increased PDGF does not necessarily accelerate remyelination, suggesting that recruitment may not be rate-limiting in a model of efficient, spontaneous remyelination (Woodruff et al. 2004). This does not imply that OPC recruitment is not rate-limiting in other scenarios. For instance, OPC recruitment might be rate-limiting if there are insufficient mitogens or migration inhibitors. In addition, the depletion or oversaturation of the OPC pool due to prolonged or repeated damage might, in theory, impair recruitment and remyelination. OPC depletion does not occur in animal models, as repeated episodes of demyelination does not impair remyelination, nor does repeated episodes of remyelination alter the ability of OPCs to repopulate the lesion site (Penderis et al. 2003). This is consistent with the high capability of adult OPCs to self-renew, even a year after birth in mice (Kang et al. 2010; Psachoulia et al. 2009; Rivers et al. 2008). OPC proliferation by

mitogens is therefore necessary for remyelination, but is not rate-limiting during spontaneous remyelination.

1.4.2.3 Several sources of OPCs

During recruitment, the origin of OPCs varies due to the injury state. In the absence of injury, adult OPCs self-maintain and are not produced by other neural precursors/progenitors (Kang et al. 2010; Psachoulia et al. 2009; Rivers et al. 2008). In the spinal cord, the ependymal zone contains neural progenitors that have the potential *in vitro* to make oligodendrocytes, astrocytes and neurons (Meletis et al. 2008). In the absence of injury, ependymal cells can self-renew *in vivo* but are restricted to the ependymal zone (Barnabe-Heider et al. 2010; Meletis et al. 2008). Following a stab injury to the spinal cord, ependymal cells exhibit marked increases in proliferation and the ability to exit the ependymal zone. However, the vast majority of oligodendrocytes are still produced by OPCs after this injury (Barnabe-Heider et al. 2010; Meletis et al. 2008). Whether ependymal cells increase their capacity to produce OPCs following a specific demyelinating injury is still unclear. In the brain, the subventricular zone also contains multipotential neural progenitor cells (Gritti et al. 1996; Reynolds and Weiss 1992; Vescovi et al. 1993), and in an undamaged state, cells within the subventricular zone rarely produce oligodendrocytes *in vivo* (Menn et al. 2006). After demyelination this propensity to produce OPCs is enhanced (Menn et al. 2006; Nait-Oumesmar et al. 1999; Picard-Riera et al. 2002). Remyelination can thus attract additional sources of OPCs.

1.4.3 OPC differentiation and myelination during the remyelination process

In development and during remyelination, OPCs are required to proliferate and differentiate as well as make contact with and ensheathe axons. During remyelination, OPC recruitment is not rate-limiting (Woodruff et al. 2004), suggesting that OPC differentiation or axonal ensheathment is the rate-limiting step. The fundamental principles of developmental myelination are mostly consistent with what occurs during remyelination, though subtle differences exist. For instance, myelin thickness is normally proportional to the axonal diameter. After demyelination, for unknown reasons, myelin is abnormally thin (Blakemore 1974). Similarly, remyelination is also associated with abnormally short internodal lengths (Gledhill et al. 1973b; Gledhill and McDonald 1977). During development, mice with elevated AKT signalling produce abnormally thick myelin (Goebbels et al. 2010; Harrington et al. 2010; Narayanan et al. 2009), but following remyelination such mice exhibit no difference in myelin thickness when compared to the myelin of wildtype controls (Harrington et al. 2010). Thus, the mechanisms controlling myelin thickness are different during remyelination from those during development.

1.4.3.1 Transcriptional control of oligodendrocyte differentiation during remyelination

While very little is known regarding the transcriptional control of oligodendrocyte differentiation during remyelination, it is likely just as important as it is during development. Many studies have assessed the expression of transcription factors during remyelination, but few evaluate the importance of each individual transcription factor during this process. Two key

transcription factors during oligodendrocyte development are the bHLH transcription factors Olig1 and Olig2. Olig2 is necessary for OPC specification and mice that are Olig2-deficient die at birth preventing assessment of remyelination in these mice (Lu et al. 2002; Park et al. 2002; Takebayashi et al. 2002). Despite the availability of a mouse line to remove Olig2 conditionally (Chen et al. 2008; Yue et al. 2006), the role of Olig2 in remyelination has yet to be explored. Olig1, which is less important in oligodendrocyte development, is necessary for remyelination (Arnett et al. 2004; Lu et al. 2002). Though no defect in OPC recruitment is observed in the absence of Olig1, remyelination is delayed, suggesting that this transcription factor is essential for oligodendrocyte differentiation during remyelination..

During development, OPC differentiation requires the suppression of inhibitory transcription factors. One way this occurs is through the deacetylation of histones; an important step in chromatin condensation that can decrease global gene expression (Li et al. 2009). One common model of demyelination involves adding cuprizone to the diet of experimental animals (Blakemore 1973; Kipp et al. 2009; Matsushima and Morell 2001). Remyelination after dietary cuprizone is preceded by an increase in HDAC1 expression, which associates with the promoter regions of transcriptional inhibitors of oligodendrocyte differentiation (Shen et al. 2008). HDAC-dependent deacetylation is likely responsible for decreasing transcriptional inhibitors, thus promoting oligodendrocyte differentiation and remyelination. When animals on a cuprizone diet are given an HDAC inhibitor, there is an increase in OPC histone acetylation and an increase in transcriptional inhibitors of differentiation resulting in impaired remyelination (Shen et al. 2008). Interestingly, insufficient elevation of HDAC1 and an impaired capacity to recruit HDAC to the

promoters of transcriptional inhibitors correlates with age-associated impairment in remyelination. Thus, age-associated decline in remyelination is due, in part, to impaired epigenetic control of oligodendrocyte differentiation (Shen et al. 2008).

1.4.3.2 Extracellular inhibitors of remyelination

Several extracellular inhibitors of oligodendrocyte development are also present following demyelination. The presence of inhibitors during remyelination does not always recapitulate development. For example, RGCs in the developing brain express the notch ligand Jagged, an inhibitor of OPC differentiation that may control the timing of oligodendrocyte differentiation (Wang et al. 1998). Both isoforms of the notch ligand, Jagged1 and Jagged2, are present following demyelination, along with Notch1. However, Jagged1 expression is observed predominantly in astrocytes and macrophages, but not along axons, suggesting that this protein does not inhibit remyelination (Stidworthy et al. 2004). Similarly, Notch1 might not be a rate-limiting step in remyelination since this process remains unchanged when Notch1 is conditionally removed from oligodendrocyte lineage cells (Stidworthy et al. 2004).

LINGO-1 is an important inhibitor of myelination and remyelination (Lee et al. 2007; Mi et al. 2007; Mi et al. 2005; Mi et al. 2009). During development, impairment of either axonal or oligodendrocyte LINGO-1 improves myelination (Lee et al. 2007; Mi et al. 2005). Anti-LINGO-1 treatments increase oligodendrocyte maturation *in vitro* (Mi et al. 2005), and increase myelination in both DRG-OPC co-cultures and cerebellar brain slice cultures (Mi et al. 2009). Similarly, anti-LINGO-1 treatment increases the extent of remyelination following focal

demyelination as well as cuprizone-induced demyelination, suggesting that LINGO-1 is an inhibitor or remyelination (Mi et al. 2007; Mi et al. 2009).

Wnt signalling inhibits both developmental myelination (Fancy et al. 2009; Fancy et al. 2011b; Ye et al. 2009) and remyelination (Fancy et al. 2009; Fancy et al. 2011b). Following demyelination, there is a specific upregulation of the Wnt pathway mediator tcf4 in OPCs but not in PLP-expressing cells, indicating that Wnt signalling is elevated during oligodendrocyte differentiation during remyelination (Fancy et al. 2009). Constitutively active β-catenin in oligodendrocyte lineage cells (Fancy et al. 2009) and deficiency of Axin2 (Fancy et al. 2011a)—a repressor of Wnt signalling that acts to promote the degradation of β-catenin (Jho et al. 2002; Lustig et al. 2002)—both inhibit oligodendrocyte differentiation and remyelination. Axin2 stabilization with an inhibitor of tankyrase (a poly-ADP-ribosylating enzyme that promotes axin degradation (Huang et al. 2009)) has the reverse effect, increasing oligodendrocyte differentiation in culture and improving remyelination in both a demyelinated cerebellar slice culture and also following focal chemical demyelination (Fancy et al. 2011a). This demonstrates that Wnt signalling may be an important regulator of remyelination.

A potential inflammation-induced inhibitor of remyelination is hyaluronan, a glycosaminoglycan that is found along cell membranes and within the extracellular matrix (Jiang et al. 2011). In the CNS, hyaluronan is produced predominantly by astrocytes (Marret et al. 1994). When OPCs are grown on hyaluronan, their ability to express MBP is robustly inhibited (Back et al. 2005). The identification of accumulated hyaluronan in MS lesions suggests that hyaluronan is a remyelination inhibitor during disease (Back et al. 2005). Hyaluronan can act as

a ligand for Toll-like receptors (TLRs) 2 and 4 (Jiang et al. 2011; Scheibner et al. 2006; Termeer et al. 2002), and TLR2 is required for hyaluronan's inhibitory effects on OPCs *in vitro* (Sloane et al. 2010). Mice that are TLR2-deficient are insensitive to infusion of additional hyaluronan, which in wild-type control animals inhibits remyelination (Sloane et al. 2010). Thus, hyaluronan is one potential inhibitor of remyelination, likely acting via TLR2.

Myelin itself might also inhibit remyelination. When OPCs are grown in the presence of myelin, they are greatly impaired in their ability to express myelin markers, and when myelin is injected following demyelination, remyelination is impaired (Kotter et al. 2006; Robinson and Miller 1999). Kotter and colleagues reported that myelin debris can occasionally observed surrounding demyelinated axons, suggesting that this debris had an inhibitory effect on OPC remyelination (Kotter et al. 2001; Kotter et al. 2005). Given the presence of myelin debris following demyelination, these findings suggest that myelin plays a role in impairing CNS remyelination. This is supported by the finding that remyelination and phagocytosis of myelin are both attenuated in animals by depleting circulating monocytes (Kotter et al. 2001; Kotter et al. 2005). Older animals, which generally have much slower remyelination (Kotter et al. 2005), also show impaired myelin uptake and the presence of excess myelin debris compared to that seen in the tissue of younger animals (Hinks and Franklin 2000). These data support the hypothesis that myelin debris removal is a rate-limiting step following demyelination.

1.4.3.3 Remyelination promoting factors

Given the high number of potential molecules that negatively regulate remyelination, it is likely that many different factors are required to establish a permissible environment for remyelination. Mitogens are necessary to drive OPC recruitment to regions of demyelination, but what factors make the CNS environment conducive to the oligodendrocyte differentiation during remyelination? Inflammation may be key (Foote and Blakemore 2005a; Ludwin 1980; Morell et al. 1998). Generally, macrophage depletion (Kotter et al. 2001; Kotter et al. 2005) or inhibiting microglial activation with agents like minocycline (Li et al. 2005), inhibits remyelination. Macrophages phagocytose inhibitory myelin debris (Kotter et al. 2001; Kotter et al. 2005), and likely also secrete remyelination promoting cytokines. Spontaneous remyelination is also impaired with the loss of CD4+ and/or CD8+ T-cells (Bieber et al. 2003). T-cells regulate the innate immune system, which might account for their role in remyelination. For example, mice lacking mature and functional T-cells have delayed phagocytocis of myelin debris and fewer phagocytic macrophages (Ghasemlou et al. 2007). Although untested, it is also possible that B-cells improve remyelination by secreting autoantibodies directed at myelin antigens, which improve myelin phagocytosis after peripheral nerve injury (Vargas et al. 2010).

These findings collectively highlight the idea that immune cells contribute to remyelination, possibly by improving the environment for this process to occur, but also by potentially secreting molecules that can improve recruitment and differentiation. Inflammation is sufficient to promote remyelination of chronically demyelinated axons. Chronic hypomyelination and astrocytosis occurs in mutant taiep, rats. After one year of age, taiep rats lack signs of

inflammation (Duncan et al. 1992; Foote and Blakemore 2005b), which in many ways models the chronic inactive lesions in clinical cases of MS. Following irradiation, these animals can be repopulated by the transplantation of genetically competent OPCs. However, this results in little remyelination, which occurs largely at the transplantation injection site. If sterile charcoal is injected into a region away from the transplantation site, it stimulates an influx of leukocytes and remyelination occurs specifically in the sites of injection. Therefore, in areas of chronic hypomyelination, inflammation is sufficient to induce remyelination. Several inflammatory cytokines are upregulated after demyelination (Biancotti et al. 2008) and may be responsible for promoting remyelination. For instance, tumour necrosis factor-α (Arnett et al. 2001; Arnett et al. 2003), interleukin 1β (Mason et al. 2001), interleukin 11 (Zhang et al. 2006a) and lymphotoxin β receptor (Plant et al. 2007) are all involved in remyelination. Other factors that arise directly or indirectly from inflammation such as IGF-1 (Mason et al. 2001), LIF (Zhang et al. 2006a) and CNTF (Skripuletz et al. 2011) also potentially promote remyelination. Taken together, inflammation is integral for promoting an environment suitable for remyelination and accounts for many factors that are generally conducive for remyelination.

1.5 Spinal cord injury: is it a model of white matter repair?

Following demyelination, oligodendrocyte support is lost and the axon is placed in a state of "virtual hypoxia" (see above), making it vulnerable to axonal degeneration. Indeed, if remyelination is impaired, there is enhanced axonal degeneration (Irvine and Blakemore 2008),

suggesting that a more rapid and efficient rate of remyelination can potentially spare more axons. The hypothesis that SCI is a target for white matter repair is based on two major findings: first, that demyelination occurs after SCI (Blight 1983; Bresnahan et al. 1976; Totoiu and Keirstead 2005) and second, that increased remyelination following neural precursor cells transplantation is correlated with improved functional recovery in animal models of SCI (Cao et al. 2005; Cummings et al. 2005; Hofstetter et al. 2005; Karimi-Abdolrezaee et al. 2006; Keirstead et al. 2005; Lee et al. 2005; Mitsui et al. 2005). This hypothesis has direct implications for clinical translation as remyelination is the major proposed mechanism of improvement following the transplantation of human embryonic stem cells derived from OPCs (which recently entered clinical trials in the USA, but is now on hold for financial reasons). In the proceeding section, I will briefly discuss the pathophysiology of SCI and highlight the features most relevant to white matter repair, before discussing the extent of demyelination and remyelination that occurs following SCI.

1.5.1 Pathophysiology of SCI

SCI is often described in terms of primary and secondary injury. The primary injury is a result of a blunt force, compression or stab from an object that directly contacts the spinal cord. (Bunge et al. 1993; Choo et al. 2007; Norenberg et al. 2004). Following this primary injury, there is a prolonged period of subsequent damage that is known as the secondary injury cascade. This secondary injury is the result of several interrelated mechanisms including free radical generation, ischemia, hemorrhage, vascular dysregulation excessive immune cell infiltration, and dysregulation of ion homeostasis (Kwon et al. 2002; Kwon et al. 2004; Norenberg et al. 2004).

With respect to white matter damage, these events culminate in oligodendrocyte loss in the weeks following injury, often in spinal cord segments distal to the injury site (Abe et al. 1999; Casha et al. 2001; Crowe et al. 1997; Emery et al. 1998; Li et al. 1999; Liu et al. 1997; McTigue et al. 2001)

1.5.1.1 Secondary damage after SCI

SCI results in impaired blood vessel perfusion and a lack of microcirculation, and is a result of both primary injury and secondary damage to blood vessels (Tator and Fehlings 1991). The ischemic damage caused by disrupted blood flow is potentially compounded by a loss of autoregulation (Senter and Venes 1979; Young et al. 1982), the mechanism maintaining tissue blood flow within a narrow range independently of perfusion pressure. The consequences of ischemia are intense: glutamate exocytosis, energetic rundown, elevated free radical production, and lipid peroxidation. Following SCI, there is also an elevation in levels of the of the excitatory neurotransmitter glutamate (Liu et al. 1991; Panter et al. 1990; Wrathall et al. 1996). Glutamate binds to membrane receptors, including N-methyl-D-aspartate (NMDA), alpha-amino-3-hydroxy-5-methylisoxazolepropionate (AMPA)/ kainate receptors ionotrophic receptors, and metabotropic glutamate receptors. The net effect of glutamate binding is an increase in intracellular Ca^{+2}, due to Ca^{+2} entry from the extracellular space and Ca^{+2} release from intracellular stores (Choi 1988). A rise in intracellular Ca^{+2} can result in many detrimental changes within a cell, and Ca^{+2} levels that are too high can result in cell death (Kroemer et al. 1998). For cells that do survive, a rise in intracellular Ca^{+2} levels is associated with an increase in cytoskeletal breakdown, production of free radicals, and nitric oxide (Gleichmann and Mattson

2011; Szydlowska and Tymianski 2010). Free radicals such as ROS are toxic, as these molecules can react with many different cellular macromolecules such as DNA, lipids and proteins. Most ROS are a by-product of mitochondrial function. Elevated ROS levels can damage mitochondria making them less efficient in the production of ATP and more prone to further free radical production (Barja 2004).

Another consequence of mitochondria damage is a reduced production of ATP. Normally, significant ATP is needed in both grey matter and white matter for the maintenance of resting membrane potentials in neurons (Harris and Attwell 2012). An impairment in ATP production can lead to fluctuations in ion homeostasis which can, in extreme examples, result in cell swelling. Specifically, energy rundown in axons can result in a reversal of the Na^{+}/ Ca^{2+} exchanger, which can promote axonal degeneration (see above) (Stys et al. 1992)

Immune cells also participate in cellular damage and repair following SCI. Hours after injury to the spinal cord occurs, there is a rapid infiltration of neutrophils which peaks as early as 12-24 hours after injury (Dusart and Schwab 1994; Stirling and Yong 2008). After neutrophil entry, monocytes are recruited from the periphery, followed by the infiltration of T-cells (Beck et al. 2010; Popovich et al. 1997; Stirling and Yong 2008). Attenuating neutrophil entry improves functional recovery in rats, indicating that neutrophils might be detrimental after SCI due to added oxidative stress (Bao et al. 2004; Gris et al. 2004). However, robust removal of circulating neutrophils in mice impairs functional recovery after SCI by altering levels of cytokines and growth factors necessary for wound healing (Stirling et al. 2009). Thus, neutrophil entry might be a repair response that becomes damaging when in excess.

Macrophages can arise from both blood-derived monocytes and by activation of CNS-resident microglia. CNS-resident microglia are self-autonomous and the major source of macrophages until there is a major disruption of the blood-brain-barrier (Ajami et al. 2007; Ginhoux et al. 2010). Monocyte-derived macrophage infiltration is necessary for the progression of symptoms in an MS animal model known as autoimmune encephalitis (EAE) (Ajami et al. 2011). In contrast to the detrimental role of blood-derived monocytes in EAE, Shechter and colleagues (2009) reported that ablation of circulating monocytes following SCI impairs functional recovery. This beneficial role of blood-derived monocytes is thought to be due to their capacity to release interleukin-10, which produces anti-inflammatory properties that dampen endogenous microglia (Shechter et al. 2009). Thus, it has been argued that macrophages have both detrimental and beneficial roles and these can potentially be differentiated by two distinct subsets of macrophage (Kigerl et al. 2009). The proinflammatory subset, M1, is activated by factors like interferon-γ and lipopolysaccharide. M1 macrophages produce high levels of oxidative species and proinflammatory cytokines; they are neurotoxic *in vitro* (Ding et al. 1988; Kigerl et al. 2009). In contrast, the anti-inflammatory M2 subtype is activated by factors like interleukin-4 and interleukin-13, and can promote angiogenesis, suppress disruptive immunity, and promote regenerative growth of sensory axons (Kigerl et al. 2009; Sica et al. 2006). It is of significant interest to establish strategies that promote the generation of M2 macrophages and determine if this generation is beneficial following SCI.

1.5.2 Oligodendrocyte death and demyelination after SCI

Several aspects of secondary damage result in the death of oligodendrocytes (McTigue and Tripathi 2008). White matter is vulnerable to ischemic damage (Pantoni et al. 1996) potentially because oligodendroglia are highly susceptible to oxidative stress (Husain and Juurlink 1995; Merrill et al. 1993; Noble et al. 1994; Oka et al. 1993). This susceptibility is due, in part, to the excess iron carried in OPCs (Thorburne and Juurlink 1996). Iron can have detrimental effects on cells as it can mediate the conversion of peroxides to strong oxidants such as hydroxyl radicals (Herbert et al. 1994). OPCs also have lower levels of glutathione and glutathione peroxidase compared to astrocytes, suggesting a reduced capacity to scavenge peroxides (Juurlink et al. 1998; Oka et al. 1993; Thorburne and Juurlink 1996). Importantly, treatments that reduce glutathione levels also reduce the capacity to scavenge ROS and compromise OPC survival in culture (Back et al. 1998; Oka et al. 1993; Yonezawa et al. 1996). The low capacity to scavenge ROS combined with elevated iron stores is thought to put OPCs in a precarious position, with the potential for "dangerously high" peroxide levels "if iron is released from iron stores" (Juurlink et al. 1998).

As OPCs differentiate there is an increased resistance to oxidative stress. As OPCs differentiate into oligodendrocytes the toxic effects of glutathione deprivation lessen, with oligodendrocytes surviving much better than OPCs and producing far fewer ROS than OPCs when glutathione is deprived (Back et al. 1998). The increased capacity to buffer ROS with oligodendrocyte differentiation is potentially linked to the tremendous metabolic demand imposed on oligodendrocytes during the production and maintenance of myelin (Connor and

Menzies 1996). Thus, increased demand to buffer ROS in oligodendrocytes is coupled to increased supply of ROS due to heightened metabolic requirements. The heightened oxidative stress during ischemia is thought to overwhelm the ROS buffering capacity and contribute to oligodendrocyte death.

Another major cause of oligodendrocyte cell death after SCI is excitotoxicity. Evidence, both *in vitro* and *in vivo* demonstrates that elevated glutamate levels preferentially kill oligodendrocytes, but not astrocytes (Li and Stys 2000; Matute et al. 1997; McDonald et al. 1998). In culture, glutamate or deprivation of oxygen and glucose cause oligodendrocyte cell death, which is largely blocked by an AMPA/Kainate glutamate channel inhibitor (McDonald et al. 1998). Oligodendrocytes *in situ* express AMPA/Kainate receptors and undergo cell death in response to both AMPA and Kainate (Li and Stys 2000; Matute et al. 1997; McDonald et al. 1998). Specific inhibitors to AMPA receptor prevent a decline in neuronal function due to elevated glutamate, suggesting a role for AMPA receptors in glutamate-mediated injury (Li and Stys 2000). More recently, the NMDA receptor has been found to be present in myelin and be responsible for $Ca+^{2}$ influx into myelin due to glutamate stimulation (Micu et al. 2006). NMDA receptor antagonism reduced ischaemic damage on myelin and, thus, glutamate-mediated damage to myelin may have a different mechanism than damage to oligodendrocytes cell bodies (Micu et al. 2006). Glutamate elevation thus results in $Ca+^{2}$-mediated damage to myelin and oligodendrocytes that results in energy rundown and free radical production, potentially causing cell death.

In addition to glutamate-mediated excitotoxicity, release of extracellular ATP also causes oligodendrocyte cell death (Wang et al. 2004). After SCI, ATP is released predominately through connexin 43 hemichannels (Huang et al. 2012) and the distribution of extracellular ATP coincides with regions whose cells are undergoing cell death (Wang et al. 2004). Agonists to the ATP receptor P2X7 promote cellular apoptosis (Wang et al. 2004). By contrast, P2X7 receptor inhibition after SCI decreases the number of cells undergoing apoptosis and increases functional recovery (Peng et al. 2009; Wang et al. 2004). Oligodendrocytes, as well as OPCs, express functional P2X7 receptors that are permeable to $Ca+^{2}$ (Fields and Burnstock 2006; Matute et al. 2007). ATP can promote oligodendrocyte death via $Ca+^{2}$ entry through the P2X7 receptor (Matute et al. 2007). The P2X7 receptor mediates oligodendrocyte cell death in several pathological conditions as P2X7 receptor antagonist prevented demyelination in an animal model of MS (Matute et al. 2007).

Another major cause of oligodendrocyte cell death is the overabundance of proinflammatory cytokines. TNF-α, IL-1β, IL-2 and interferon γ have all been demonstrated to induce cell death in culture (Curatolo et al. 1997; Li et al. 2008; Sherwin and Fern 2005; Steelman and Li 2011; Vartanian et al. 1995). Of these cytokines, both TNF-α and IL-1β, but not IL-2 and interferon γ, have drastically elevated levels that peak within 24 hours in a rat model of SCI before returning to near baseline levels (Stammers et al. 2012). By 36 hours after human SCI, levels of TNF-α, IL-1β, IL-2 and interferon γ in CSF are below detectable levels (Kwon et al. 2010) and thus unlikely to mediate prolonged periods of oligodendrocyte apoptosis.

The most prominent mechanisms causing oligodendrocytes death after SCI are still unclear, but likely multifaceted. Apoptosis after SCI is widespread in segments adjacent to the injury site and peaks a week after injury, but occurs also for weeks later (Abe et al. 1999; Casha et al. 2001; Crowe et al. 1997; Emery et al. 1998; Li et al. 1996; Li et al. 1999; Liu et al. 1997; McTigue et al. 2001). Apoptosis has been observed as late as 60 days after SCI in humans (Emery et al. 1998). Oligodendrocytes account for much of the delayed cell death after SCI, which is enhanced in regions of active degeneration (Casha et al. 2001; Crowe et al. 1997; Li et al. 1999; Liu et al. 1997). The loss of oligodendrocytes after SCI is severe: 93% of the oligodendrocytes die at the lesion epicenter by 7 days after SCI (McTigue et al. 2001). With the demand for axonal survival cues in development (Barres and Raff 1999), it may be presumed that much of the oligodendrocyte cell death is related to loss of axonal support, but surprisingly axonal degeneration is not sufficient to induce oligodendrocyte apoptosis (Sun et al. 2010). Thus, the loss of axonal support potentially may contribute to oligodendrocyte apoptosis, but general secondary damage is required for oligodendrocyte death.

1.5.2.1 Demyelination after SCI

Associated with the oligodendrocyte loss, demyelination has been demonstrated in several animal models of traumatic SCI (Blight 1983; Blight 1985; Bresnahan et al. 1976; Gledhill et al. 1973a; James et al. 2011; Lasiene et al. 2008; Powers et al. 2012; Siegenthaler et al. 2007; Totoiu and Keirstead 2005). Demyelination is also apparent within 2 weeks of naturally-occurring SCI in dogs and cats, but uncommon after that time (Smith and Jeffery 2006). Far less is reported about demyelination after human SCI (Bunge et al. 1993; Guest et al.

2005; Kakulas 1999; Norenberg et al. 2004). Demyelination is reported in all studies in the acute stages of SCI in humans. However, there is considerable debate as to the extent of demyelination after human SCI in a chronic setting. There are reports of sparse or absent chronic demyelination (Kakulas 1999; Norenberg et al. 2004), but also other reports with persistent and, in some cases, substantial demyelination when residual compression of the spinal cord was present (Bunge et al. 1993; Guest et al. 2005).

Some of the variability in observed demyelination might be due to differences in the mechanism of injury. For example, hemisection injuries has less demyelination compared to contusive injuries as indicated by microscopy of spinal cord cross-sectional tissue (Siegenthaler et al. 2007). One major limitation in measuring demyelination from spinal cord cross sections is that it is not possible to distinguish between spared axons and severed axons that persist near the lesion. Distal segments of severed axons do persist adjacent to the injury site with presumably no function. Currently, only two studies have accounted for this important confound; both studies find no persisting demyelination 3 months following contusive injuries in mice (Lasiene et al. 2008) and rats (Powers et al. 2012). By measuring the myelin status of spared axons at levels distal (Lasiene et al. 2008; Powers et al. 2012) or adjacent to the lesion site (Powers et al. 2012) . it can be concluded that (a) remyelination is complete in descending spinal cord tracts at a chronic setting and (b) measuring demyelination in cross sectional tissue will yield false positive demyelinated axons (Lasiene et al. 2008; Powers et al. 2012). Therefore, axons that appear demyelinated in a cross section of the chronically injured spinal cord of rats and mice are suggested to be injury-severed axons that persist adjacent to the lesion site.

Remarkably, the extent of remyelination—and by proxy demyelination—of axons that traverse the lesion site is very high. By one report, after SCI a majority of spared descending rubrospinal axons are remyelinated; this remyelination was complete by 3 months after injury (Powers et al. 2012). Although remyelination is demonstrated to be complete in a chronic injury setting, this does not necessarily mean that improving remyelination in the acute setting will not improve function after SCI. Demyelinated axons are hypothesized to be vulnerable to the protracted secondary damage following SCI. According to this hypothesis, those axons that remain demyelinated, even for short periods, are at increased risk of axonal degeneration. In fact, the robust demyelination suggested by Powers and colleagues demonstrates the importance of promoting remyelination in the acute setting. A more rapid remyelination may spare neurological circuitry from the secondary damage that continues in the days and weeks following SCI.

1.5.3 Cellular transplantation as a means to improve remyelination

Cellular transplantation is a means to replace lost or dysfunctional oligodendrocytes after injury, disease or developmental defects. There are several cellular sources for remyelination after SCI including Schwann cells, neural progenitor cells and fate-restricted neural or glial precursors (Tetzlaff et al. 2011). Transplanted Schwann cells typically are located in or just adjacent to the lesion site, and typically remyelinate axons within the lesion epicenter. Schwann cell transplantation is associated with several benefits, in addition to remyelination (Biernaskie et al. 2007). Thus, it is not known whether transplanted Schwann cell improve function by increasing remyelination or by providing other potential benefits.

From a recent systematic review that considered all research published before the summer of 2008, transplantation of neural progenitor cells and fate-restricted neural or glial precursors is reported in fifty published studies (Tetzlaff et al. 2011). In 23 of the 28 studies with behavioural assessments, transplantation improved functional recovery after SCI (Tetzlaff et al. 2011). Transplantation of neural progenitor cells or fate-restricted precursor cells both produce astrocytes and oligodendrocytes in the injured spinal cord, despite often having the potential to produce neurons in culture. Therefore, the environment after SCI favours gliogenesis and not neurogenesis.

Enhancing the capacity of transplanted cells to differentiate into oligodendroglia by genetic modification improves functional recovery (Cao et al. 2005; Hofstetter et al. 2005). Similarly, the transplantation of cells with robust oligodendroglial potential is also associated with enhanced recovery, typically correlated with improved remyelination (Karimi-Abdolrezaee et al. 2006; Keirstead 2005; Parr et al. 2008). From this body of research it is concluded that "more white matter sparing and (re)myelination of host axons after transplantation of rodent and human oligodendrocyte precursors at one week after thoracic contusions" correlates with improvements in behavioural recovery (Tetzlaff et al. 2011). However, a limitation of this work from a translational perspective is that a variety of cells are transplanted and often transplantation experiments occurs without replication; the optimal cell for transplantation is yet to be determined. Another important facet to cellular transplantation is that it is likely to have several other benefits in addition to remyelination. These benefits are more specifically addressed in Chapter 2 (see discussion).

1.5.4 Therapies targeting endogenous cell repair following spinal cord injury

In contrast to transplantation, it might be possible to accelerate remyelination by improving the ability of endogenous OPCs to be recruited and differentiated into oligodendrocytes. One common strategy to promote cellular regeneration is to block the influence of an inhibitory molecule. Blocking the influence of these inhibitory compounds is a novel strategy to promote myelin repair. The expression of many inhibitors of myelination/remyelination such as PSA-NCAM, hyaluronan, and notch ligands delta or jagged has not, to my knowledge, been measured after SCI. However, after SCI myelin debris is present in the extracellular space for at least 8 weeks in rodents (Buss and Schwab 2003) and 3 years in humans (Buss et al. 2005). OPCs in contact with myelin have an impaired capacity to express immature oligodendrocyte markers (Baer et al. 2009; Robinson and Miller 1999; Syed et al. 2008) and myelin infusion after demyelination impairs oligodendrocyte differentiation and subsequent remyelination (Kotter et al. 2006). The capacity to phagocytose myelin debris is impaired as part of the natural aging process (Zhao et al. 2006), as is the capacity to remyelinate (Hinks and Franklin 2000; Ruckh et al. 2012; Shen et al. 2008; Shields et al. 1999). Remyelination can be rejuvinated with heterochronic parabiosis of an aged animal with a young animal (Ruckh et al. 2012). Rejuvinated remyelination is correlated to an increased rate of phagocytosis; if the improved phagocytosis is prevented, so is much of the improvement in remyelination. Likewise, impairement of phagocytosis by depleting macrophages also impairs remylination (Kotter et al. 2001; Kotter et al. 2005). Taken together, the myelin debris phagocytosis is one rate-limiting step during spontaneous remyelination. Extracellular myelin

debris after SCI contains several inhibitors of axonal outgrowth is thought to inhibit of axonal regeneration (Filbin 2003; Liu et al. 2006b; Schwab 2004; Yiu and He 2006). However, given the slow Wallerian degeneration myelin is one potential inhibitor of remyelination. The heightened oligodendrogenesis at the lesion border (Tripathi and McTigue 2007)—where phagocytosis of myelin debris surrounding spared axons is likely greatest—is consistent with myelin debris inhibiting remyelination after SCI.

1.6 Experimental overview and hypothesis

Remyelination is a means to restore oligodendrocyte support of axons and normalize axonal energetics. Remyelination is therefore suggested to prevent axonal degeneration after demyelination. SCI is associated with prolonged oligodendrocyte death and many spared axons are demyelinated. Thus, improving remyelination after SCI might spare axons and improve functional recovery. This can be accomplished by the transplantation of a neural progenitor or glial precursor after injury; however, the optimal cell is yet to be determined. In Chapter 2, PDGF-responsive neural precursor cells (PRPs) were transplanted after clinically relevant animal model of spinal cord injury (Chojnacki and Weiss 2004). PRPs are multi-potential cells that are capable of self-renewal *in vitro*. **I hypothesize that transplantation of PRPs after SCI will remyelinate denuded axons and improve the amount of remyelination as well as functional recovery.** I demonstrated that transplanted PRPs integrate with host tissue and differentiate into remyelinating oligodendrocytes. The capacity of transplanted PRPs to produce compact myelin

was also confirmed in MBP-deficient mice lacking compact myelin. To my surprise, PRP transplantation was not associated with improvement in the number of myelinated axons at the lesion epicenter, nor was it associated with improvement in functional recovery in two separate transplantation experiments.

Given that we did not find significant increases in total myelin or behavioural recovery after PRP treatment, one may speculate that the endogenous precursors provide a sufficient level of spontaneous remyelination in our injury model. An alternative possibility is that the same extrinsic factors that block remyelination by endogenous precursors may also block the remyelination by transplanted PRPs or their progeny. Myelin is present following SCI and known to inhibit the expression of immature markers *in vitro*. Therefore, **I hypothesize that myelin inhibits remyelination after SCI.** To begin testing this hypothesis, I developed a myelin spot assay and demonstrated that OPCs maturation was robustly inhibited when cultured on myelin, characterized by decreased expression of immature and mature oligodendrocyte markers, impaired production of myelin gene products, as well as stalled morphological complexity. The expression of two transcription factors known to prevent OPC differentiation and maturation were increased in contact with myelin: ID2 and ID4. Overexpression of ID2 and ID4 in OPCs was previously reported to decrease the percentage of cells expressing mature oligodendrocyte markers. Hence the increased expression of ID2 and ID4 in oligodendroglia on myelin is sufficient to block their maturation and may inhibit endogenous remyelination in the injured or demyelinated CNS.

Chapter 2

Platelet-Derived Growth Factor-Responsive Neural Precursors give rise to Myelinating Oligodendrocytes after Transplantation into the Spinal Cords of Contused Rats and Dysmyelinated Mice[1].

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[1] A version of this chapter has been published. Plemel JR, Chojnacki A, Sparling JS, Liu J, Plunet W, Duncan GJ, Park SE, Weiss S, Tetzlaff W. 2011. Platelet-derived growth factor-responsive neural precursors give rise to myelinating oligodendrocytes after transplantation into the spinal cords of contused rats and dysmyelinated mice. Glia 59(12):1891-910.

2.1 Introduction

Spinal cord injury (SCI) results in substantial oligodendrocyte death occurring over a course of weeks after the primary trauma (Abe et al. 1999; Casha et al. 2001; Crowe et al. 1997; Li et al. 1999; Liu et al. 1997) and resulting in demyelination in murine models of SCI ((Blight 1983; Bresnahan et al. 1976; Totoiu and Keirstead 2005) as well as in human SCI patients (Guest et al. 2005; Kakulas 1999; Norenberg et al. 2004; Smith and Jeffery 2006). Cell replacement strategies represent one promising approach for replacing lost oligodendrocytes and remyelinating denuded axons, thereby restoring impaired impulse conduction, as well as preventing axonal degeneration. A variety of cell sources have been explored as potential transplant candidates for replacing lost oligodendrocytes following SCI, including: fetal-derived epidermal growth factor (EGF)/ fibroblast growth factor (FGF)-responsive neural precursor/progenitor cells (Cummings et al. 2005; Hooshmand et al. 2009; Iwanami et al. 2005; Lowry et al. 2008; Nakamura et al. 2005; Ogawa et al. 2002; Tarasenko et al. 2007), adult-derived EGF/FGF-responsive neural precursor cells (Hofstetter et al. 2005; Karimi-Abdolrezaee et al. 2006; Karimi-Abdolrezaee et al. 2010; Parr et al. 2007; Pfeifer et al. 2004), pre-differentiated embryonic stem cells (Keirstead et al. 2005; McDonald et al. 1999), oligodendrocyte precursor cells (OPCs) (Bambakidis and Miller 2004; Lee et al. 2005) or glial restricted precursor cells (Cao et al. 2010; Cao et al. 2005; Han et al. 2004; Hill et al. 2004; Mitsui et al. 2005). However, despite over a decade of research, the optimal precursor population to achieve oligodendrocyte replacement post-SCI has yet to be determined. There are many advantages to the use of embryonic stem cells (ESCs), as they can proliferate indefinitely *in vitro*

and can be pre-differentiated into glial precursors to achieve a sufficient number of cells for transplantation (Nistor et al. 2005). However, the transplantation of ESC-derived cells poses a substantial risk given that transplantation of a single undifferentiated, multipotent stem cell may be enough to cause the formation of embryonic tumors (Reubinoff et al. 2000; Thomson et al. 1998). Importantly, the tumor risk of human ESCs may not be fully revealed in rodent studies, as xenotransplants of stem cells show far fewer tumor formations than allotransplants (Erdo et al 2003).

With a substantially lower risk of tumor formation, fetal and adult neural tissues could be an attractive alternative source for oligodendrocyte precursors, although it must be noted that these sources (particularly the fetal) are not without logistical, ethical and legal concerns that may hinder their clinical translation. Various neural and glial progenitor cells have been transplanted into the injured spinal cord (Cao et al. 2002; Hill et al. 2004; Lepore et al. 2005; Mitsui et al. 2005; Mujtaba et al. 2002) as well as into the CNS in a variety of disease models (Eftekharpour et al. 2007; Hammang et al. 1997; Isacson et al. 1986; Kordower et al. 1995; Zhang et al. 1999). These studies commonly generate cells via classical neurosphere culture techniques using the growth factors EGF and/or FGF, at times with additional enrichment steps (Zhang et al. 1999). Although EGF- and/or FGF-responsive precursors consistently generate oligodendrocytes from rodent tissue, human EGF- and/or FGF-responsive precursors have a much lower capacity for generating oligodendrocytes *in vitro* (Chojnacki et al. 2008; Horiguchi et al. 2004; Ostenfeld et al. 2002) than human PDGF-responsive neural precursors (PRPs) (Chojnacki et al. 2008). This PRP population exists in rodents as well, and rodent PRPs are

multipotent, capable of producing neurons, oligodendrocytes and astroctyes *in vitro* (Chojnacki and Weiss 2004; Gregg et al. 2007). PRPs differ from the more commonly recognized OPCs, which also express platelet derived growth factor receptor α (PDGFRα), in that OPCs predominantly produce oligodendrocytes in culture, as well as astrocytes under certain conditions, but never produce neurons (Raff et al. 1983; Temple and Raff 1985). Here we investigated whether PRPs could differentiate into remyelinating oligodendrocytes and improve functional recovery after SCI. PRPs from embryonic day 14 green fluorescent protein (GFP)-expressing mice were transplanted 7-14 days after a contusion of the thoracic spinal cord in adult rats. Transplanted PRPs integrated into host tissue, and differentiated into branched oligodendrocytes that ensheathed multiple axons. These GFP-positive sheaths were closely associated with myelin basic protein (MBP) and normal paranodal and juxtaparanodal structures, as defined by the pattern of cell adhesion molecules Caspr and Kv1.2 potassium channels, respectively. PRP-derived oligodendrocytes were also demonstrated to produce compact myelin with normal periodicity following transplantation into dysmyelinated Shiverer mice, a well characterized model to test the myelinating potential of CNS transplant candidate cells (Archer et al. 1994; Eftekharpour et al. 2007; Gansmuller et al. 1986; Lachapelle et al. 1983; Liu et al. 2000; Maire et al. 2009; Mothe and Tator 2008; Nistor et al. 2005; Vitry et al. 2001; Warrington et al. 1993; Windrem et al. 2004; Windrem et al. 2008). Thus, PRP-derived oligodendrocytes are able to generate mature myelin sheaths on nude/denuded CNS axons. To our surprise, although transplanted PRPs efficiently produced oligodendrocytes in the injured spinal cord, there was no significant increase in the total number of myelinated axons in PRP-transplanted versus media

control animals, and there was no improvement in behavioural recovery following transplantation in two separate experiments.

2.2 Materials and methods

2.2.1 Animals

A total of 83 rats and 3 Shiverer mice were used in this study. All procedures were approved by the University of Calgary and the University of British Columbia, in accordance with the guidelines of the Canadian Council on Animal Care.

2.2.2 Preparation of PRPs.

PDGF-responsive neural precursors (PRPs) were cultured as described previously (Chojnacki and Weiss 2004). The anterior entopeduncular area was removed from Embryonic Day 14 mouse (Tg(GFPU)5Nagy/J) embryos and mechanically dissociated with a fire-polished Pasteur pipette in minimal hormone medium (MHM). Cells were plated at a density of 4.0 X 10^4 cells/ml. MHM was composed of DMEM/F-12 (1:1) including HEPES buffer (5mM), glucose (0.6%), sodium bicarbonate (3mM), glutamine (2mM), insulin (25 μg/ml), transferrin (100 μg/ml), progesterone (20nM), putrescine (10μM), sodium selenite (30nM), and 2% B27 (all from Sigma, Oakville, Canada; except glutamine and B27 from Invitrogen, Burlington, Canada). For the generation of primary neurospheres 100ng/ml of PDGF-AA (Peprotech, Rocky Hill, NJ) and 100ng/ml of NT-3 (Peprotech) were added to the MHM. All subsequent passages were cultured

in PDGF + NT-3 + 20ng/ml of FGF2 (R&D Minneapolis, MN) + 2μg/ml heparan sulfate (Sigma).

2.2.3 Spinal cord contusion injury.

In order to establish behavioural baselines, adult male (250 ±30g) and female (277±13g) Sprague Dawley rats (Charles River Laboratories, Wilmington, MA) were acclimatized to the behavioural testing environment (see below). Animals were anesthetized with a mixture of ketamine hydrochloride (72mg/kg; Bioniche, Belleville, Canada) and xylazine hydrochloride (9mg/kg; Bayer, Toronto, Canada) via intraperitoneal injection. The skin of the back was shaved, disinfected and, under aseptic conditions, the dorsal aspect of the spinal column exposed at the T8-T10 vertebrae. Body temperature was maintained throughout the surgical period and blood oxygenation level and heart rate were continuously monitored. Using a fine pair of rongeurs, a laminectomy of the ninth thoracic vertebra (T9) was performed taking care not to damage the dura. Rats were transferred to the stage of the Ohio State University spinal cord impactor and were held in place by Allen clamps that were connected to the dorsal processes of the T10 and T8 vertebrae while 50% of the animals body weight was supported from underneath. Using the vibrator mode to determine the point of initial contact, the impactor tip was lowered onto the dorsal surface of the spinal cord. Once at the point of initial contact, the impactor tip was displaced 1.5mm into the spinal cord with a 6ms dwell time. Immediately following the injury, rats were hydrated with a subcutaneous injection of Ringer's solution (10ml). Buprenorphine (0.03mg/kg; Scherling-Plough, Welwyn Garden City, UK) was administered subcutaneously twice daily for two days following injury to alleviate pain. Antibiotics (Enrofloxacin, 5mg/kg,

i.p. or 50mg/L orally; Bayer, Toronto, Canada) were given as needed to treat/prevent infection. Bladders were expressed twice daily until the rats regained spontaneous micturition.

Transplantation of cells in our pilot study was conducted at 14 days following contusion (Table 2.1). At this point cartilaginous tissue was present on the vertebra at the laminectomy site and caused difficulties with the transplantation surgery. To ensure ease of access to the spinal cord, the timing of cell transplantation was changed to seven (male study) and 12 days post-contusion (female study) in the subsequent groups. Immediately before surgery PRP spheres were gently dissociated into single cells by titration using MHM, except for cells used in the 'cell plus growth factor' group, which were dissociated in the growth medium. Cells were suspended at a final concentration of $2.0x10^5$ cells/µl (pilot study), $1.0x10^5$ cells/µl (male study), or $8.0x10^4$ cells/µl (female study). In our pilot study, cell integration and differentiation potential was assessed by injecting $1.0x10^6$ cells directly into the lesion site as well as $1.0x10^5$ cells into 4 sites that were 2.5-4mm rostral or caudal to the lesion epicentre, and 1.5mm apart, on either side of the dorsal midline. Subsequent histological analysis indicated poor survival of cells inside the lesion cavity, and led us to omit the injection of cells directly into the lesion cavity in subsequent experiments. As such, rats in subsequent experiments only received the 4 rostral and caudal cell injections adjacent to the lesion (as described above). For long-term behavioural and histological analysis (male study), animals were placed into 4 groups: PRPs (n=18), PRPs with trophic factors (TF, which included 312ng/µl FGF and 62.5ng/µL PDGF-AA; n=15), media injection (DMEM: F12 at 1:1; n=8), and media injection with trophic factors (TF; n=11) (Table 2.1). A

second study was conducted in female rats that included two groups: PRPs (n=16) and media injection (as above; n=11).

To control for unwanted variability in injury severity among the various treatment groups in each experiment, the animals were grouped according to peak force of contusion as well as their early scores on the open field locomotor scale. All animals received subcutaneous injections of cyclosporine A (15mg/kg, s.c.; Novartis, East Hanover, NJ) beginning 2 days prior to transplantation and continuing until sacrifice (pilot study) or until 14 days post transplantation (male and female studies) at which time the animals received a similar dose of oral cyclosporine (150mg/L; Neoral, Novartis). In our hands, whole blood samples measured using therapeutic substance monitoring (TSM) revealed therapeutic levels of 225.6 ±25.6ug/L when Neoral was administered to uninjured animals (n=4). In the pilot and male studies, all of the animals were also treated with Minocycline (Genpharm, Etobicoke, Canada) around the time of transplantation, because this drug has been shown to improve transplant survival of oligodendrocyte progenitors in Long Evans Shaker rats (a myelin mutant that shows progressive microglial activation; (Zhang et al. 2003). In our pilot experiment, Minocycline was administered for 3 days prior to transplantation (90mg/kg, i.p.) until 6 days after transplantation (45mg/kg, i.p. for the first 3 days following transplant and 22.5mg/kg, i.p. for 3 additional days). In order to minimize animal suffering due to repeated injections, animals in the long-term male study were given minocycline in their diet for 8 days (20mg minocycline/g dried food) starting 2 days prior to transplantation and persisting until 5 days after transplantation. This dose was found to yield whole blood levels of 33.6± 3.6μg/ml when ingested by uninjured animals (n=2).

This is in the same order as the levels we measured 5-6 hours after the last intravenous injection of minocycline (60mg/kg/day for 5 days) in whole blood: 57.0±4.6μg/ml (unpublished data).

2.2.4 Behavioural assessment.

Both open field and CatWalk (Noldus; Leesburg, USA) assessments were conducted to determine the degree of behavioural recovery at various time points after injury. Animals were habituated to the CatWalk apparatus by running them across the walkway 4-6 times per session for 5-6 sessions prior to injury and data collected during the last session was used to establish pre-injury baselines. Open field locomotor testing was conducted prior to injury and at 2, 4, and 6 or 7 days post-injury and on a weekly basis thereafter until 8 weeks post injury (male study) or 12 weeks post injury (female study). Post-injury CatWalk assessment was delayed until the animals had regained plantar placement with weight support. In the long-term male study, 5 weeks post injury animals were habituated to the apparatus and then assessed at 7 weeks post injury. In the long-term female study, animals were assessed every two weeks between week 4 and 12 post injury. All behavioural testing was conducted and quantified by researchers blind to the experimental treatments.

2.2.5 Open field test.

Open field locomotor assessment was conducted by placing animals in a large plexiglass open field (150cm long x 120cm wide x 15cm high) for 4 minutes while assessing their performance using the Basso, Beattie, and Bresnahan (BBB) scale (Basso et al. 1995). Briefly, the BBB is a 22-point (0-21) scale that provides a gross indication of hindlimb locomotor

function. Scores from 0 to 7 indicate improving degrees of movement of the ankle, knee, and hip joints on each limb, whereas scores from 8 to 11 indicate various stages of plantar weight support and stepping with no coordination. Scores between 12 and 14 indicate differing degrees of forelimb-hindlimb coordination, and scores from 14-21 take paw position, toe clearance, trunk stability, and tail position into account. All animals included in the present study scored a 21 on the BBB prior to SCI.

2.2.6 CatWalk quantitative gait analysis.

The CatWalk system (Noldus) measures many fine features of gait as an animal walks across a 100cm long translucent platform (Hamers et al. 2001; Vrinten and Hamers 2003). The platform has black, opaque walls running lengthwise on either side to guide the animal across. All data acquisition was done in a darkened room. An LED light source spans the length of the platform and acts as a source of internally reflected illumination, which is reflected externally to varying degrees when the platform is distorted by pressure applied to the surface. As a result, when an animal's paw applies pressure to the platform, light exits the glass to an extent proportional to the amount of pressure applied and this illumination was recorded by a CCD camera (Specs) housed beneath the platform, and the analog image taken by the camera was then converted to a digital file with a frame grabber for subsequent analysis using the CatWalk software. With this software, areas that make contact with the platform are assigned an identity, for instance right or left forepaw or hindpaw. Four runs across the platform from each animal were analyzed and averaged in order to determine the regularity index, hindlimb base of support and hindlimb stride length at each time point and only crossings that involved continuous

movement were assessed. Regularity index is a measure of the percentage of steps completed that are considered a regular stepping sequence and provides a measure of forelimb-hindlimb coordination. Hindlimb base of support is the average distance between the two hindpaws and hindlimb stride length is the average distance between successive placements of either hindpaw along the direction of travel.

2.2.7 Sensory testing.

We used the plantar radiant heat test (Ugo Basile, Camerio, Italy) to test for thermal allodynia. Animals were placed on a glass surface and each paw was stimulated independently from the beneath the glass with a movable infrared light source that provided gradually increasing heat intensity. In order to ensure that the animals would suffer no tissue damage, even at the maximum duration of the heat application, the infrared intensity was set at 50%. All four paws were stimulated and upon a withdrawal response the device would automatically cease and record the withdrawal latency. A total of 2-3 measurements were recorded for each paw to give an average recording. Those animals that had not yet regained wait support were omitted from analysis.

We used the Dynamic Plantar Aesthesiometer (Ugo Basile, Comerio, Italy) to test for mechanical allodynia. This device basically allows one to conduct an automated version of the von Frey hair test. Briefly, a thin metal filament raises and makes contact with the paw during plantar placement, and increasing force is gradually applied until a withdrawal response was achieved, at which point the device records the latency of the response. Animals were positioned

above the device on a wire mesh surface in which there was access to all four paws. Average recordings were composed of 2-3 measurements for each paw, where the device was set at a load rate of 5 g/s up until a maximum load of 50g. Those animals that had not yet regained weight support were omitted from analysis.

2.2.8 Tissue processing, immunohistochemistry, and Luxol fast blue staining.

Tissue processing and immunohistochemistry followed that described in our previous work (Biernaskie et al. 2007; Plemel et al. 2008). Animals were euthanized with a lethal dose of chloral hydrate (Sigma) and perfused transcardially with 0.01M PBS followed 4% paraformaldehyde at a variety of days post injury (Table 2.1). The spinal cords were removed, post fixed in 4% paraformaldehyde at 4˚C overnight after which tissue was cryoprotected in 24% sucrose. The spinal cord were frozen and spinal cord sections, 20μm in thickness, were cut in either the coronal or transverse planes using a cryostat and mounted on Superfrost Plus slides (Fisher). For immunodetection of myelin basic protein (MBP), tissue sections were delipidized prior to any staining procedures by washing sections 2 minutes into a number of ascending and descending concentrations of ethanol. For immunohistochemistry, 0.2% triton-X 100 was used along with blocking serum, primary antibodies and secondary antibodies to permeabilize cell membranes. Non-specific binding sites were blocked with 10% normal donkey serum, except when using Alexa fluor 647 goat anti-rabbit secondary in which case non-specific binding sites were blocked with 5% NDS and 5% normal goat serum. The tissue was incubated overnight at room temperature with primary antibodies, washed and then incubated with fluorescent-conjugated secondary antibodies for 1-2 hours at room temperature. The following primary

antibodies were used: mouse monoclonal anti-MBP (1:10, Chemicon, Temecula, CA), goat polyclonal anti-MBP (1:500, Santa Cruz Biotechnology, Santa Cruz CA) chicken polyclonal anti-GFP (1:1000, Chemicon), rabbit polyclonal anti-neurofilament-200 (1:400, Serotec, Oxford, UK), rabbit polyclonal anti-β-III tubulin (1:400, Covance, Princeton, NJ), rabbit polyclonal anti-NG2 (1:200, Chemicon), rabbit polyclonal anti-PDGFRα (1:100, Santa Cruz Biotechnology), goat polyclonal anti-PDGFRα (R&D systems, Minneapolis, MN), mouse monoclonal anti-NeuN (1:100, Chemicon), mouse monoclonal anti-CC1 (APC, Calbiochem Immunochemicals, San Diego, CA), mouse monoclonal anti-glial fibrillary acidic protein (GFAP, 1:400, Sigma, St. Louis, MO, USA) or rabbit polyclonal anti-GFAP (1:500, DakoCytomation, Carpinteria, CA), mouse anti-ki67 (1:10, BD Biosciences, Mississauga, Canada), mouse monoclonal anti-Caspr (1:300, a generous gift from Dr. J. Trimmer, University of California, Davis, CA) mouse monoclonal anti-kv1.2 (1:200, Dr. J. Trimmer), mouse monoclonal anti-fibronectin (1:200, Sigma), rabbit anti-laminin(1:1000, Sigma) and mouse monoclonal anti-neurocan (1:, Developmental Hybridoma bank). Secondary antibodies used were Cy3-conjugated, FITC-conjugated AMCA-conjugated donkey anti-mouse, -rabbit, or -chick (Invitrogen, Carlsbad, CA) and Alexa fluor 647-conjugated donkey anti-rabbit (Invitrogen). The images from figures 3 (A,B), 4(L-O), and 5 as well as Supplementary figures 1 and 2 use the following secondary antibodies: Dylight 405-conjugated, Dylight 488-conjugated, Dylight 594-conjugated or Dylight 649-conjugated donkey anti-mouse, -rabbit, -goat or –chick (Jackson ImmunoResearch, West Grove, PA) as well as Dylight 488 goat anti-mouse IgG1, Dylight 647 goat anti-mouse IgG2b and Dylight 594 goat anti-mouse IgG1. To visualize nuclei, following immunohistochemistry

tissue was incubated with for 2-5 minutes with ToPro3 (1:1000, Invitrogen). Luxol fast blue was used to determine global myelination. To ensure consistent staining, slides were split so that each staining dish had a constant number of slides from each experimental group in order to normalize animal groups. Tissue was dehydrated in ascending concentrations of ethanol before being placed into 1% Luxol fast blue diluted in a solution containing 95% ethanol and 5% acetic acid at 60˚C overnight. The extent of Luxol fast blue was regulated by washing slides in 0.05% Lithium Carbonate and 70% ethanol prior to a second dehydration in ascending concentrations of ethanol, which was followed by a xylene wash before coverslipping with Entellan. A Zeiss Axioplan 2 microscope fitted with deconvolution software (Northern Eclipse, Empix, Mississauga, Canada) was used to visualize immunofluorescence and Luxol fast blue staining. Labelled sections were also examined with a Nikon C1 Laser Scanning Confocal Microscope, a Zeiss Axio Observer equipped with a Yokogawa X-1 Spinning Disk (Fig. 2) or a Quorum WaveFX Spinning Disc Confocal Microscope using Volocity (Improvision) software (Fig. 3). Image files were processed for analysis using Photoshop 7.0 (Adobe, Toronto, Canada) and ImageJ software (National Institutes of Health, Bethesda, MD).

2.2.9 Histological quantification.

Animals from the male study were sacrificed at 9 weeks post injury and randomly split according to open field score to determine which tissue would be cut longitudinally and used in cell count and which would be cut coronally and used in myelin measurements. To estimate the survival of transplanted GFP-positive PRPs we used unbiased stereological techniques to measure the transplant area and cell density from longitudinal sections taken every 200μm

throughout the extent of the graft area and applied the following formula: Cell count= Σ [transplant area x 200μm x density] + Σ [dispersed cells x 200μm]. In order to minimize the density differences between animals, cells that were tightly intermingled (transplant area) were measured separately from those cells that were distinguishable and separate from one another at 5x primary magnification (dispersed cells). Cell density was measured by taking 2-3 confocal micrographs at 60x primary magnification as stacks that spanned the thickness of the sections from randomly sampled fields in each of the included animals. As density measurements were not significantly different between groups or over time post–transplantation, density measurements were averaged across all groups in order to calculate cell counts. At 8 weeks post-transplantation 2 of the 9 animals assessed completely lacked cells and so were not included in the quantification, one of these animals was from the group transplanted with cells and trophic factors and the other was from the group receiving cells alone. At 2 and 14 days post-transplant, all animals assessed still had transplanted cells.

The total amount of myelin was quantified using images of transverse sections stained with Luxol fast blue. Those images were taken at the epicenter of injury and every 400μm rostrally and caudally. Spared white matter was determined by quantifying the area with high Luxol fast blue staining (tissue that was both dark and consistent in texture) using SigmaScan Pro 5.0 (SPSS Inc., Chicago, IL).

To determine the fate of transplanted cells confocal microscopy was used to count the percentage of GFP-positive cells that co-labelled with various neural markers, as published previously (Eftekharpour et al. 2007; Karimi-Abdolrezaee et al. 2006). This quantitative analysis

was performed using animals from group 1 (n=4; Table 2.1). For each animal, the two spinal cord sections containing the greatest area of transplanted cells were used and sections were taken no closer than 200μm to ensure that cells were not double counted. Ten random fields were assessed per spinal cord section and those fields contained 60 cells on average.

To measure the number of myelinated and unmyelinated axons tiled confocal images were taken of the entire lesion epicentre section at high power magnification. Each image within the larger confocal tile had a sample region that had an area of $100\mu m^2$ that was digitally enhance which was used to count the number of myelinated and unmyelinated axons. This measurement was very precise, with an average coefficient of error of 8.57%.

2.2.10 Shiverer mouse transplantation

Shiverer mice (Cha.SWV(C3Fe)-MBP^{shi}/J), which completely lack compact myelin, were transplanted with PRPs (n=3) in order to confirm compact myelination. Prior to surgery PRP spheres were gently dissociated into single cells by titration and resuspended into MHM at a final concentration of $1.0x10^5$ cells/μl. Three-week-old Shiverer mice weighing 9.1± 0.1g were anesthetized with inhalant isoflurane mixed with carboxygen (95% O2 / 5% CO2). The skin of the back was shaved and disinfected and the dorsal aspect of the spinal column exposed at the C3/4 vertebrae. Cells were transplanted into the spinal cord at 3 sites: one in the dorsal column and two in the dorsal lateral funiculus, the latter of which were separated by a distance of approximately 1mm. 1μl of cells was injected into the dorsal column and 0.5μl of cells were injected into each site in the dorsal lateral funiculus, resulting in a total of $2.0x10^5$ cells injected

per animal. To alleviate pain, Buprenorphine (0.05mg/kg) was given twice daily for two days. Animals received subcutaneous injections of cyclosporine A (20mg/kg, s.c.; Novartis, East Hanover, NJ) until 14 days post transplantation at which time the animals were placed on oral cyclosporine (200mg/L of water; Neoral, Novartis). Animals were euthanized 4 weeks after transplantation.

2.2.11 Electron microscopy

Shiverer mice were euthanized with a lethal dose of ketamine/xylazine at 4 weeks post transplantation, perfused transcardially with 4% glutaraldehyde in a 0.1M cacodylate buffer (pH 7.4) and postfixed for an additional week. Tissue was incubated in 1% osmium in a 0.1M cacodylate buffer (pH 7.4) for one hour, dehydrated in increasing concentrations of ethanol, and embedded in a 1:1 Spurr/Epon resin. Semithin sections (0.5μm) were cut in the transverse on an ultramicrotome (Leica Ultracut E, Richmond Hill, Canada), stained with toluidine blue, dehydrated in increasing concentrations of ethanol, coverslipped with Entellan and imaged with a Zeiss Axioplan 2 microscope using Northern Eclipse software. Blocks were further trimmed and sectioned at 50-100nm on an ultramicrotome, placed on carbon coated copper grids, stained with uranyl acetate and lead citrate and imaged with a Hitachi H7600 transmission electron microscope (Mississippi State, MS).

2.2.12 Statistical analysis

All behavioural and histological analyses were conducted by observers blinded to the treatment of individual animals. Data are presented as mean ± standard error of mean (SEM) or

in case of the co-labelling analysis as mean ± Standard Deviation (SD), as indicated. Total myelin, open field assessments and the Catwalk assessments in the female study were all analyzed using Students T-test across time points with a Bonferroni correction. The Catwalk data in the male study and all sensory data were assessed using Student T-test. In measuring the drop in PRP counts with time, ANOVAs were conducted followed by Fisher's least significant difference (LSD) post hoc assessments. All statistics were analyzed using SPSS (Chicago, IL) and all statistics are reported as one-tailed. The significance level for all tests was set at $p < 0.05$.

2.3 Results

2.3.1 PRPs integrated into host tissue, differentiated into oligodendrocytes and myelinated axons

PRPs are a self-renewing, multipotential population of cells that are distinct from the EGF/FGF-responsive population of precursor cells (Chojnacki et al. 2008; Chojnacki and Weiss 2004; Gregg et al. 2007). PRPs isolated from the anterior entopeduncular area of embryonic day 14 GFP mice and cultured in the presence of PDGF expand as spheres in both primary cultures and subsequent passages. Previously we demonstrated that PRPs from primary cultures were robust in their capacity to produce oligodendrocytes (Chojnacki and Weiss 2004). Here we tested PRP oligodendrocyte production *in vitro* after three to four passages, by dissociating and differentiated PRPs in mixed hormone media. After one day *in vitro*, 42% of the cells expressed the oligodendrocyte precursor marker PDGFR-α (Figure 2.1 A) and after three days *in vitro*,

20% of cells labeled for the immature oligodendrocyte marker O4 (Figure 2.1 B). This indicates that PRPs remain a source of oligodendrocytes *in vitro* after multiple passages.

Passage 3 to 4 PRPs were transplanted between one and two weeks following a contusive spinal cord injury to assess their potential to integrate and mature into myelinating oligodendrocytes (Table 2.1). Eight weeks after transplantation, animals were euthanized for subsequent histological examination. Transplanted PRPs integrated well into the host white matter parenchyma and GFP-positive cells were found near the border of the lesion cavity as well as in the spared white matter; they were rarely found in the grey matter. In the white matter, the GFP-positive cells displayed small round cell bodies that were dispersed amongst the spared axons (Figure 2.2 A, arrowheads). Many of these round cells extended thin elongated processes running in parallel to the axons in the rostro-caudal direction (Figure 2.2 A). There was a noticeable paucity of myelin staining in white matter regions where PRPs integrated, as evidenced by weak MBP immunohistochemistry despite the presence of axons (Figure 2.2 C). Given that the myelin sheaths of remyelinated axons are thinner than the original sheaths (Blakemore 1974; Ludwin 1978), this weaker MBP signal may result from thin myelin sheaths indicative of remyelination. Very few GFP-positive cells survived in the lesion site itself, but a portion of the transplanted PRPs surrounded the lesion cavity (Figure 2.2 A, B, arrows; Figure 2.4) and intermingled with astrocytes and astrocytic processes that make up the glial scar (Figure 2.4). To assess the fate and the extent of differentiation of the transplanted PRPs we determined the percentage of cells expressing GFP and co-expressing other cellular markers. At 8 weeks post-transplantation, no expression of the early progenitor marker nestin was detected. The

oligodendrocyte precursor markers PDGFRα (Figure 2.3 A) and NG2 (Figure 2.3 B) were expressed in 35.0±3.2% and 34.5±3.7% of the transplanted cells, respectively. Another 31.5±3.3% of GFP-expressing cells co-labeled with CC1, a marker of mature oligodendrocytes (Figure 2.3 C). PRP-derived CC1-positive cells extended multiple branches, each with numerous processes that often contacted axons (Figure 2.3 C, E), a morphology characteristic of mature oligodendrocytes. Some GFP-positive PRP-derived cells expressed the astrocyte-specific marker GFAP (Figure 2.3 D), although the percentage of PRP-derived astrocytes was quite low (8.9±2.2%). No transplanted PRP-derived cells displayed a neuronal phenotype, as there was no co-labelling of GFP with the neuron-specific marker NeuN. PRP-derived cells contributed to the glia scar, as astrocytes and as NG2 positive cells (Figure 2.4). PRPs also appeared to migrate following their transplantation (Figure 2.5), although the extent of migration is confounded by the fact that some of the rostral-caudal spreading could result from the initial injection of the cells.

We found that only those PRP-derived cells in close proximity to axons differentiated into mature oligodendrocytes. MBP interacts with the negatively charged plasma membrane surface to increase lipid compaction of the oligodendrocyte plasma membrane (Fitzner et al. 2006; Simons and Trotter 2007). As such, MBP plays an essential role in the compaction of the myelin sheath (Privat et al. 1979; Roach et al. 1983) and is expressed in both mature and immature myelin sheaths. PRP-derived oligodendrocytes extended GFP-positive processes and ensheathed axons surrounded by thin layers of MBP expression. In both longitudinal (Figure 2.6 B-G) and cross sectional tissue samples (Figure 2.6 H-K) we found evidence of a close

association (Figure 2.6 B-F, H-J, L, M) between PRP-derived GFP and MBP, indicating that PRP-derived cells produce myelin proteins. We used deconvolution and 3D rotation, to confirm that MBP was limited to the outside of the axonal ensheathment, with GFP labelling between the MBP and the adjacent axon, indicating that MBP is produced by PRP-derived cells (Figure 2.6 C). PRP-derived cells that ensheathe axons and produce myelin markers around axons also express the mature oligodendrocyte marker CC1 (Figure 2.6 L,M), giving further evidence that PRP-derived cells mature into myelin-producing oligodendrocytes. Many axons that were ensheathed by a thick GFP process were associated with thin MBP-positive sheaths (Figure 2.6 D-I, arrows), which may be interpreted as evidence that these particular sheaths were still immature. During compaction the majority of the cytoplasm is removed, along with our cytoplasmic marker GFP, leaving the myelin sheath nearly void of GFP-labelling and merely surrounded by a thin GFP-positive cytoplasmic layer with a GFP-positive stalk connecting it to the oligodendrocyte cell body (Figure 2.6 N,O). We encountered some thicker MBP-positive myelin sheaths that were in close association with a thin GFP-positive process, likely representing mature myelin produced by PRP-derived cells (Figure 2.6 H-J). However, the majority of the axons that were surrounded by thicker MBP immunoreactivity were GFP-negative, representing either spared myelin or mature myelin made by endogenous remyelinating cells. Hence, the patterns of GFP, CC1, and MBP co-labelling observed, suggest that transplanted PRP-derived cells differentiate into mature, myelinating oligodendrocytes in the injured spinal cord.

One characteristic of abnormal myelin is the spreading of paranodal and juxtaparanodal molecules from the Ranvier node along the axon (Arroyo et al. 2002). This pathological expansion of paranodal and juxtaparanodal ion channels and adhesion molecules is observed following demyelination after SCI (Karimi-Abdolrezaee et al. 2004; Lasiene et al. 2008). As remyelination proceeds and myelin matures, the normal (i.e., compact) pattern of expression of the paranodal and juxtaparanodal molecular architecture (e.g., Caspr and Kv1.2, respectively) returns (Eftekharpour et al. 2007; Lasiene et al. 2008), thus providing further evidence for the maturation and functional state of the myelin sheath around a given axon. We found that GFP-positive PRP processes were associated with, and ensheathed axons containing compact paranodal Caspr (Figure 2.7 A-M) and juxtaparanodal Kv1.2 (Figure 2.7 A-F). GFP-positive processes originate from oligodendrocytes, as indicated by the co-expression of GFP and CC1 (Figure 2.7 G-M). This provides further evidence that the transplanted PRP-derived oligodendrocytes were producing mature myelin as well as indicating that PRP-derived cells contribute to the restoration of the paranodal and juxtaparanodal molecular architecture.

To demonstrate the ability of PRP-derived cells to produce mature, compact myelin, PRPs were transplanted into Shiverer mice (Bird et al. 1978; Chernoff 1981; Privat et al. 1979). These animals carry a mutant MBP gene resulting in major hypomyelination; i.e., sheaths consisting of a few loose cytoplasmic wrappings but no compact myelin. This model has been used extensively to determine the myelinating potential of transplanted candidate cells, as any compact myelin sheaths present must be produced by donor cells (Archer et al. 1994; Eftekharpour et al. 2007; Gansmuller et al. 1986; Liu et al. 2000; Maire et al. 2009; Mothe and

Tator 2008; Nistor et al. 2005; Vitry et al. 2001; Warrington et al. 1993; Windrem et al. 2004; Windrem et al. 2008). Thus, we transplanted PRPs into the intact spinal cord of 3-week-old Shiverer mice and euthanized the animals 4 weeks later. We observed GFP in the intact spinal cords in all three animals using lower power fluorescent light microscopy. Myelin sheaths were observed in semi-thin (~0.5 μm) sections of these transplanted Shiverer mice stained with toluidine blue, a basic and lipophilic stain (Figure 2.8 A-D). These myelin sheaths were present throughout the ventral lateral funiculus, a region that coincided with the location where GFP was observed in the tissue blocks of this particular animal (data not shown). Many compact myelin sheaths were found by electron microscopy of thin sections (~0.1 μm) stained with uranyl acetate and lead citrate (Figure 2.8 E-G). These myelin sheaths had normal spacing and periodicity, with appropriately arranged main period lines and inter-period lines. Taken together our data indicate that transplanted PRPs differentiated into oligodendrocytes that produced mature myelin in the injured rat spinal cord and compact myelin sheaths in Shiverer mice.

2.3.2 Transplantation of PRPs did not increase the amount of myelinated axons or functional recovery following SCI

Other studies have shown that transplanting cells with the capacity to produce myelinating oligodendrocytes following SCI results in an increase in global myelination as well as improvement in functional recovery (Cao et al. 2010; Hofstetter et al. 2005; Karimi-Abdolrezaee et al. 2006; Keirstead et al. 2005). Accordingly, we sought to determine whether transplantation of cells capable of myelination would increase the amount of white matter or improve functional recovery. To test this we transplanted PRPs into animals 7 days after SCI. In

order to maximize PRP survival all animals were given the immunosuppressant cyclosporine A (first 2 weeks: 15 mg/kg, s.c.; orally 150 mg/L of drinking water thereafter) as well as the neuroprotective agent minocycline (20mg minocycline/g dried food) (Zhang et al. 2003). Following transplantation, the number of PRPs in the spinal cord dropped to half (Figure 2.9 A) between 2 and 14 days, but remained constant thereafter with an average of 62,569 GFP+ PRP cells at 8 weeks post transplantation. That number of grafted PRPs is comparable to or higher than the graft survival shown in other cell transplantation studies after SCI (Hofstetter et al. 2005; Karimi-Abdolrezaee et al. 2006; Parr et al. 2007; Parr et al. 2008; Tarasenko et al. 2007). We also found a low level of proliferation (<1%) at 56 days post–transplantation, as indicated by PRP expression of the cell proliferation marker Ki-67 (Figure 2.10).

In an attempt to improve survival/proliferation of transplanted PRPs, we co-injected the cells with trophic factors (312ng/µl FGF-2 and 62.5ng/µL PDGF-AA). Following the example of Karimi-Abdolrezaee and colleagues, who reported significant numbers of transplanted cells myelinating in the injured spinal cord after transplanting adult neural stem cells infused with a trophic factor cocktail of PDGF, FGF-2 and EGF (2006), we choose PDGF-AA and FGF-2 because PRPs are known to be responsive to those factors, but left out the EGF because E14 PRPs do not express the EGF receptor *in vivo* (Chojnacki and Weiss 2004). As expected, PRPs transplanted with trophic factors showed enhanced survival with no drop in the number of cells present at 14, or even 56 days post-transplantation (Figure 2.11). This increase in the number of GFP-labeled cells was not associated with an improvement in behavioural recovery and did not induce mechanical or thermal allodynia (Figure 2.11). However, PRP transplantation with

trophic factors induced hyperplasia in 2 out of 5 animals, widely in one animal and in focal regions in the second animal (Figure 2.12). Hyperplasia was never observed with PRP transplantation alone (>15 animals).

To assess that amount of total myelin, coronally sectioned tissue from PRP transplanted animals and their media injected controls were stained with Luxol fast blue. The area of white matter was measured at 400μm intervals rostral and caudal to the lesion epicenter (Figure 2.9 B). This analysis demonstrated that there was no significant difference in total white matter comparing transplanted to media injected control animals at any measured anatomical location. To complement this analysis, we also measured the number of myelinated and unmyelinated axons at the lesion epicenter using confocal microscopy (Figure 2.9 C-G). We found no differences in the number of myelinated or unmyelinated axons in the PRP transplanted animals compared to the media control group. This indicates that PRP transplantation did not increase the amount of overall myelination following SCI.

To measure behavioural improvement in rats following SCI, animals were subjected to both open field assessments with the BBB scale (Figure 2.13 A), as well as quantitative assessments using the CatWalk (Figure 2.13 B-D). In the open field, a clear deficit was apparent in motor function that was nearly identical between treatment groups at 2 and 6 days post-injury, indicating a consistent degree of injury between the groups. Both groups showed some functional recovery, eventually regaining weight supported stepping with some degree of forelimb-hindlimb coordination, but PRP transplantation did not improve functional recovery beyond that observed in the media injected controls. The CatWalk was also used to study functional recovery

following SCI as it provides a quantitative assessment of numerous gait parameters. Despite analysis of several different parameters, including hindpaw base of support (Figure 2.13 B), hindpaw stride length (Figure 2.13 C), and regularity index (Figure 2.13 D), the CatWalk data did not reveal any significant differences between the treatment and control groups.

In our initial long-term behavioural experiment outlined above, we chose to use male rats in order to reflect the fact that the male to female ratio for human SCI worldwide is approximately 4 to 1 (Wyndaele and Wyndaele 2006). We felt this was an important decision in light of growing evidence that sex differences may differentially affect the processes of repair and functional recovery from SCI, and therefore the outcomes of transplantation therapies. To examine whether females would respond better to PRP treatment than males, we transplanted PRPs into young female rats 12 days post-injury and assessed locomotor recovery using the open field (Figure 2.13E) and CatWalk measures (Figure 2.13F). Similar to our results using male rats, there was no significant improvement in functional recovery (compared to media injected control animals) after PRP transplantation in contused female rats on either the BBB or CatWalk assessments.

2.3.3 Transplantation of PRPs did not induce mechanical or thermal allodynia

In previous work, neural stem/progenitor cell transplantation following SCI resulted in aberrant axonal sprouting and forepaw allodynia; i.e., a pain response to normally non-noxious stimuli (Hofstetter et al. 2005; Macias et al. 2006). Accordingly, we tested whether transplantation of PRPs promoted allodynia. PRP-transplanted animals and their media injected

controls were subjected to thermal and mechanical sensitivity measurements of both hindpaws and forepaws, using infrared heat and an automated von Frey hair device (Figure 2.14). There was no decline in withdrawal latency (i.e. the time for an animal to remove their paw in response to stimuli) for either mechanical or thermal stimuli in forepaws or hindpaws up to 8 weeks post-injury, indicating that the transplantation of PRPs did not induce the lowered thermal or mechanical sensory thresholds indicative of allodynia that is commonly associated with enhanced pain.

2.4 Discussion

Cell transplantation with the intent to repair myelin is an exciting clinical possibility and clinical trials have been initiated with human embryonic stem cell-derived oligodendrocytes (Geron, Menlo Park, CA). However, the ideal source of cells for clinical translation still needs to be determined. PRPs are a population of cells with a very low risk of producing teratomas following transplantation since PRPs are not derived from pluripotent embryonic stem cells. Moreover, unlike the more commonly used EGF/FGF-responsive precursors used in neural stem/progenitor cell (NSPC) transplants, human PRPs retain the ability to produce large numbers of oligodendrocytes *in vitro*, whether isolated from adult or fetal tissue. As such, PRPs represent a source of myelinating CNS precursor cells that would be available from multiple sources for clinical application. Here, we found strong evidence that transplanted PRPs were able to integrate into host tissue, make contact with denuded axons and produce compact myelin.

Following their transplantation into the injured spinal cord, PRPs integrated into host tissue and differentiated into oligodendrocytes that produced mature myelin, demonstrating their suitability as a candidate cell to replace lost oligodendrocytes post-SCI. To better assess the capacity of PRPs to produce compact myelin, we transplanted PRPs into Shiverer mice and confirmed the generation of myelin with normal periodicity using electron microscopy. Despite this capacity to remyelinate, PRP transplantation did not increase the total myelin content in the injured spinal cord nor improve locomotor recovery compared to media injected control animals.

2.4.1 PRP transplantation and behavioural recovery

Several studies to date have transplanted a progenitor/precursor cell with the capacity to myelinate following SCI and found evidence of remyelination as well as some degree of functional behavioural improvement (Cao et al. 2005; Cummings et al. 2005; Hofstetter et al. 2005; Karimi-Abdolrezaee et al. 2006; Keirstead et al. 2005; Mitsui et al. 2005). Unlike these previous precursor studies, we did not see significant enhancement of functional recovery following the transplantation of a myelin-competent precursor, indicating that the transplantation of myelinating cells was not sufficient to improve locomotor recovery in our injury model.

Several other studies have demonstrated functional improvement following SCI though transplantation of progenitor/precursor cells and this often correlated with the capacity of the transplanted cells to integrate, differentiate and myelinate axons at sufficient numbers (Cao et al. 2005; Cummings et al. 2005; Hofstetter et al. 2005; Karimi-Abdolrezaee et al. 2006; Keirstead et al. 2005; Lee et al. 2005; Mitsui et al. 2005). Why then would the transplantation of PRPs not

also promote locomotor recovery following SCI? The transplanted PRPs did integrate well with host tissue and approximately one third differentiated into mature oligodendrocytes. This robust degree of differentiation was comparable to several other transplantation studies using adult neural precursor cells (Karimi-Abdolrezaee et al. 2006), genetically modified glial restricted precursors (Cao et al. 2005) and genetically modified adult neural progenitor/stem cells (Hofstetter et al. 2005), which all reported improved functional outcomes following transplantation. The latter two studies had to use genetic modification to attain similar levels of oligodendrocyte differentiation (Cao et al. 2005; Hofstetter et al. 2005), as those reported here with the PRPs without such modification. This suggests that the lack of enhanced functional recovery following PRP transplantation is not due to a lack of sufficient differentiation.

Regarding survival, we observed roughly 60,000 cells without co-injection of trophic factors and 100,000 cells with co-injection of trophic factors 56 days after transplantation. This number of transplant-derived cells is comparable or greater than that seen in other studies showing functional efficacy (Hofstetter et al. 2005; Karimi-Abdolrezaee et al. 2006; Parr et al. 2007; Parr et al. 2008; Tarasenko et al. 2007), indicating that a lack of survival of the PRPs is unlikely to account for the lack of improved locomotor recovery. We also found a comparable number of transplant-derived mature oligodendrocytes as other studies (Hofstetter et al. 2005; Karimi-Abdolrezaee et al. 2006), so the lack of behavioural efficacy for the PRPs cannot be explained by a lack of appropriate differentiation either. Finally, we demonstrated that transplanted PRP-derived cells produce mature compact myelin, just as other studies (Cao et al. 2005; Cummings et al. 2005; Karimi-Abdolrezaee et al. 2006) demonstrating significant

functional improvement. Despite all of the similarities in cell behaviour between this study and previous work transplanting progenitor cells, we have found no significant improvement in total myelin content or locomotor recovery, both of which were common findings in early work by other groups.

One explanation for the lack of ameliorated functional recovery in our study is that PRPs may be less competitive than the endogenous host cells with respect to myelination, particularly in comparison to the myelinating cells used in other studies. Following SCI there is a drastic loss of oligodendrocytes resulting in substantial loss of myelin (Crowe et al. 1997; Totoiu and Keirstead 2005). There is significant repair of myelin by natural, endogenous mechanisms during the first weeks following SCI, yet there are likely still a number of demyelinated axons at the time of transplantation (Lasiene et al. 2008; Totoiu and Keirstead 2005). The number of demyelinated axons is confounded by the presence of dystrophic axons that rarely remyelinate (Franklin and ffrench-Constant 2008; Lasiene et al. 2008) calling into question the degree of chronic demyelination. Thus, there are endogenous cells within the spinal cord that remyelinate axons following injury, and this endogenous response competes with the transplanted cells. Given that remyelination protects axons from degradation (Griffiths et al. 1998; Irvine and Blakemore 2006; Irvine and Blakemore 2008; Kassmann et al. 2007; Kornek et al. 2000; Lappe-Siefke et al. 2003; Nguyen et al. 2009; Totoiu et al. 2004; Wilkins et al. 2003), it is possible that a faster rate of remyelination can potentially spare more axons from secondary degeneration. As such, improved function might result from the transplantation of a cell that myelinates axons in a more competitive manner by remyelinating axons more rapidly than endogenous cells.

Alternatively, transplanted cells might differ in their ability to promote endogenous remyelination (Einstein et al. 2009). Hence, it is conceivable that PRPs failed to show functional efficacy because they do not myelinate as quickly as the cells used in other transplantation studies or because they fail to enhance endogenous remyelination.

The fact that the transplantation of a myelinating cell failed to provide functional recovery in the present study may carry interesting implications for the nature of chronic demyelination in our model of SCI. If chronically demyelinated axons exist and the remyelination of those axons fails due to a deficit in the endogenous OPCs, then the addition of exogenous precursors that myelinate would be expected to improve total myelin content, and presumably behavioural performance. Given that we did not find significant increases in total myelin or behavioural recovery after PRP treatment, one may speculate that chronic demyelination is not an issue with our injury model (at least in the timeframe of the present experiment). This would imply that endogenous precursors provide a sufficient level of spontaneous remyelination in our injury model. An alternative possibility is that the same extrinsic factors that block remyelination by endogenous precursors may also block the remyelination by transplanted PRPs or their progeny. There are many known factors that impair myelination, including: LINGO (Mi et al. 2007; Mi et al. 2004; Mi et al. 2005), BMPs (Cheng et al. 2007; Gao et al. 2006), Notch ligands (Givogri et al. 2002; Wang et al. 1998), and even myelin itself (Baer et al. 2009; Kotter et al. 2006). As many of these factors are present in the spinal cord after injury, it is likely that transplanted PRPs would be exposed to such signals. Given that the majority of these factors are thought to inhibit the early stages of oligodendrocyte

development, the PRPs may respond to these signals as an endogenous OPC would, and therefore simply fail to mature into myelinating oligodendrocytes.

There could also be species-specific differences in the ability of precursor cells to differentiate and produce myelin efficiently, particularly in the context of the injured rat spinal cord. According to that logic, one might expect mouse cells to be less efficient than endogenous OPCs at remyelinating the injured rat spinal cord. Speaking to this notion, a recent systematic review of cellular therapies for SCI summarized 50 studies testing cell transplantation of neural, embryonic, or glial restricted precursors following traumatic SCI(Tetzlaff et al. 2011). Of those 22 studies, only two involved the transplantation of mouse fetal precursor/ progenitor cells into rats, and in both of those studies, the transplanted mouse NSC alone failed to elicit improvements in behavioural recovery (Macias et al. 2006; Teng et al. 2002), despite the ability of those cells to myelinate following transplantation in Shiverer mice (Yandava et al. 1999). In addition to species-specific differences, the age at which precursor cells are isolated may also play a role in their ability to myelinate, as Windrem et al. (2004) demonstrated that human fetal glial progenitor cells are much less efficient at myelinating than cells from an adult source(Windrem et al. 2004). Both of these factors may have contributed to limiting myelination by transplanted cells in the present study, as PRPs were derived from fetal mice, and transplanted into injured adult rats. Given that endogenous OPCs are known to be present around lesion sites (Tripathi and McTigue 2007) and throughout the parenchyma (Horner et al. 2000; McTigue et al. 2001) following SCI in the rat, our transplanted fetal mouse PRPs would have been competing directly with adult rat OPCs to myelinate rat host axons. As such, our results could be interpreted

as evidence that fetal mouse precursors are less efficient at myelination than endogenous adult precursors in the injured rat spinal cord.

Alternatively, differences in neuroanatomical recovery in transplantation studies could be the result of other attributes of the transplanted cells and not necessarily their ability to myelinate. For instance, the neural precursors cells that promote improved behavioural recovery used in other transplantation studies were derived from EGF/FGF-responsive precursor cells (Hofstetter et al. 2005), embryonic stem cell derived oligodendrocyte precursor cells (Keirstead 2005) or glial restricted precursor cells (Cummings et al. 2005). As these cells are isolated from different populations of cells compared to PRPs, it would be expected that they also secrete a different subset of trophic factors. These trophic factors could be the mechanism by which the transplanted cells promote neuroanatomical and locomotor recovery following SCI. In this capacity, neural cells transplanted following SCI might improve locomotor recovery simply by acting as a neurotrophin pump. Previous work in at least one other lab supports this notion, as the transplantation of a glial restricted precursor was found to promote functional recovery only after it was genetically modified to produce a neurotrophin (Cao et al. 2005). Likewise, some of the benefits of embryonic stem cell derived oligodendrocyte precursor cells could be attributed to the neurotrophins that they secrete (Sharp et al. 2010; Zhang et al. 2006b)

The degree of immunomodulation could also vary between cell transplantation studies and partially account for differences in behavioural recovery. Transplantation of neural precursor cells into a model of multiple sclerosis, results in remyelination, similar to that seen in SCI (Pluchino et al. 2003). These neural precursor cells migrate to regions of pathology and induce

apoptosis of encephalitogenic T-cells, thus acting in a neuroprotective manner (Pluchino et al. 2005). In SCI, neural precursor cells have been demonstrated to act in conjunction with T-cells that are induced by CNS antigens (Ziv et al. 2006). Together, they dampen microglia activation, which is associated with decreased inflammation and smaller lesion size.

Spinal cord injury models could also account for differences observed in behavioural results among different transplantation studies. For instance, in rats there is an increase in the number and diffusion of demyelinated axons in a hemisection as compared to a contusion injury (Siegenthaler et al. 2007). This seems relevant to human SCI patients, as chronic cord compression appears to increase the amount of demyelination (Bunge et al. 1993). If that is the case in the rat, then it may explain the difference between our results transplanting PRPs after OSU contusion injuries and those found by others. The OSU impactor is a relatively fast contusion apparatus with no enduring compression. Given the visco-elastic properties of axons, the velocity of that injury may lead to more axonal damage (Sparrey et al. 2008) whereas the lack of compression may lead to less demyelination. In other words, our injury model may leave fewer intact, but denuded axons available for remyelination. In that case, one would expect less benefit from the transplantation of a myelinating precursor cell such as the PRPs.

2.4.2 Differentiation of PRPs following transplantation after SCI

In our study, PRPs were isolated from the embryonic day 14 anterior entopeduncular area, a time and region which corresponds to high expression of PDGFRα neural precursor cells (Chojnacki and Weiss 2004). At that time and location, the PRPs are a separate, non-overlapping

population of cells compared to the EGF-/FGF-responsive neural progenitor cells used in other transplantation studies (Chojnacki and Weiss 2004). With regard to their pattern of differentiation, PRPs either take on a parvalbumin-expressing interneuron phenotype or produce OPCs and their lineage cells. The differentiation of these two cell types can be altered in a mutually exclusive manner by the presence of thyroid hormone or BMP-2, respectively (Chojnacki and Weiss 2004). Alternatively, in the presence of BMP-2 and CNTF, PRPs can also take on an astrocytic fate. PRPs also possess a certain capacity for self-renewal when in a supportive environment. For instance, PDGF, FGF and Sonic Hedgehog in combination promote PRP self-renewal (Chojnacki and Weiss 2004). Taken together, this evidence indicates that *in vitro* PRPs are multipotential precursor cells capable of self-renewal.

In contrast to their *in vitro* potential, following their transplantation PRPs took on a more limited fate in the environment of the injured spinal cord. Thus, even though PRPs are capable of neuronal differentiation *in vitro*, they did not assume this fate following transplantation after SCI. This is consistent with other studies using the EGF-/FGF-responsive precursors, which are capable of producing neurons *in vitro*, yet only rarely express neuronal markers following transplantation post-SCI (Hofstetter et al. 2005; Parr et al. 2007; Parr et al. 2008; Setoguchi et al. 2004) unless genetically modified (Hofstetter et al. 2005). PDGFRα and NG2 are typical OPC markers that are expressed during early oligodendrocyte development and are downregulated during oligodendrocyte differentiation (Butt et al. 1997; Hall et al. 1996; Nishiyama et al. 1999). Accordingly, we found that roughly one-third of cells expressed the mature oligodendrocyte marker CC1 and another third expressed OPC markers. Hence, our data indicate that a majority

of transplanted PRP-derived cells express markers indicative of an oligodendrocyte lineage, similar to other transplantation studies (Cao et al. 2001; Hofstetter et al. 2005; Karimi-Abdolrezaee et al. 2006; Lowry et al. 2008; Parr et al. 2007; Parr et al. 2008).

2.4 Conclusion

Our study demonstrates that PRPs are a novel source of precursor cells that have the ability to integrate into host tissue and myelinate following transplantation into the injured spinal cord. Despite clear integration and myelination, the mouse-derived PRPs failed to promote functional recovery beyond that observed with media injection in the rat contusion model used. Future work should focus on delineating species-specific differences in cell behaviour in order to fully assess the potential of these cells for clinical translation following SCI. In that regard, it is important to note that human PRPs retain their ability to produce large numbers of oligodendrocytes *in vitro.* In conclusion, PRPs are a promising source of oligodendrocyte precursors for potential clinical application in cell replacement therapies, and thus warrant continued investigation using other models of demyelination related to both injury and disease.

Table 2.1 Summary of experimental details

Experiment	Groups	Group size	Endpoint	Transplantation location	Time of transplantation	Minocycline	Cyclosporine
Pilot study: PRP cell co-labelling	1. PRP transplant	n=4	10 weeks post injury	$1.0x10^6$ cells into lesion and $1.0x10^5$ cells into 4 sites Rostral/ Caudal	14 days post injury	Intraperitoneal injections (see methods)	Subcutaneous injections (15 mg/kg)
Short term experiment: Survival	1. PRP transplant	n=3	9 days post injury: 2 days post transplant	$1.0x10^5$ cells into 4 sites Rostral/ Caudal	7 days post injury	Added into food (20mg minocycline per gram of dried food) starting 2 days prior to transplantation and lasting until 5 days post transplantation	First 2 weeks following transplantation *via* subcutaneous injections (15 mg/kg) added to drinking water (150mg/L) thereafter
	2. PRP transplant with TF (Trophic Factors)	n=3					
	3. PRP transplant	n=3	4 weeks post injury: 2 weeks post transplant				
	4. PRP transplant with TF	n=3					
Long-term Experiment: behavioural and histological analysis in male rats	1. PRP transplant	n=12	9 weeks post injury	$1.0x10^5$ cells into 4 sites Rostral/ Caudal	7 days post injury	Added into food (20mg minocycline per gram of dried food) starting 2 days prior to transplantation and lasting until 5 days post transplantation	First 2 weeks following transplantation via subcutaneous injections (15 mg/kg) added to drinking water (150mg/L) thereafter
	2. Media injected	n=8					
	3. PRP transplant with TF	n=9					
	4. Media injected with TF	n=11					
Long-term Experiment: behavioural analysis in female rats	1. PRP transplant	n=16	14 weeks post injury	$8.0x10^4$ cells into 4 sites Rostral/ Caudal	12 days post injury	None given	First 2 weeks following transplantation via subcutaneous injections (15 mg/kg) added to drinking water (150mg/L) thereafter
	2. Media injected	n=11					

Figure 2.1 Passage 3 PRPs possessed a large capacity for oligodendroglial differentiation. Passage 3 PRP neurospheres were dissociated and cultured for 1 (A) or 3 (B) days *in vitro*. (A) After 24 hours of differentiation, 42% of the live cells stained for PDGFRα (red). The PDGFRα was largely internalized due to its activation by the PDGF ligand present in the medium during the expansion phase prior to dissociation. After 3 days of differentiation, 20% of the cells labeled for the immature oligodendrocyte marker O4 (red) (B). Scale bar is 25μm (A,B).

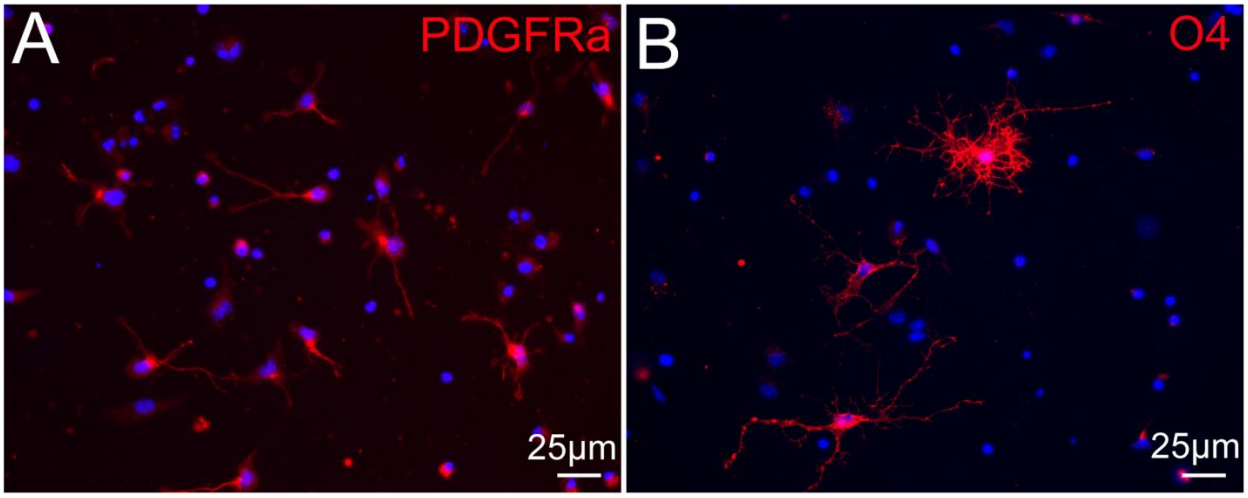


Figure 2.2 Transplanted PRPs surrounded the sub-acute spinal cord injury site and integrated well with host tissue. (A-C) Representative photomicrographs of a longitudinal section taken from an animal 8 weeks following transplantation of mouse PRPs demonstrated that some cells were found on the edge of the lesion cavity, but the majority of PRP-derived cells (green; GFP) integrated into host parenchyma. PRP-derived cells were found densely dispersed among axons (blue; NF200 and β-III tubulin) (A) in myelinated (red; MBP) white matter (B). Cells in close proximity to the lesion cavity are denoted by arrows (A,B). The arrowheads point to dispersed cells that possessed processes running parallel to axons (A). Scale bar is 125μm (A-C).

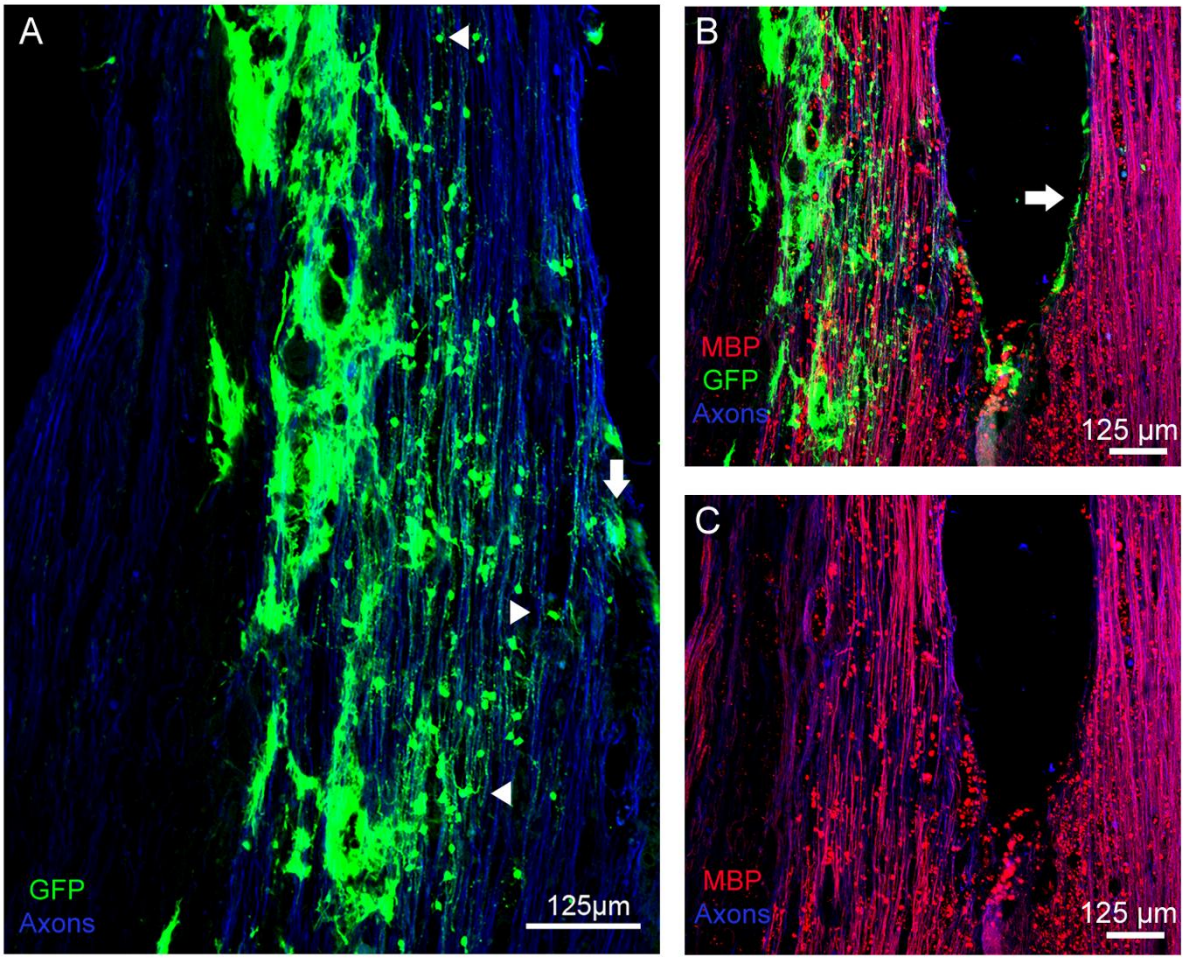
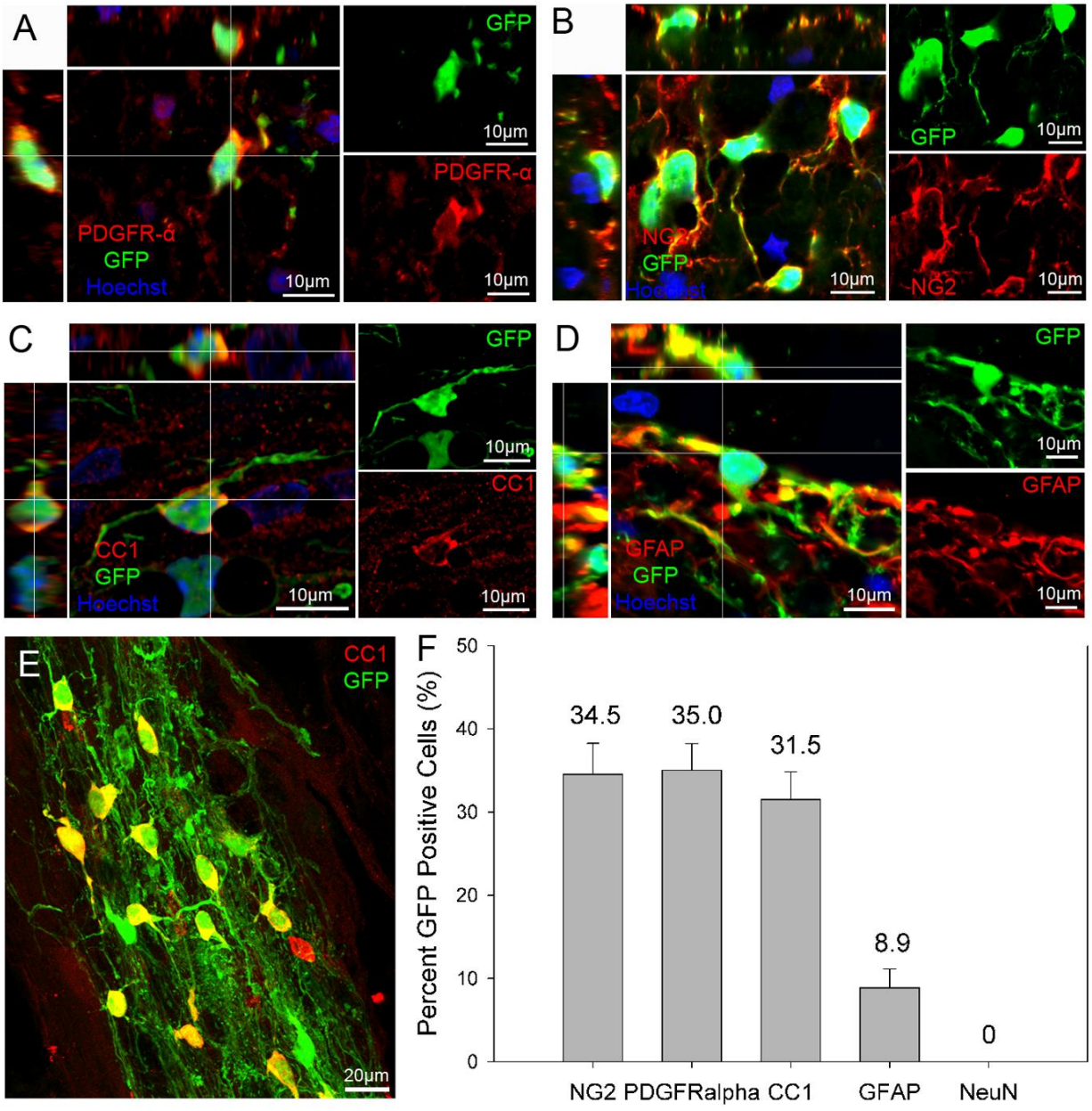

A
GFP
Axons
125μm
B
MBP
GFP
Axons
125 μm
C
MBP
Axons
125 μm

Figure 2.3 Transplanted PRP-derived cells displayed phenotypic markers of glial cells, predominantly of an oligodendrocytic fate. (A-E) Representative confocal images (with and without orthogonal views) taken from longitudinal sections of animals 8 weeks post transplantation immunostained for the following lineage restricted markers (red): PDGFR-α (A), NG2 (B), CC1 (C, E) and GFAP (D). Quantification of the percent of parenchymal GFP-positive PRP-derived cells (green) that co-express those markers indicated that roughly one third of transplanted cells in the parenchyma maintained a precursor fate (co-expression of GFP and PDGFR-α/NG2; A,B,F), whereas 31.5±3.3% became mature oligodendrocytes (co-expression of CC1 and GFP; C,E,F). A smaller proportion (8.9±2.2%) became astrocytic; as indicated by stellate morphology and the co-expression of GFAP and GFP (D,F). No transplanted PRP-derived cells were found to express the neuronal marker NeuN (image not shown; F). Scale bar is 10μm (A-D) 20μm (E) and error bars are standard deviation (F). Images obtained from both a Zeiss Axio-Observer equipped with a Yokogawa Spinning Disk Confocal Microscope (A-D) as well as a Nikon C1 Laser scanning confocal microscope (E).


A
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10μm
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CC1
CC1
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Hoechst
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10μm
D
GFP
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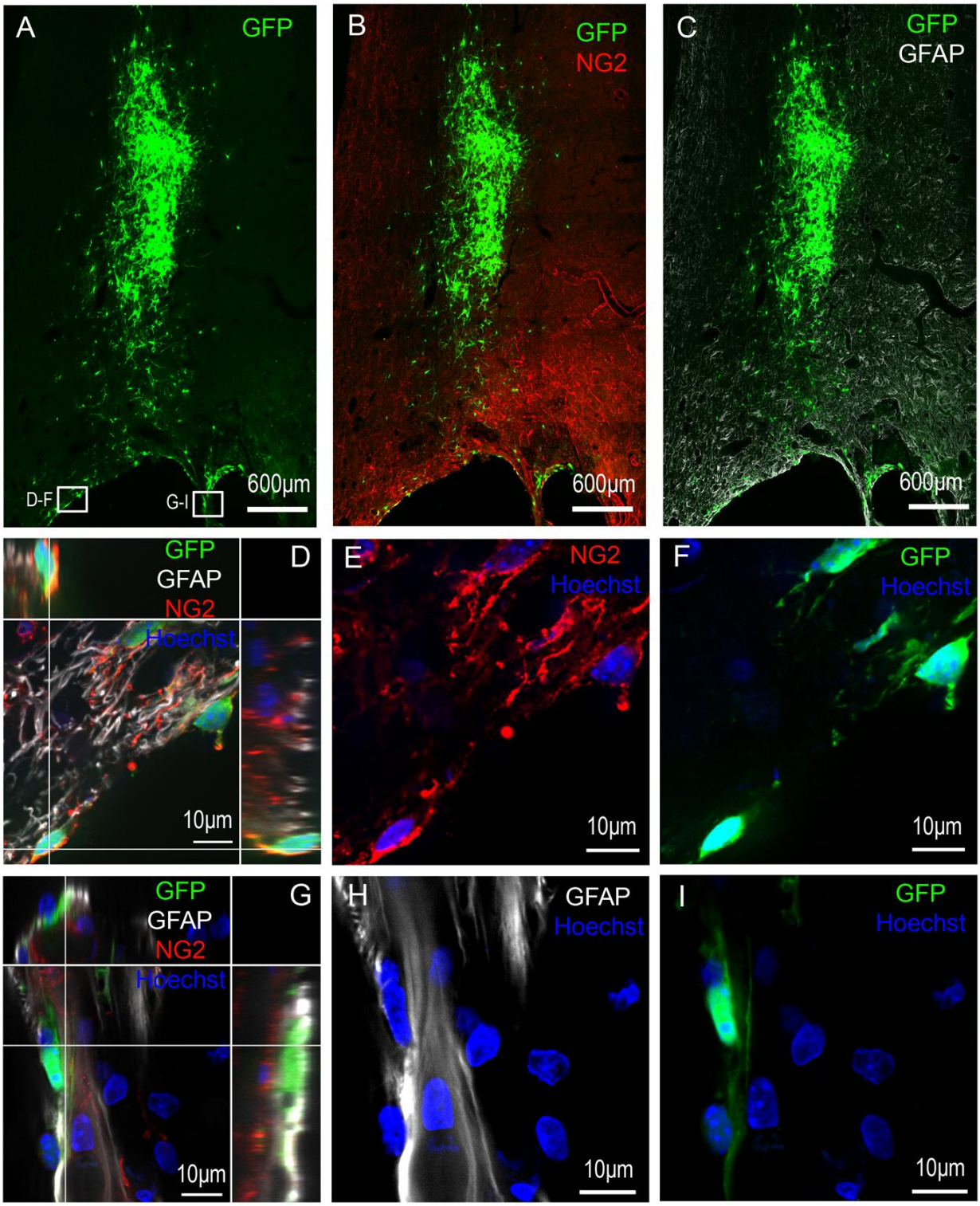
GFAP
GFAP
GFP
Hoechst
10μm
10μm
E
CC1
GFP
20μm
F
Percent GFP Positive Cells (%)
50
40
30
20
10
0
34.5
35.0
31.5
8.9
0
NG2
PDGFRalpha
CC1
GFAP
NeuN

Figure 2.4 Transplanted PRP-derived cells adjacent to the lesion site contributed to the glial scar and express either NG2 or GFAP. (A-C) PRP-derived cells (green; GFP) are located at the edge of the lesion epicentre indicated by a lack of NG2- positive cells (red) or astrocytes (GFAP; white). At higher magnification, PRP-derived cells adjacent to the lesion epicenter expressed either the precursor marker NG2 (red;D-F) or the astrocyte marker GFAP (white; G-I). Nuclei are labeled by Hoechst (blue; D-I). Images obtained from an animal that was 14 days post-transplantation. Scale bar is 600μm (A-C) 10μm (D-I).


A
GFP
D-F
G-I
600μm
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10μm

Figure 2.5 PRP-derived cells migrated following transplantation. (A) At 2 days post transplantation the extent of rostral-caudal spreading from the lesion site of PRP-derived cells (green; GFP) is at most 3016μm (section with greatest extent of rostral- caudal spreading in this group where n= 3). (B) At 14 days post transplantation, the extent of rostral-caudal spreading of PRP-derived cells is greater, and reached a maximum of 4390μm (n=3). Given the extent to which PRP-derived cells integrated into the parenchyma and the fact that the extent of rostral-caudal spreading is greater at 14 days as compared to 2 days post-transplantation, this provides evidence that PRP-derived cells migrated through host parenchyma. However, it must be acknowledged that our assessment of this migration is potentially confounded by spreading of the cells caused by the initial injection.

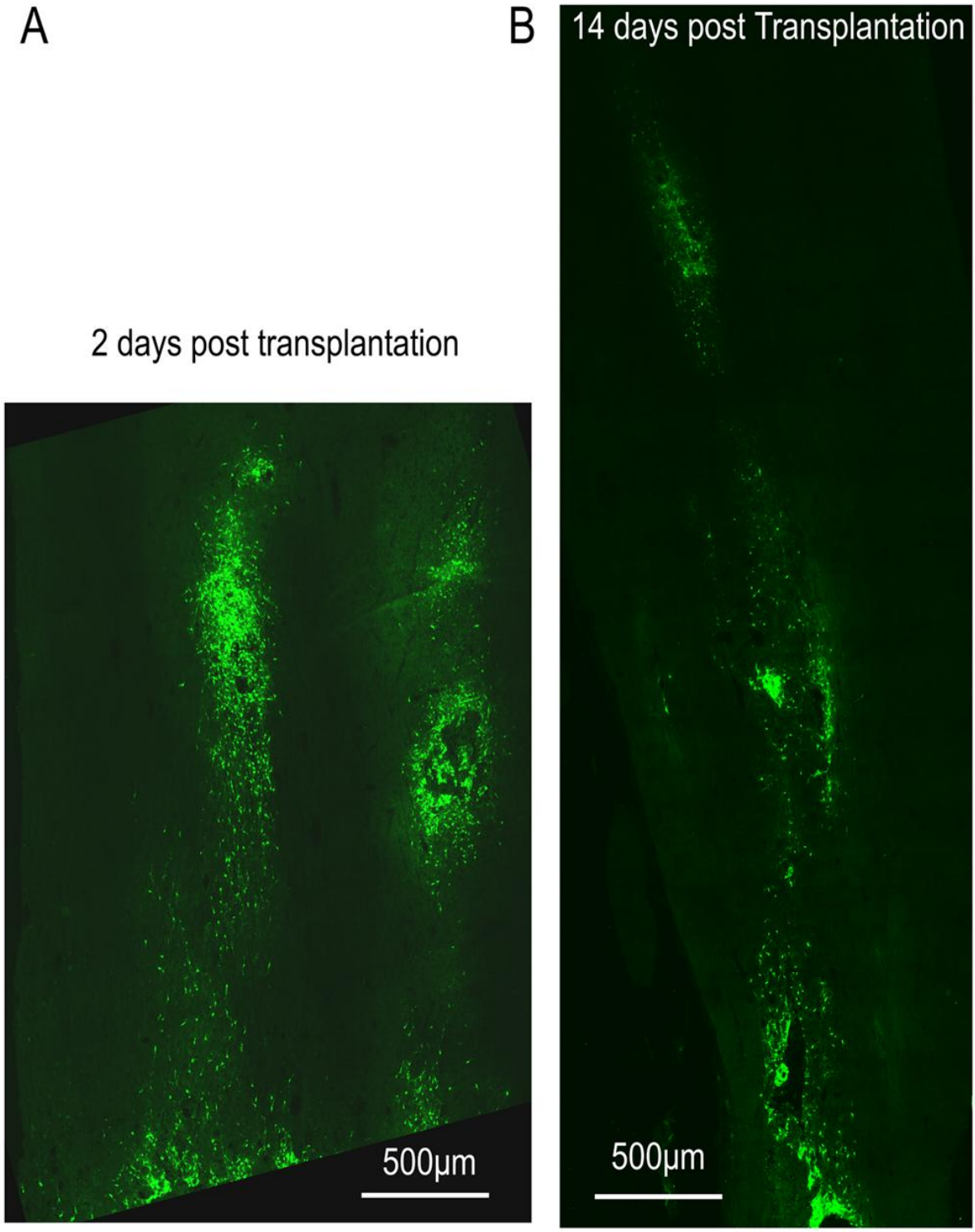

A
2 days post transplantation
500µm
B
14 days post Transplantation
500µm

Figure 2.6 Following PRP transplantation in the injured spinal cord, PRP-derived cells expressed mature oligodendrocyte markers and send out projections that encircle denuded axons and produce myelin. PRP-derived cells (green; GFP) closely associated with myelin (red; MBP) and ensheathed axons (blue; NF200 and β-III tubulin), as imaged from longitudinal (B-G,L-O) and cross sections (H-K). Arrows illustrate regions of close association between GFP and MBP (B, C, D, H). (A) Only those PRP-derived cells that were well integrated and located within parenchyma expressed MBP. (D-G) Representative orthogonal view confocal photomicrograph (B) illustrated in separate channels. (C) That same image was subjected to deconvolution and reorientation to demonstrate further the close association of GFP and MBP. (H-K) Cross sectional confocal photomicrographs depicting a projection from a PRP-derived cell ensheathing an axon and expressing MBP. (L,M) PRP-derived cells that ensheathed axons, expressed the mature oligodendrocyte marker CC1 (white); denoted by arrows. (N,O) At times PRP-derived mature oligodendrocytes contacted numerous myelinated axons, yet only the stalk of the cells associate with MBP; likely reflecting the loss of GFP from the PRP-derived myelin sheath during compaction. Scale bar is 33μm (A), 5μm (B,D-G) 2μm (C,H-K), 10μm (L-O). Images obtained from both a Quorum WaveFX Spinning Disc confocal microscope (A-G), a Nikon C1 Laser scanning confocal microscope (H-K) and a Zeiss Axio-Observer equipped with a Yokogawa Spinning Disk confocal microscope (L-O).

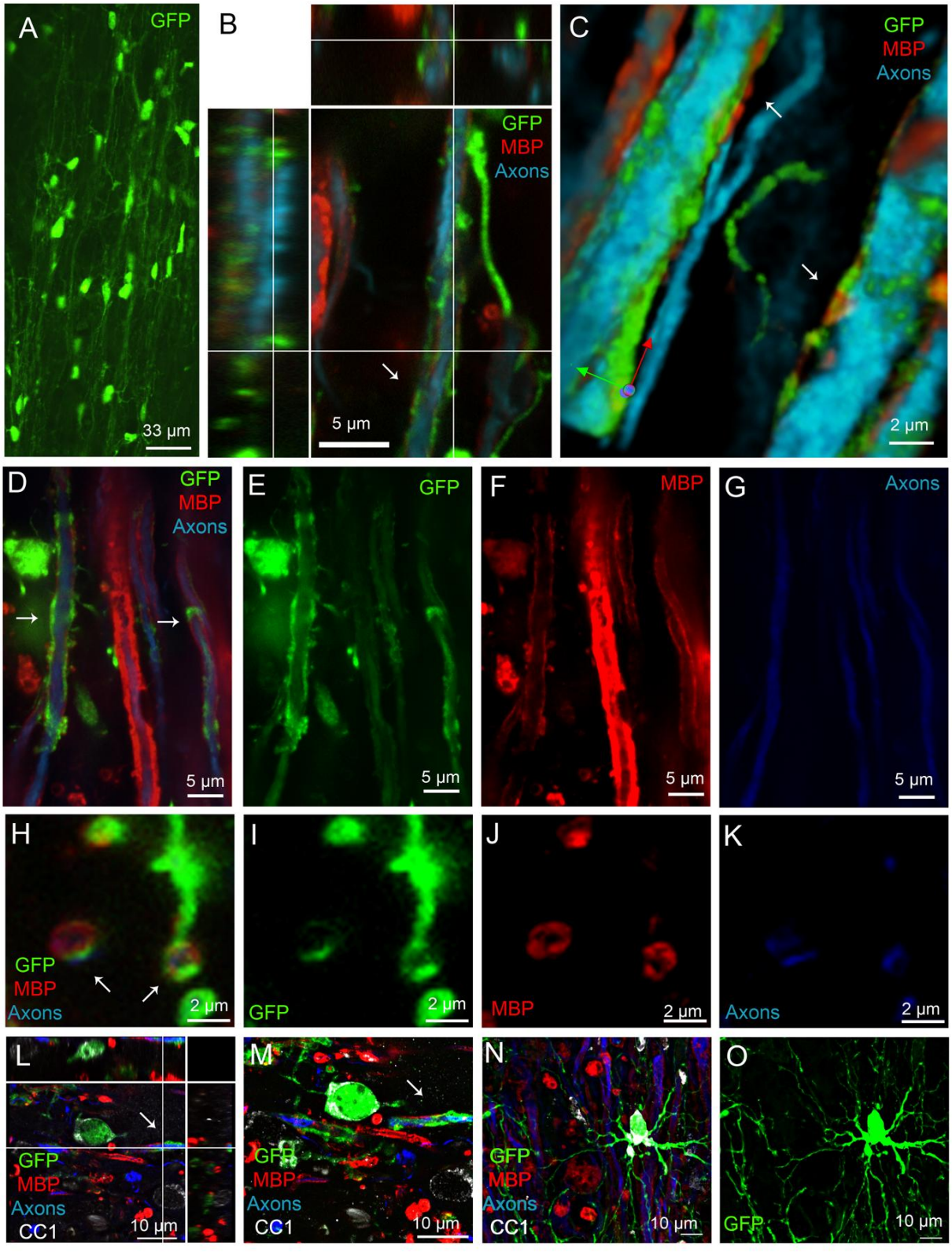

A
GFP
33 µm
B
GFP
MBP
Axons
5 µm
C
GFP
MBP
Axons
2 µm
D
GFP
MBP
Axons
5 µm
E
GFP
5 µm
F
MBP
5 µm
G
Axons
5 µm
H
GFP
MBP
Axons
2 µm
I
GFP
2 µm
J
MBP
2 µm
K
Axons
2 µm
L
GFP
MBP
Axons
CC1
10 µm
M
GFP
MBP
Axons
CC1
10 µm
N
GFP
MBP
Axons
CC1
10 µm
O
GFP
10 µm

Figure 2.7 Transplanted PRP-derived cells expressed mature oligodendrocyte markers ensheathed axons and induced non-pathological nodes of Ranvier. Demyelinated axons display dispersed paranodal (Caspr) and juxtaparanodal (Kv1.2) markers, which compacted following remyelination by PRP-derived cells. Confocal photomicrographs from longitudinal sections indicate that PRP-derived cells (green) were found to associate with compact Caspr (red; A-M) and Kv1.2 (white; A-F) expression along axons (blue; β-III tubulin/ NF-200; A-M) indicating that PRP-derived cells induce normal (i.e., non-pathological) nodes of Ranvier. PRP-derived cells that expressed the mature oligodendrocyte marker CC1 (white; G-M) ensheathed axons and associated with compact Caspr. (G-I) Maximum projection image demonstrates the extent to which each PRP made contact with numerous axons, including an axon that is further digitally magnified from a confocal slice (J-M). Arrow in G and J represent the same axon in both a maximum projection image (G) as well as in a thin optical confocal slice (J). Regions of interest from A and J were digitally magnified in B-F and K-M, respectively. Scale bar is 10μm (A,G-J) and 5μm (B-F,K-M).

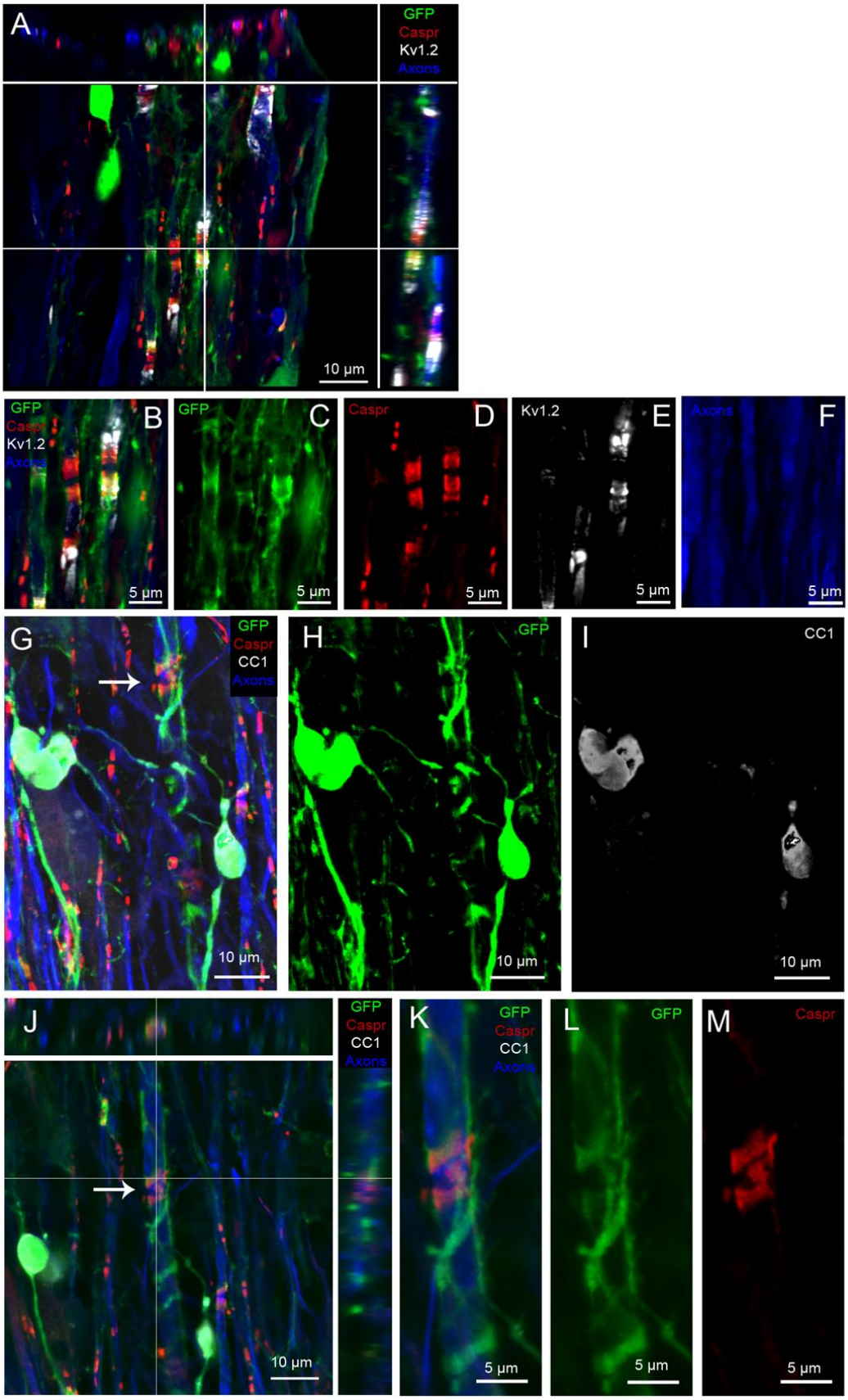

A
GFP
Caspr
Kv1.2
Axons
10 µm
B
GFP
Caspr
Kv1.2
Axons
5 µm
C
GFP
5 µm
D
Caspr
5 µm
E
Kv1.2
5 µm
F
Axons
5 µm
G
GFP
Caspr
CC1
Axons
10 µm
H
GFP
10 µm
I
CC1
10 µm
J
GFP
Caspr
CC1
Axons
10 µm
K
GFP
Caspr
CC1
Axons
5 µm
L
GFP
5 µm
M
Caspr
5 µm

Figure 2.8 PRPs transplanted into myelin deficient Shiverer mice integrated well, differentiated and produced compact myelin. (A-D) Representative light micrographs of semi-thin sections of Shiverer mouse spinal cord after PRP transplantation and staining with toluidine blue membrane marker. (A) The large area of densely stained dark rings of toluidine blue, indicative of myelin sheaths, was found to include much of the lateral portion of the ventral funiculus, and higher magnification images from medial (B) and lateral (C,D) areas confirmed the presence of more myelin structures laterally. (E-G) Representative electron photomicrographs of increasing magnification depicting thin sections of Shiverer mouse spinal cord transplanted with PRPs and stained with uranyl acetate and lead citrate to enhance contrast. These images provide definitive evidence that PRP-derived cells produced compact myelin in the Shiverer mouse spinal cord, as several examples of compact myelin are visible (E, F) and demonstrate regular periodicity (G). Scale bar is 100μm (A), 15μm (B-D), 1μm (E), 500nm (F), 50nm (G).

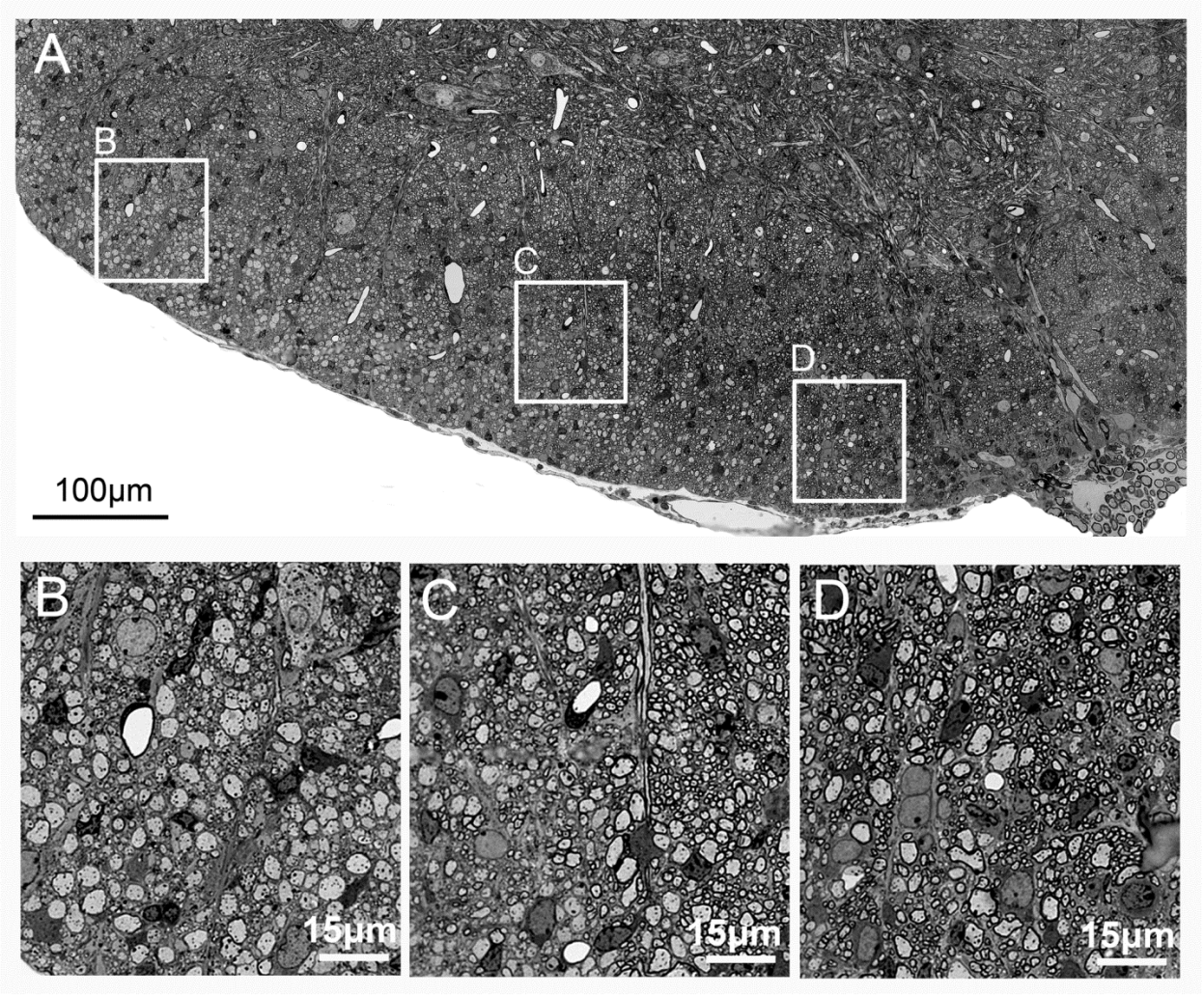

A
B
C
D
100μm
B
15μm
C
15μm
D
15μm
E
1μm
F
G
500nm
G
50nm

Figure 2.9 Total myelin content as well as the number of myelinated axons was not significantly improved by PRP transplantation. PRP-derived cells survived up to 56 days after transplant into the contused rat spinal cord, despite a significant loss of cells within the first 2 weeks post-transplantation. (A) Cell counts of transplanted PRP-derived cells conducted 2, 14 and 56 days post-transplantation demonstrated that the number of PRP-derived cells significantly drops between 2 and 14 days. The cell counts changed significantly over time (ANOVA) and were significantly lower at 14 and 56 days compared to 2 days (Fisher's LSD; $p<0.05$). (B) On average PRP-transplanted rats did not show an increase total myelin content compared to media injected control rats. (C-G) Confocal micrographs taken at the lesion epicenter were imaged at high power (C-F) and digitally enhanced (D, F) to measure the number of myelinated and unmyelinated axons. The PRP transplanted (C, D) and media controls (E, F) did not differ significantly in terms of the number of myelinated or unmyelinated axons at the injury epicenter (G). Error bars represent SEM. Scale bar is 10μm (C,E), 1μm (D,F).

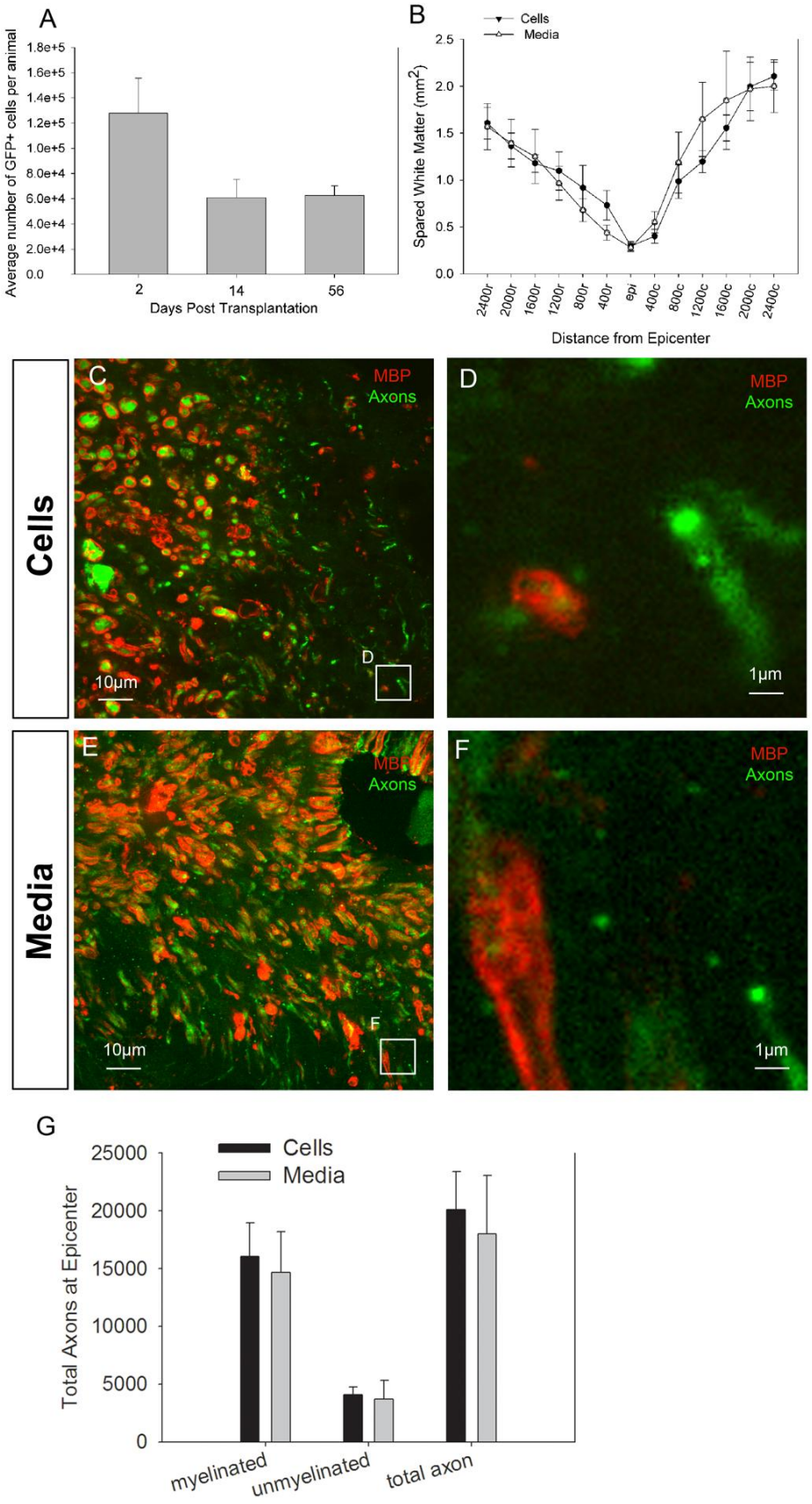

A
Average number of GFP+ cells per animal
1.8e+5
1.6e+5
1.4e+5
1.2e+5
1.0e+5
8.0e+4
6.0e+4
4.0e+4
2.0e+4
0.0
2
14
56
Days Post Transplantation
B
Cells
Media
Spared White Matter (mm2)
2.5
2.0
1.5
1.0
0.5
0.0
2400r
2000r
1600r
1200r
800r
400r
epi
400c
800c
1200c
1600c
2000c
2400c
Distance from Epicenter
Cells
C
MBP
Axons
D
10μm
D
MBP
Axons
1μm
Media
E
MBP
Axons
F
10μm
F
MBP
Axons
1μm
G
Cells
Media
Total Axons at Epicenter
25000
20000
15000
10000
5000
0
myelinated
unmyelinated
total axon

Figure 2.10 Transplanted PRPs continued to proliferate even 8 weeks following their transplantation. (A-D) PRP (green; GFP) co-labeled with the cell proliferation marker Ki-67 (red) and the nuclear marker Hoechst (blue). Scale bar is 40μm (A) and 10μm (B-D).

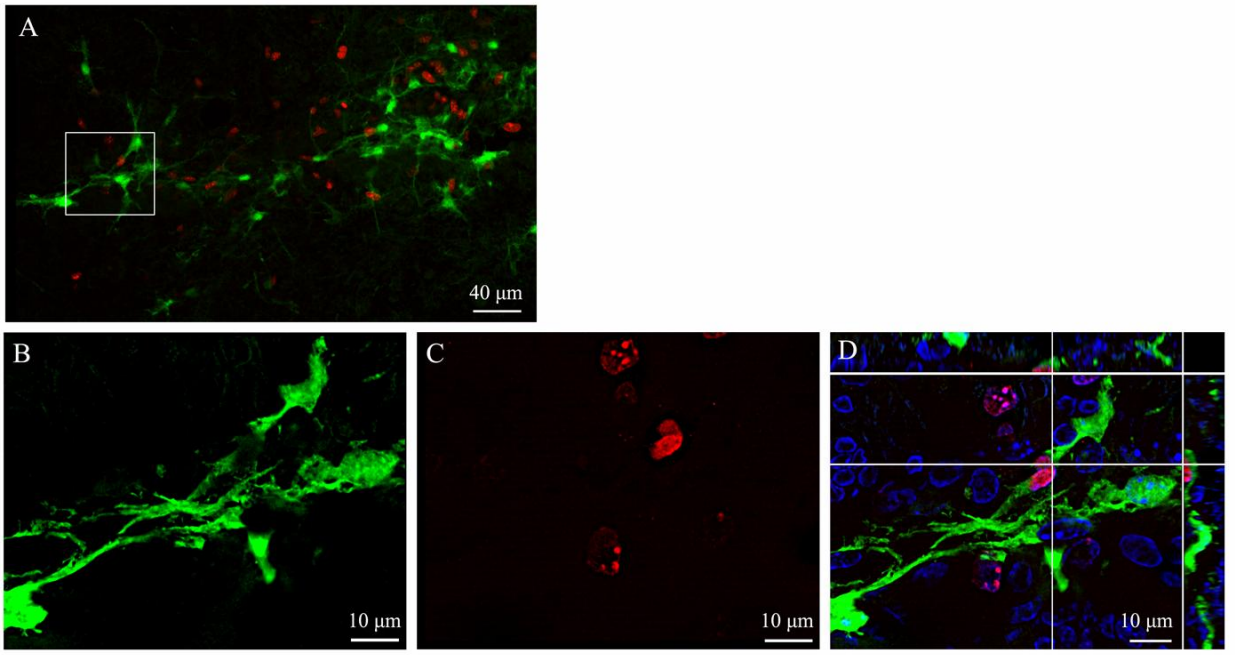


Figure 2.11 Transplantation of PRPs along with trophic factors into rats after contusive spinal cord injury increased the number of surviving transplanted cells, but did not improve behavioural recovery or induce mechanical or thermal allodynia in forepaws or hindpaws. In contrast to PRPs transplanted in the absence of trophic factors, cell counts demonstrate that the number of PRP-derived cells remained relatively constant over time when trophic factors were included with the cells (A). Despite that improved cell survival/proliferation, when compared to a media control group also treated with trophic factors, PRP transplantation with trophic support did not increase the amount of spared white mater (B), or the degree of hindlimb locomotor recovery on the BBB (C) or CatWalk (D; p=0.1, E). This combinatorial treatment did not induce mechanical (F, H) or thermal (G, I) allodynia in hindpaws (F,G) or forepaws (H, I).

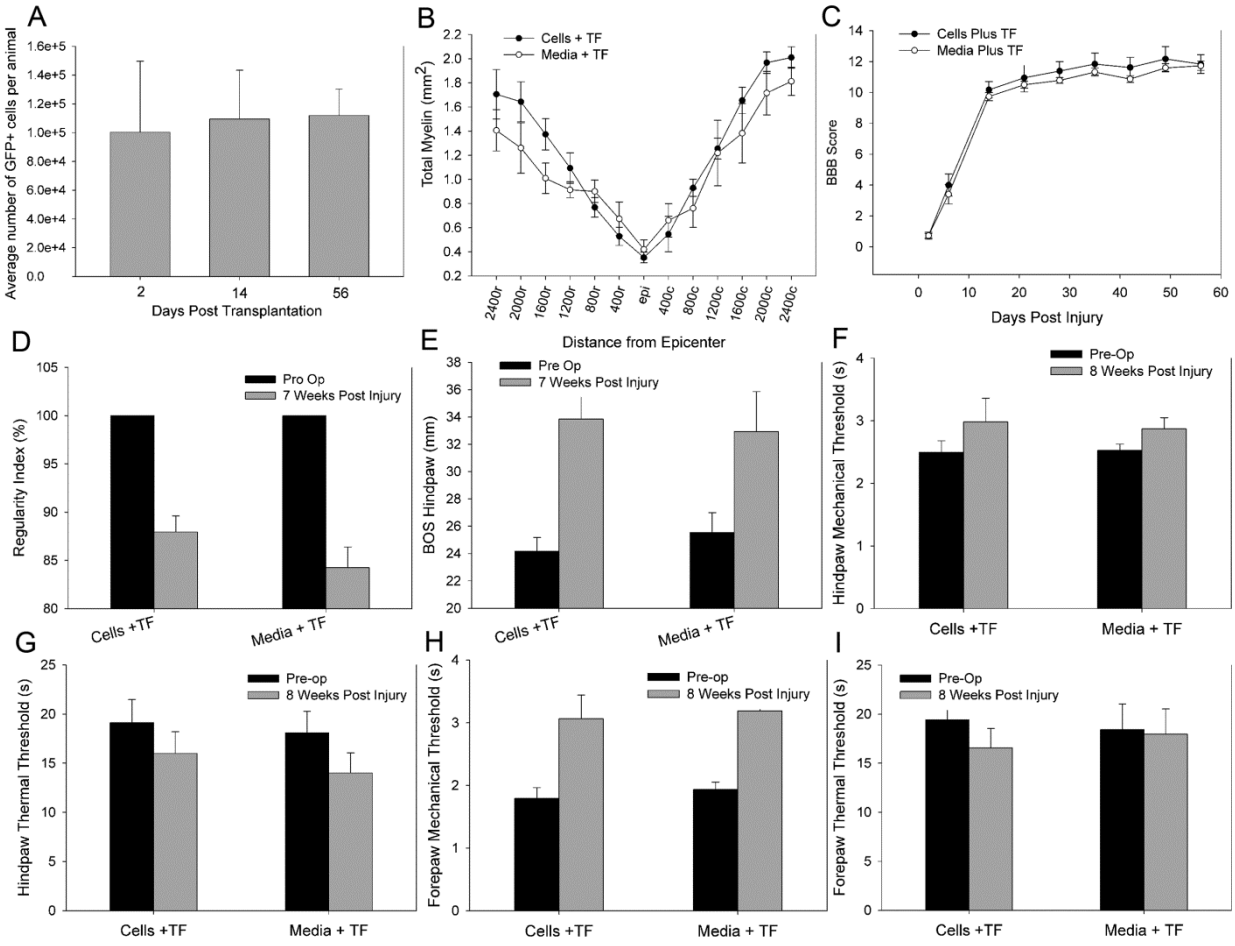

A
Average number of GFP+ cells per animal
1.6e+5
1.4e+5
1.2e+5
1.0e+5
8.0e+4
6.0e+4
4.0e+4
2.0e+4
0.0
2
14
56
Days Post Transplantation
B
Cells + TF
Media + TF
Total Myelin (mm2)
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
2400r
2000r
1600r
1200r
800r
400r
epi
400c
800c
1200c
1600c
2000c
2400c
C
Cells Plus TF
Media Plus TF
BBB Score
14
12
10
8
6
4
2
0
0
10
20
30
40
50
60
Days Post Injury
D
Pro Op
7 Weeks Post Injury
Regularity Index (%)
105
100
95
90
85
80
Cells +TF
Media + TF
E
Distance from Epicenter
Pre Op
7 Weeks Post Injury
BOS Hindpaw (mm)
38
36
34
32
30
28
26
24
22
20
Cells +TF
Media + TF
F
Pre-Op
8 Weeks Post Injury
Hindpaw Mechanical Threshold (s)
4
3
2
1
0
Cells +TF
Media + TF
G
Pre-op
8 Weeks Post Injury
Hindpaw Thermal Threshold (s)
25
20
15
10
5
0
Cells +TF
Media + TF
H
Pre-op
8 Weeks Post Injury
Forepaw Mechanical Threshold (s)
4
3
2
1
0
Cells +TF
Media + TF
I
Pre-Op
8 Weeks Post Injury
Forepaw Thermal Threshold (s)
25
20
15
10
5
0
Cells +TF
Media + TF

Figure 2.12 In some instances, transplantation of PRPs with trophic factors promoted hyperplastic growth. (A-G) Evidence for hyperplastic growth comes from an increased density of GFP-positive grafted cells (green), as well as the dense infiltration of host cells (Hoescht staining; blue; F,G) surrounding the regions with transplanted cells. Illustrative photomicrographs from longitudinal sections of the spinal cord of an animal treated with PRPs plus trophic factors. (A) The PRP-derived cells formed a dense graft area that bridged the lesion site in this particular case. However, such areas had atypical and deficient astrocytic cytoarchitecture and enhanced expression of the inhibitory chondrotin sulfate proteoglycan, neurocan (B,C). We also found the graft area to be partially encapsulated by laminin with several laminin tubes projecting through the graft area (D, E). Surrounding the graft site there was a greatly enhanced cell density (hyperplasia; F,G), that was never seen in proximity to grafted PRPs in the absence of trophic factors. Photomicrographs were taken at the lesion border (B,D,F) as well as at a proximal graft site (C,E,G). Scale bar is 250μm (A-G).

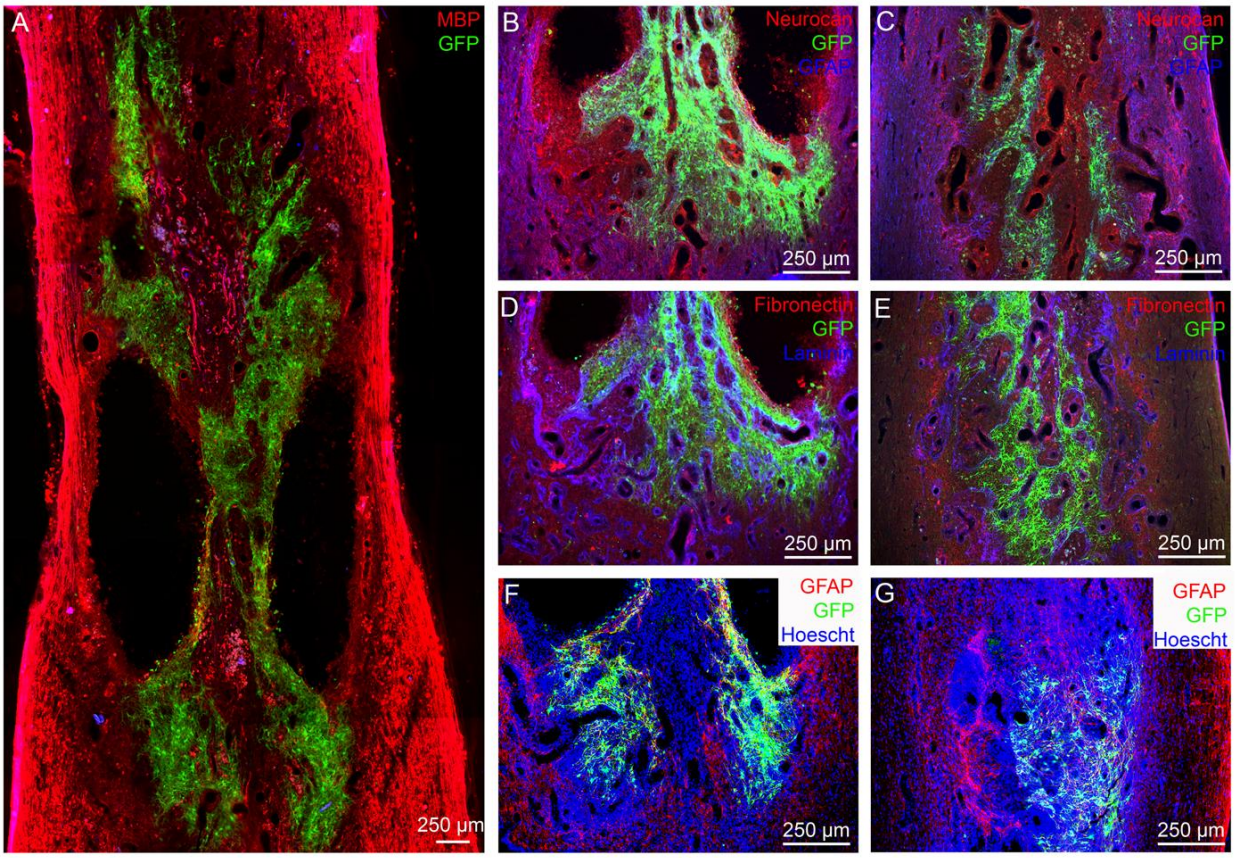

A
MBP
GFP
250 µm
B
Neurocan
GFP
GFAP
250 µm
C
Neurocan
GFP
GFAP
250 µm
D
Fibronectin
GFP
Laminin
250 µm
E
Fibronectin
GFP
Laminin
250 µm
F
GFAP
GFP
Hoescht
250 µm
G
GFAP
GFP
Hoescht
250 µm

Figure 2.13 Transplantation of PRPs into male (A-D) or female (E, F) rats after a contusive spinal cord injury, failed to improve behavioural recovery when compared to media injected controls. Open field testing (A) failed to show any significant differences between groups of male rats transplanted with PRPs versus media at 7 days post-injury. CatWalk assessment of hindpaw base of support (BOS; B; p=0.07), hindlimb stride length (C) and regularity index (D) also failed to demonstrate differences between PRP treated and control groups. In a separate study, female rats transplanted with PRPs following contusive spinal cord injury also failed to show any significant differences compared to media injected controls on open field locomotion (E; day56 p=0.05, not significant with Bonferroni correction) and CatWalk analysis of hindpaw BOS (F). All error bars represent SEM.

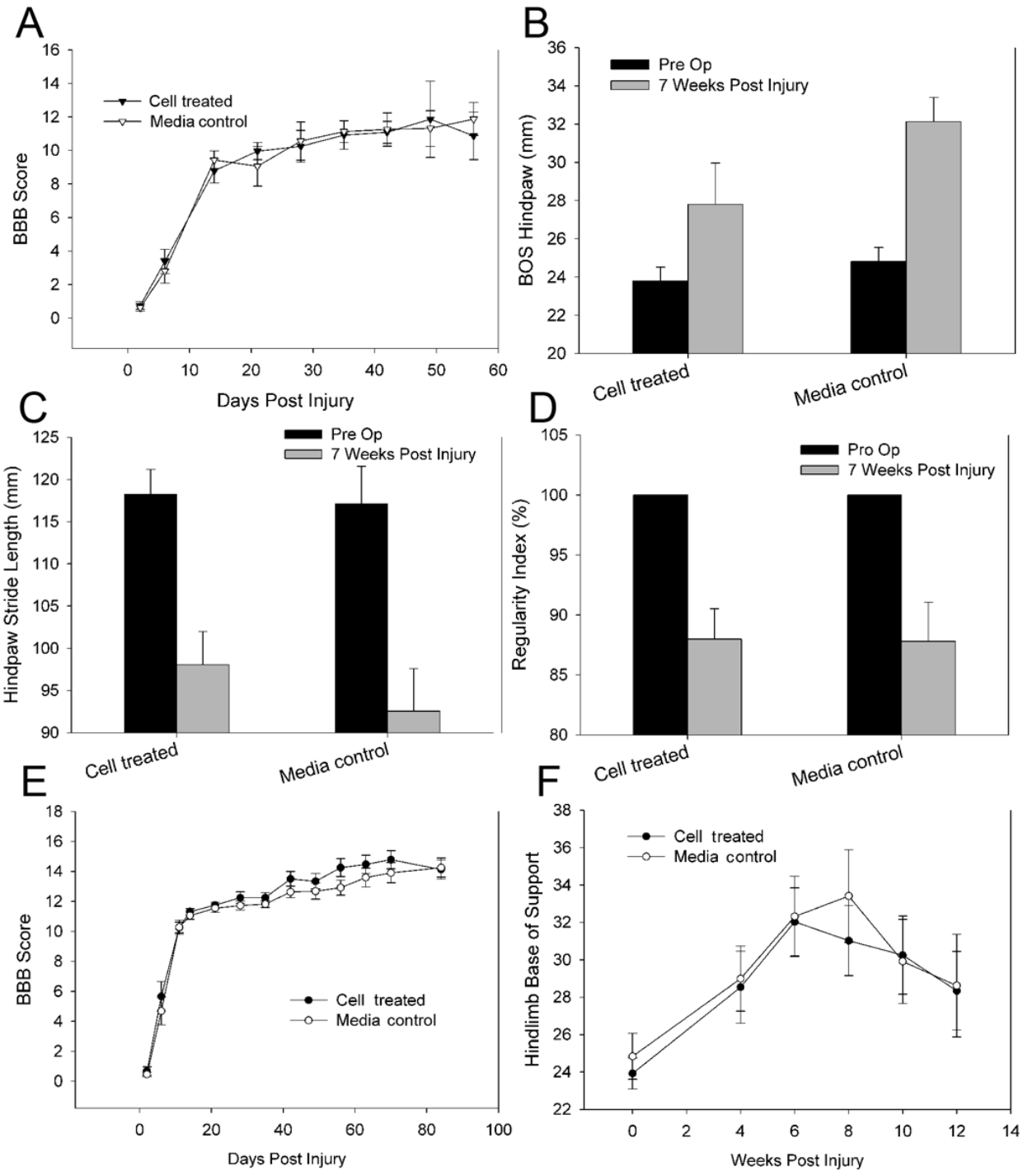
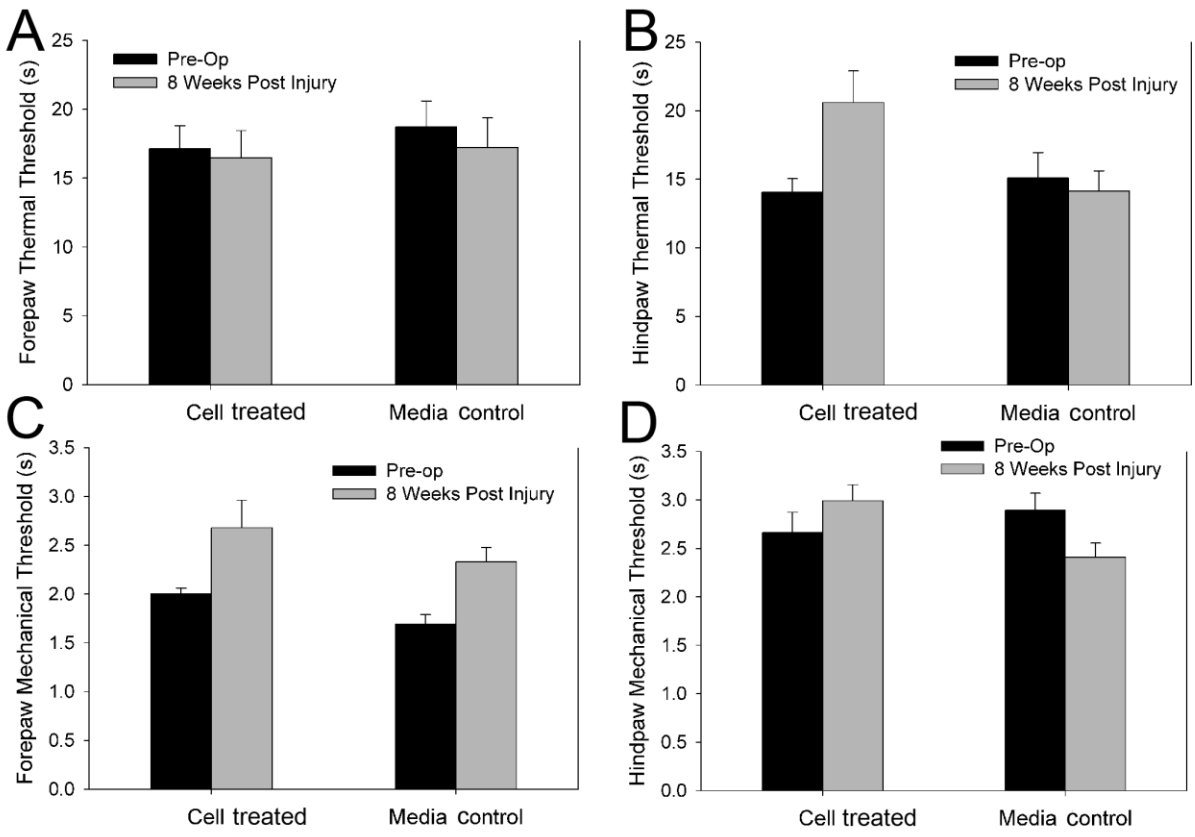

A
BBB Score
Days Post Injury
Cell treated
Media control
B
BOS Hindpaw (mm)
Pre Op
7 Weeks Post Injury
Cell treated
Media control
C
Hindpaw Stride Length (mm)
Pre Op
7 Weeks Post Injury
Cell treated
Media control
D
Regularity Index (%)
Pro Op
7 Weeks Post Injury
Cell treated
Media control
E
BBB Score
Days Post Injury
Cell treated
Media control
F
Hindlimb Base of Support
Weeks Post Injury
Cell treated
Media control

Figure 2.14 Transplantation of PRPs, into rats 7 days after a contusive spinal cord injury did not induce thermal (A,B) or mechanical (C, D) allodynia in forepaws (A, C) or hindpaws (B, D), as the PRP treated group showed withdrawal latencies (threshold in seconds) that were longer or not significantly different from pre-op measurements. All error bars are SEM.



Chapter 3

Myelin Inhibits Oligodendroglial Maturation by Increasing Transcription Factors in the Inhibitors of the DNA Binding Family

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[2] A version of this chapter has been submitted for publication. Plemel JR, Manesh S, Tetzlaff W. 2011. Myelin inhibits oligodendroglial maturation by increasing trascription factors in the inhibitor of DNA binding family.

3.1 Introduction

The hallmarks of multiple sclerosis (MS) are demyelination and degeneration of axons. Myelin is not only essential for rapid axonal conduction, but is also necessary for axonal support (Nave 2010b; Nave and Trapp 2008). For example, myelin proteins PLP or CNP are not necessary for the initial production of the myelin sheath, but in their absence there is axonal degeneration (Griffiths et al. 1998; Lappe-Siefke et al. 2003). Genetic ablation of oligodendrocytes produces axonal damage, indicating that oligodendrocyte support is vitally important for axons (Ghosh et al. 2011; Locatelli et al. 2012; Pohl et al. 2011; Traka et al. 2010). Similarly, blocking remyelination increases axonal degeneration (Irvine and Blakemore 2008). Aging is associated with a slower rate of remyelination (Ruckh et al. 2012; Shields et al. 1999; Sim et al. 2002) and this also correlates with more axonal loss following demyelination (Hampton et al. 2012; Irvine and Blakemore 2006). Thus, rapid and efficient remyelination is hypothesized to spare more axons and decrease the extent of axonal degeneration, which is thought to be the cause of permanent neurological disability in MS (Trapp and Nave 2008).

Remyelination is thought to largely recapitulate developmental myelination whereby endogenous oligodendrocyte precursor cells (OPCs) proliferate and are recruited to sites of demyelination and differentiate into myelinating oligodendrocytes (Fancy et al. 2011a). In humans with MS, remyelination often fails, resulting in chronic demyelinated lesions (Barkhof et al. 2003; Patrikios et al. 2006; Prineas and Connell 1979). The presence of OPCs at sites of chronic demyelination (Chang et al. 2000; Kuhlmann et al. 2008; Wolswijk 1998) indicates that there is a quiescent population of OPCs apparently unable to differentiate. Premyelinating oligodendrocytes can also be present at sites of chronic demyelination, suggesting that in certain

circumstances oligodendrocyte production occurs, but the maturation of premyelinating immature oligodendrocytes into myelinating oligodendrocytes fails (Chang et al. 2002). One potential explanation for the impaired OPC differentiation and/or maturation is the presence of inhibitory molecules at the site of demyelination. Several inhibitors of oligodendrocyte development have been discovered, some of which are present following demyelination. For instance hyaluronan accumulates in chronically demyelinated MS lesions and is known to inhibit oligodendrocyte lineage cell maturation as well as remyelination (Back et al. 2005; Sloane et al. 2010). Likewise, BMPs (Cheng et al. 2007; Mabie et al. 1997), Wnts (Azim and Butt 2011; Fancy et al. 2009; Fancy et al. 2011b; Feigenson et al. 2009; Ye et al. 2009), LINGO-1 (Mi et al. 2004; Mi et al. 2005) and PSA-NCAM (Charles et al. 2000; Fewou et al. 2007) can inhibit OPC development. The antagonism of many of these molecules *in vivo* increases remyelination (Fancy et al. 2009; Fancy et al. 2011b; Mi et al. 2007; Mi et al. 2009; Sabo et al. 2011). Myelin is present following demyelination and contact with myelin impairs the expression of immature oligodendrocyte markers *in vitro* (Baer et al. 2009; Robinson and Miller 1999; Syed et al. 2008). Addition of purified myelin after demyelination impairs spontaneous remyelination *in vivo* (Kotter et al. 2006). Hence, myelin debris removal could be an important component regulating remyelination. Interestingly, aging is associated with both an impairment in the phagocytocis of myelin debris (Zhao et al. 2006) as well as an impairment in remyelination (Hinks and Franklin 2000; Ruckh et al. 2012; Shen et al. 2008; Shields et al. 1999). Age-related decline in remyelination efficiency can be reversed through heterochronic parabiosis, i.e. by sharing blood circulation between an old and a young mouse. Heterochonic parabiosis rejuvinates remylination in part via improved phagocytosis of myelin debris by the juvenile monocytes (Ruckh et al.

2012). Thus, during focal demyelination, the efficiency of phagocytosing myelin debris correlates with the rate of spontaneous remyelination. In conditions where myelin debris is not adequately phagocytosed—such as occurs during aging (Zhao et al. 2006) or following trauma to the CNS (Becerra et al. 1995; Buss et al. 2004; Buss et al. 2005; Buss and Schwab 2003; George and Griffin 1994; Griffin et al. 1992; Miklossy and Van der Loos 1991)—blocking the inhibitory influence of myelin might improve remyelination. However, this requires an understanding of the mechanisms by which myelin impairs oligodendrocyte development.

In this study, we report that myelin inhbits the extent to which oligodendrocyte lineage cells express immature and mature oligodendroycte markers as well as the production of myelin gene products. When in contact with myelin, OPCs in differentiation medium stop proliferating and downregulate OPC markers to the same extent as cells on a control substrate, indicating that their differentiation, per se, is not inhibited. Instead, the majority of cells have a multipolar simple morphology indicative of immature oligodendrocytes, but simply fail to upregulate stereotypic immature and mature oligodendrocyte markers. In the presence of myelin, oligodendrocyte lineage cells upregulate ID2 and ID4, which are both known to block the expression of immature/mature oligodendrocyte markers *in vitro* (Wang et al. 2001), providing a mechanistic explanation as to how myelin blocks oligodendrocyte lineage cell maturation at an intermediate stage.

3.2 Materials and methods

3.2.1 Oligodendrocyte precursor cell cultures

The cortices of P1/2 Sprague-Dawley (SD) rat were isolated and diced and digested in papain (Worthington) at 37°C for 90 minutes, and then gently dissociated as outlined previously (Dugas et al. 2006). Dissociated cells were resuspended in high- glucose Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Burlington, Canada) supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 100U/ml penicillin, 100µg/ml streptomycin (all from Invitrogen) and 20% fetal bovine sera (Paa Laboratories, Toronto, Canada). Cells were plated on poly-D-lysine (PDL; Sigma) coated T75 tissue culture flasks for 10-12 days. To purify OPCs, confluent T75 flasks were shaken for 1 hour at 200rpm, the media replaced, and then shaken for an additional 18-20 hours at 37˚C. To remove adherent cells, the supernatant was incubated for 45 minutes to 1 hour on a Petri plate. Cells were expanded at 37˚C at 10% CO_2 for 1 week in oligodendrocyte growth medium (OGM) composed of high-glucose DMEM supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 100U/ml penicillin, 100µg/ml streptomycin (all from Invitrogen), Trace Elements B (1X, Cellgro, Manassas, VA),5 µg/ml N-acetyl cysteine, 10ng/ml d-Biotin, 5 µg/ml Insulin, 100 µg/ml transferrin, 100 µg/ml Albumin from bovine serum, 16 µg/ml putrescine, 60 ng/ml progesterone, 40 ng/ml sodium selenite (all from Sigma, Oakville, Canada) with the addition of 4.2 µg/ml Forskolin (Sigma), 2ng/ml NT-3 (kindly provided by Regeneron Pharmaceuticals Inc.) and 10 ng/ml PDGF-AA (Peprotech, Rocky Hill, NJ) to promote proliferation. Following 1 week of expansion, 15,000-20,000 cells were then pre-plated onto 12mm coverslips coated with PDL (Sigma) containing dried spots of myelin (Figure 3.1) and grown in 24-well culture plates containing OGM without trophic factors to

promote differentiation. For measurements of survival, cells were plated onto 25mm coverslips (on/off myelin 200,000 cells per well; transfection of ID2/ID4 100,000 cells per well) and grown in 6-well culture plates containing OGM. This protocol yields, on average, 90% purity as assessed by expression of NG2 or O4 at 2 days *in vitro* (Figure 3.2)

3.2.2 Substrate preparation

Myelin was prepared from SD rat brains using a standard protocol (Norton and Poduslo, 1973). Briefly, brains were homogenized in a cold 0.32M sucrose and put on a layer of 0.85M sucrose. Following ultracentrifugation (75,000g for 30 minutes), the crude myelin that forms at the interface of the two sucrose solutions was collected, resuspended in cold water and centrifuged at 75,000g for 15minutes. Twice, isolations underwent osmotic shock by removing supernatant, adding fresh cold water, and centrifuging the sample (12,000 g for 10 minutes). The myelin pellets were suspended into cold 0.32M sucrose, layered over 0.85M sucrose and centrifuged at 75,000g for 30 minutes. The purified myelin was isolated from the interface of the two sucrose solutions and washed in water. Liver membrane fractions were prepared by removing the liver and homogenizing it in a 0.85M sucrose solution and layering this homogenate over a 1.23M sucrose solution. After centrifugation at 75,000g for 30 minutes, the interface was collected and washed in water (75,000g for 15 minutes) before resuspending in water. The protein content of both myelin and liver fractions was measured with a BCA assay (Pierce, Rockford, IL). To prepare the culture substrate, myelin or liver was diluted in DMEM to 500 μg/ml protein, unless stated otherwise. The myelin spot assay consisted of 3-4 x 1μl drops of myelin added to PDL-coated coverslips and dried overnight (Figure 3.2).

3.2.3 Immunocytochemistry

Cultured cells were fixed for 5-7min in 4% paraformaldehyde and incubated with 10% normal donkey serum and 1% bovine serum albumin (BSA) for 20 to 40 minutes to block non-specific binding. Coverslips that were being immunostained with cyclic nucleotide phosphodiesterase1 (CNP1), Ki-67, glial fibrillary acid protein (GFAP), and myelin basic protein (MBP) were permeabilized with 0.2% Triton-X added to the blocking solution, whereas coverslips that were being immunostained with neuron-glial antigen 2 (NG2), platelet-derived growth factor receptor alpha (PDGFR-α) or O4 were incubated in a blocking solution without Triton-X. Coverslips were incubated overnight with 1% bovine serum albumin (BSA) using the following primary antibodies: rabbit polyclonal anti-NG2 (OPCs; 1:200, Millipore, Billerica, MA), goat polyclonal anti-PDGFR-α (OPC; 1:100, RD systems, Minneapolis, MN), mouse monoclonal anti-O4 (immature oligodendrocytes; 1:200, Millipore and mouse monoclonal anti-CNP1:200,Millipore), mouse monoclonal anti-ki-67 (proliferation; 1:50, BD Bioscience), goat polyclonal anti-MBP (mature oligodendrocytes; 1:500, Santa Cruz, Santa Cruz, CA), rabbit anti-GFAP (astrocytes; 1:1000, Dako, Burlington, ON). Coverslips were incubated for 1-2 hours using the following secondary antibodies: Dylight 405-, Dylight 488- or Dylight 594- conjugated donkey anti-mouse, -rabbit or -goat (Jackson ImmunoResearch, West Grove, PA) with the addition of Hoechst (2μg/ml) to stain nuclei. Survival was determined by labeling live cells with calcein acetoxymethyl (AM) (1-2μM, Invitrogen) and dead cells with propidium iodide (PI) (10-20μg/ml, Invitrogen). Images were acquired with a Zeiss Axio Observer equipped with a Yokogawa X-1 Spinning Disk confocal microscope with Axiovision software. During survival measurements, the CO_2 and temperature were controlled using a heated insert with a cover.

To measure marker expression, blinded observers used immunoflourescent microscopy (Zeiss Axioplan 2) to sample 5-10 field (200 plus cells per coverslip) and counted healthy nuclei, as indicated by Hoechst staining. Cells that contained marker expression within cellular branches were considered positive. Percentage difference is defined as the difference between "on myelin" and "off myelin" expression divided by the average of the "on myelin" and "off myelin". This is presented as a percentage. Morphology was measured as reported previously (Huang et al. 2011), simple cells were defined as those with multiple processes that had branching in the primary process, as indicated with O4 immunostaining (Figure 3.4 A-C) or calcien AM immunofluorescence (Figure 3.4 D,E). Complex cells were defined as those containing more complex branching of primary processes as well as tertiary branching. Membrane cells were defined as those containing MBP-positive membrane sheet structures. Mono/bipolar cells were those cells containing one or two prominent processes. All experiments were conducted with a minimum of 2-3 separate cultures (3 to 4 coverslips/culture). After transfection, survival was measured in 12-16 fields that were systematically random sampled (50-200 cells per coverslip; 6-9 coverslips / treatment/ time point). Survival on and off of myelin was measured in 5 fields independently random sampled (300 or more cells per coverslip; 11-12 coverslips / treatment/ time point).

3.2.4 RNA purification and quantitative PCR

Total RNA was isolated, and genomic DNA digested, using illustra RNAspin Mini Isolation Kit (GE Healthcare, Baie d'Urfe, Canada) according to manufacture's instructions. For gene expression measurements, 6-well culture plates were with PDL for the "off myelin" group and then with 50μg/cm^2 or 100μg/cm^2 myelin for the "on myelin" group. Total RNA was isolated after 2 days *in vitro*. The samples plated on 50μg/cm^2 and 100μg/cm^2 myelin were grouped

together because there was no difference in *mob* or *mbp* expression (data not shown). To determine gene expression during development, OPCs were expanded for 1 week in OGM supplemented with Forskolin, PDGF and NT-3 (as above), before changing the media to OGM supplemented with 40ng/ml tri-iodo-thyronine (Sigma), 20ng/ml CNTF (Peprotech) and 4.2 µg/ml Forskolin (Sigma) to induce differentiation. RNA was isolated from OPCs and 2 or 4 days *in vitro* after being placed into differentiation conditions. RNA was reverse transcribed with Superscript III using oligo $(dT)_{20}$ primers (invitrogen). Gene expression was measured by quantitative PCR with Fast SYBR Green (Applied Biosystems, Carlsbad, CA) incorporation using an Applied Biosystems Viia7 Real-time PCR system with the following primers:

Hes1: Forward CACGCTCGGGTCTGTGCTGAGAGC

reverse ATGCCAGCTGATATAATGGAG

Hes5: Forward GTGGAGATGCTCAGTCCCAAG

Reverse TGTAGTCCTGGTGCAGGCTC S

ox6: Forward TGGTATGAAGATGGACGGCG

Reverse TGTTGTTGTTGGGGAAAGGGAG

YY1: Forward AGCTTGCCCTCATAAAGGCTGCAC

Reverse GGACTCTGGGACCGTGGGTGT

ID4: Forward ACTGTGCCTGCAGTGCGATATGAA

Reverse TGCAGGATCTCCACTTTGCTGACT

ID2: Forward ATGGAAATCCTGCAGCACGTCATC

Reverse ACGTTTGGTTCTGTCCAGGTCTCT

Sox 10: Forward GTCGCTCAGTCAGTCTCGGGCT

Reverse GAAGAGCCCAACGCCCACCTCC

MRF: Forward CGGCGTCTCGACAGCCTCAA

Reverse GACACGGCAAGAGAGCCGTCA

PDGFR-α: Forward CCTCACAGGGCCGAGCCTCAT

Reverse GTGCTGCCAGCTCACTTCGCT

MBP: Forward TTCTTTAGCGGTGACAGGGGTG

Reverse GACTACTGGGTTTTCATCTTGGGTC

MOG: Forward GGCCTGATCCTGCTGTCAGGGA

Reverse TCTGAACTGTCCTGCGTAGCTGCA

GADPH: Forward ACAGCAACAGGGTGGTGGAC

Reverse TTTGAGGGTGCAGCGAACTT

All primers were designed using NCBI primer-BLAST. Primers were validated by measuring the size of the PCR product and determining their PCR efficiency. Samples were normalized to GADPH levels and presented relative to the average off myelin control value, the

average OPC value or the average pooled control siRNA value, as appropriate, using the ΔΔCT method (Livak and Schmittgen 2001).

3.2.5 Cell transfections

Cells were expanded in OGM supplemented with Forskolin, NT-3 and PDGF for a week prior to transfection in T150 tissue culture flasks. Cells were washed with equilibrated Earle's Balanced Salt Solution (EBSS) (Invitrogen), treated with 0.025%Trypsin with ethylenediaminetetraacetic acid (EDTA) (Invitrogen) and suspended in 20% FBS/DMEM. For each culture, cells were combined and split into three equal parts containing $2.5x10^6$ to $4.5x10^6$ cells each. Suspensions were centrifuges and the cells were resuspended into 100µl of Neucleofector solution (Lonza, Basil, Switzerland) and 10µl of siRNAs (20µM; Non-Targeting siRNA Pool #2, pooled siGENOME SMARTpool rat ID2 or rat ID4) and transfected with the Amaxa Neucleofector device (Lonza) using the program O-017. After transfection, cells were plated for maturation (40,000 cells per well on 12mm coverslips), survival ($1.0x10^5$ cells per well on 25mm coverslips) or for RNA isolation ($1.0x10^6$ to $1.5x10^6$ cells) and grown in OGM. RNA was isolated after 1 day *in vitro* as described above.

3.2.6 Statistics

All data was first subjected to the Shipiro-Wilk test of normality. The Kruskal-Wallis Test or the Mann-Whitney U tests assuming independent samples were used when normality was rejected as with the following experiments: all measurements of gene expression, the percentage of O4 positive cells on/off myelin, morphology with O4/MBP expression on/off myelin, the percentage of cells expressing PDGFRα or NG2 on/off myelin and the percentage of O4 cells on

different concentrations of myelin. For all other analyses, statistical evaluation was conducted with the student's T-test assuming independent samples or analysis of variance (ANOVA) that was followed up using Tukey's Honestly Significant Difference (HSD).

3.3 Results

3.3.1 Myelin inhibited production of immature and mature markers as well as morphological complexity

Myelin is known to inhibit oligodendrocyte development (Robinson and Miller 1999). Increasing the myelin load following demyelination with the stereotaxic injection of purified myelin inhibits remyelination (Kotter et al. 2006). Given the presence of myelin debris following demyelination—which in the case of trauma can be present for years after the initial trauma in humans CNS (Becerra et al. 1995; Buss et al. 2004; Buss et al. 2005; Miklossy and Van der Loos 1991)—this mechanism seems likely to play a role in impairing CNS remyelination in multiple disease conditions. To understand how myelin inhibits oligodendrocyte development, we plated oligodendrocyte precursor cells (OPCs) from neonatal rat cultures on coverslips containing spots of myelin isolated from cortical tissue of adult rats (Robinson and Miller 1999) (Figure 3.1). In culture, OPCs can be readily expanded in the presence of PDGF-AA and NT-3 (Barres and Raff 1993; Dugas et al. 2007; Dugas et al. 2006). Upon mitogen removal, OPCs stop dividing, spontaneously differentiate, and begin to produce early immature oligodendrocyte markers such as O4 and CNP as well as later oligodendrocyte markers such as Myelin Basic Protein (MBP) (Bansal et al. 1989; Baumann and Pham-Dinh 2001; Dugas et al. 2010; Dugas et al. 2006;

Temple and Raff 1986). The degree of maturation can be assessed by determining the percentage of cells expressing early lineage markers such as O4 and CNP and later markers such as Myelin Basic Protein (MBP). Following a week of expansion, OPCs were plated onto poly-D lysine (PDL) coverslips containing myelin spots and cultured in the absence of mitogens to induce differentiation. Following 2 days *in vitro*, 70%, 69% and 50% of cells expressed the markers O4, CNP and MBP, respectively (Figure 3.3 A, C, E, G). By contrast, cells located on myelin exhibited a robust impairment in their maturation with one-half to one-third of the cells expressing these same markers (Figure 3.3 B, D, F, G). Gene expression of *mbp* and Myelin oligodendrocyte glycoprotein (*mog*) was measured with quantitative PCR 2 days *in vitro* after OPCs were plated on PDL coated wells with or without a myelin coating (Figure 3.3 H). In the absence of myelin, cells cultured on myelin had nearly a 5-fold reduction in *mbp* and *mog* gene expression.

Given that myelin is rich in both lipid and protein, it is possible this microenvironment, and not myelin-components, per se, inhibits maturation. To test this possibility, we plated OPCs on slides containing liver membrane extract spots. After 2 days *in vitro*, we found no difference in the proportion of O4-positive cells located on liver membrane substrates and on control substrates. In agreement with others (Baer et al. 2009; Robinson and Miller 1999; Syed et al. 2008), this indicates that components within the myelin are inhibiting maturation (Figure 3.3 I-K).

To determine if the morphological maturation is impaired for oligodendrocyte lineage cells in contact with myelin, we measured the maturation state of these cells based on their morphology. OPCs *in vitro* are predominantly bipolar cells that increase the number of processes

as they begin to differentiate into immature oligodendrocytes (Huang et al. 2011; Pfeiffer et al. 1993). As these cells continue to mature, they increase their branching complexity and eventually lay down membrane sheets. OPCs were plated on PDL coated coverslips containing myelin spots. After 2 days *in vitro* in the absence of mitogens, cells were labelled with immature and mature oligodendrocyte markers O4 and MBP (Figure 3.4 A). Cells were characterized as simple, complex or membranous to distinguish the extent of their complexity (Figure 3.4 B). The vast majority of cells on myelin did not contain any O4-positive branches, whereas most cells not in contact with myelin were O4-positive (Figure 3.4 C). Cells in contact with myelin had a significant reduction in the percentage with complex morphologies. There was a significant increase in the percentage of O4-positive cells with a simple morphology and there was a significant decrease in the percentage of O4-positive cells with a complex morphology when cells were in contact with myelin (Figure 3.4 D). As the majority of cells located on myelin spots do not to express O4, we measured the complexity of oligodendrocyte lineage cells with the fluorescent live cell indicator calcein AM (Figure 3.4E). We found, as before, that there was a significant reduction in the percentage of cells with a complex morphology and a significant increase in the percentage of cells with a simple morphology when in contact with myelin (Figure 3.4 F). The vast majority of cells in contact with myelin were morphologically simple, which is indicative of immature oligodendrocyte lineage cells or late OPCs. Thus, contact with myelin inhibited the production of immature and mature oligodendrocyte markers, and by morphological criteria, these cells remained as immature oligodendrocytes.

3.3.2 Most OPCs in contact with myelin stopped proliferating in the absence of mitogens and downregulated OPC markers

Myelin did not inhibit oligodendrocyte development by maintaining cells as OPCs. The percentage of cells expressing OPC markers NG2 and PDGFR-α after 2 days *in vitro* in the absence of mitogens (Figure 3.5) was not different when cells were located on or off myelin (Figure 3.5 G). To differentiate between OPCs and immature oligodendrocytes, OPCs were considered as expressing either NG2 or PDGFR-α, but not the immature oligodendrocyte marker O4. Likewise, myelin did not affect the percentage of living cells that possessed the morphological characteristics of OPCs (or those cells described as mono or bipolar) when in contact with myelin (Figure 3.4 E). As PDGFR-α and NG2 can label subpopulations of OPCs (Aguirre et al. 2004; Belachew et al. 2003; Chojnacki and Weiss 2004), we measured the percentage of cells that were single or double labelled for NG2 and PDGFR-α. We found that the vast majority of OPCs expressed both PDGFR-α and NG2, with a small percentage expressing only one of these two markers (Figure 3.5 H). For those cells residing on myelin, there was no change in the percentage of OPCs labelled for PDGFR-α. However, when in contact with myelin there was a small (3-4%), albeit significant, decrease in the percentage of OPCs expressing NG2 and PDGFRα as well as an increase in the percentage of OPCs positive for NG2. This indicates that there was no major shift in OPC subpopulations when in contact with myelin. To determine if there was a change in the gene expression of OPC markers, we measured the amount of *pdgfr-α* mRNA with qPCR and found no difference in the amount of *pdgfr-α* in cells cultured on or off myelin (Figure 3.5 I). This indicates that oligodendrocyte lineage cells located on myelin are not maintained as OPCs, but instead downregulate OPC markers as well as *pdgfr-α* mRNA.

Myelin might inhibit the ability of OPCs to proliferate. To examine this possibility, OPCs were plated on coverslips containing myelin spots in the presence or absence of the mitogens PDGF and NT-3 for 2 days *in vitro*. To measure proliferation, we counted the percentage of cells expressing the cell cycle marker ki-67 (Kee et al. 2002). During oligodendrocyte development, the downregulation of OPC markers such as NG2 coincides with the inability to divide (Baumann and Pham-Dinh 2001). Consistent with this idea, we found that ki-67 was found almost exclusively in NG2-positive OPCs. In the absence of mitogens, the vast majority of cells stop expressing ki-67 and NG2 and spontaneously differentiate (Figure 3.6 A, B, E). There was no difference in the proliferative OPCs grown in the absence of mitogens when in contact with myelin. In contrast, the vast majority of OPCs were proliferating OPCs when in the presence of PDGF and NT-3 (Figure 3.6 C-E). The extent of proliferation was unaffected by contact with myelin. Thus, myelin does not grossly impair the ability of OPCs to enter the cell cycle, nor does it maintain OPCs in a proliferative state.

3.3.3 Contact with myelin did not increase astrogenesis or oligodendroglia death

Taken together, myelin impaired the maturation of oligodendrocyte lineage cells. OPCs in contact with myelin downregulated OPC markers and stopped dividing in a comparable manner to those cells not in contact with myelin. Many of these cells did not express the immature oligodendrocyte marker O4 or the OPC marker NG2 (Figure 3.7 A, B), but displayed a multipolar morphology indicative of an immature oligodendrocyte (Figure 3.7D, E). There was no difference in the percentage of GFAP-positive astrocytes when cells were located on or off myelin spots at 2 days *in vitro* (Figure 3.7 C, D). However, at 4 days *in vitro* the percentage of astrocytes present on myelin spots was significantly decreased. There was also no indication of

enhanced cell death for those cells in contact with myelin. Imaging unfixed cells and visualizing them with a live cell indicator (calcein AM) and a dead cell indicator (propidium iodide), we found no difference in the percentage of living cells that were in contact with myelin at 1 day *in vitro* (Figure 3.7 E-J). After 3 days *in vitro* the percentage of living cells increased and the percentage of dead cells decreased when in contact with myelin. Thus, increased astrogenesis or decreased survival cannot account for the inability of OPCs grown on myelin to express immature or mature oligodendrocyte markers. Since oligodendrocyte lineage cells located on myelin stopped proliferating and downregulated OPC markers to comparable levels as cells on control substrates, but failed to upregulate immature oligodendrocyte markers, we can conclude that oligodendroglial maturation was stalled in a pre-immature oligodendrocyte stage.

Based on the hypothesis that components of myelin bind to receptors on oligodendrocyte lineage cells it is expected that increasing the concentration of myelin will increase the degree to which maturation is inhibited. To test whether myelin inhibits oligodendroglia maturation in a dose-dependent manner, OPCs were plated on coverslips containing myelin spots of increasing concentration and after 2 days *in vitro* in differentiation-inducing media, we measured the percentage difference in O4 expression for cells located on or off myelin spots (Figure 3.8). Those cells located on 5μg/ml myelin spots showed a 7% decrease in the percentage of O4-positive cells compared to those cells located adjacent to the myelin spots (Figure 3.8 A, B, I). The suppression of O4 expression increased in a step-wise manner as those cells located on 250μg/ml and 500μg/ml myelin spots exhibited a 108% and 165% decrease in the percentage of O4-positive cells, respectively, compared to those cells located adjacent to the myelin spots (Figure 3.8 C-F, I). We did not see an increase in the percentage of O4-positive cells in any of

the conditions (data not shown). The amount of O4 suppression saturated at 500μg/ml of myelin with no further increase at 1000μg/ml (Figure 3.8 G-I). As the concentration of myelin within the spot increased, there was no significant change in the expression of NG2 for cells that were in contact with myelin (Figure 3.8 J). This indicates that myelin inhibits the maturation of oligodendrocyte lineage cells in a dose-dependent manner.

3.3.4 Contact with myelin increased expression of ID family members

To gain insight as to how myelin inhibits the maturation of oligodendrocyte lineage cells, we measured the gene expression of transcription factors that are known to have a role in the development of oligodendrocytes. OPCs were plated on PDL coated wells with or without myelin and after 2 days *in vitro* in the absence of mitogens, we isolated the total RNA. Importantly, we found no RNA within myelin. We first measured several transcription factors that are known to be involved in repressing myelin gene expression because we found a robust decrease in MAG and MOG expression (Figure 3.3 H), (Emery 2010); namely, we measured Hes1, Hes5, Sox6, ID2 and ID4 (Figure 3.9 A). While the expression of Hes1, Hes5, Sox6 was unchanged, the members of the inhibitors of differentiation family (ID), showed a 2.5- fold increase in *id2* and a 2-fold increase in *id4* mRNA for those cells located on myelin. Overexpression of ID2 (Wang et al. 2001) and ID4 (Kondo and Raff 2000) is known to powerfully block the development of oligodendrocytes. ID2 and ID4 are known in oligodendrocyte lineage cells to bind the bHLH transcription factors Olig1 and Olig2 (Chen et al. 2009; Samanta and Kessler 2004), which are important in the specification of oligodendrocytes as well as the production of mature oligodendrocytes (Lu et al. 2002; Yue et al. 2006; Zhou and Anderson 2002). Olig1 deficiency delays remyelination (Arnett et al. 2004) and decreases many

oligodendrocyte genes during development such as MBP, G-protein coupled receptor 17, MOG, Sox10 and Myelin gene regulatory factor (MRF, also known as GM98) (Chen et al. 2009; Emery et al. 2009). We found decreased Sox10 and MRF expression in cells cultured on myelin. This is consistent with an elevated ID2/ID4. Given that ID2 and ID4 expression decrease with development (Dugas et al. 2006; Kondo and Raff 2000), we wanted to determine if OPCs plated on myelin are simply maintaining a high level of *id2* and *id4* expression compared to cultures that are grown off myelin. Thus, we measured *id2* and *id4* mRNA during oligodendrocyte development. Total RNA was isolated from OPC cultures as well as cultures grown for 2 or 4 days *in vitro* on a PDL in differentiating conditions. We found that neither *id2* nor *id4* were downregulated at 2 days *in vitro*. However, *id*2 was significantly down-regulated at 4 days *in vitro* (Figure 3.9 C), a time point that corresponds to a spike in *mbp* (Figure 3.9 D) as well as numerous other myelin gene products (Dugas et al. 2006). Thus, contact with myelin increases the concentration of *id2* and *id4,* and this is sufficient to block the maturation of oligodendrocyte lineage cells

ID4 expression of can be regulated by the transcription factor YY1 (He et al. 2007). To determine if this was occurring, we measured *yy1* mRNA expression for OPCs grown on myelin or off myelin for 2 days *in vitro*. We found no increase in *yy1* expression (data not shown), indicating that myelin increases *id2* and *id4* expression independently of YY1.

Since myelin increases *id2* expression in oligodendrocyte lineage cells and ID2 is sufficient to block their maturation, we wanted to determine if ID2 and ID4 stimulation by myelin is necessary for impairment in the maturation of oligodendrocyte lineage cells, we transfected a nontargeting pool of siRNA or a pool of siRNA against *id2* or *id4* into OPCs.

Knockdown of ID4 resulted in impaired survival of oligodendrocyte lineage cells (Figure 3.10 A), consistent with increased cell death in the ID4 knockout animal (Marin-Husstege et al. 2006). Knockdown of ID2 decreased *id2* expression after 1 day *in vitro* by approximately 60% (Figure 3.10 B), but despite this lowered expression there was no increase in the percentage of cells expressing O4 (Figure 3.10 C) or MBP (Figure 3.10 D) on myelin compared to OPCs transfected with a non-targeting pool of siRNA. This indicates that the myelin-dependent increase in *id2* expression was not necessary for myelin inhibition of oligodendroglia maturation. Myelin, thus, inhibits oligodendrocyte lineage cells by additional mechanisms, in addition to the ID2, or by redundancy of ID family members.

3.4 Discussion

Strategies that improve remyelination are likely to be beneficial in several diseases including Multiple Sclerosis (Franklin 2002; Franklin and ffrench-Constant 2008), spinal cord injury (McTigue and Tripathi 2008) and developmental leukodystrophies (Duncan et al. 2011). Remyelination is considered to be critical because demyelinated axons not only lack the myelin necessary to achieve normal conduction velocity, but are also at increased risk of degeneration (Nave 2010a; Nave 2010b; Nave and Trapp 2008; Trapp and Nave 2008). The presence of a quiescent population of OPCs at sites of chronic demyelination highlights the need to promote OPC differentiation/maturation (Chang et al. 2000; Chang et al. 2002; Kuhlmann et al. 2008), e.g. by blocking inhibitors of differentiation/maturation. The hypothesis that myelin debris phagocytosis is a rate-limiting step in the onset of remyelination is supported by the findings that

myelin inhibits remyelination (Kotter et al. 2006) and the rate of remyelination correlates with the rate of myelin clearance (Ruckh et al. 2012; Shields et al. 1999; Zhao et al. 2006). Still, little is known about how contact with myelin inhibits oligodendrocyte development (Baer et al. 2009; Robinson and Miller 1999). Here, we found that myelin impairs the ability of oligodendrocyte lineage cells to express immature and mature myelin markers, to complete morphological differentiation as well as to express myelin gene products. Contact with myelin did not affect OPC proliferation and OPC markers were similarly downregulated when in contact with myelin. The majority of the cells in contact with myelin were multipolar cells with a simple morphology that is normally associated with immature oligodendrocytes. However, these cells did not express the typical immature oligodendrocyte marker or OPC markers and were not proliferating, indicating that they were not maintained as precursors but instead were stalled in a pre-immature oligodendrocyte stage. To understand how myelin inhibits the developmental progression of oligodendrocyte lineage cells we measured the gene expression of key transcription factors involved in oligodendrocyte development. Cells plated on myelin upregulated *id2* and *id4* expression; both of these transcription factors are sufficient to block maturation (Kondo and Raff 2000; Wang et al. 2001). ID2 and ID4 bind and antagonize Olig1 and Olig2 transcription factors (Chen et al. 2009; Samanta and Kessler 2004), suggesting that elevated ID2 and ID4 likely impair the function of Olig1 and Olig2. Consistent with this role, OPCs grown on myelin have decreased *mrf, sox10* and *mbp* expression, three genes that are known to be regulated by Olig1 (Chen et al. 2009). Taken together, OPCs in contact with myelin normally stop proliferating and down-regulate OPC-specific markers. Post-mitotic, pre-immature oligodendrocytes on myelin

have elevated *id2/id4* expression that likely antagonizes Olig1/Olig2 function resulting in impaired maturation of oligodendrocyte lineage cells.

The impairment of maturation of oligodendrocyte lineage cells due to myelin occurred in a dose-dependent manner, indicating that molecules within myelin bind to receptors on oligodendrocyte lineage cells and inhibit their developmental progression. The finding that myelin substrate treated with lipases does not change its inhibitor properties demonstrates that it is protein in myelin that is inhibitory and not lipids (Syed et al. 2008). Moreover, classic inhibitors of axonal regeneration, NOGO-A, MAG and OMgp do not inhibit oligodendrocyte lineage cells (Syed et al. 2008), indicating that a yet-to-be identified myelin-associated protein(s) inhibits oligodendroglial maturation. The identification of this protein and its receptor is likely to yield new targets in myelin repair.

3.4.1 Myelin inhibits oligodendrocyte lineage cells maturation in part by increasing ID transcription factors

IDs are helix-loop-helix proteins that lack a DNA binding site and preferentially bind to basic helix-loop-helix (bHLH) transcription factors (such as Olig1 and Olig2) forming transcription factor complexes (Abe et al. 1999; Benezra et al. 1990; Chen et al. 2012; Sun et al. 1991; Yokota 2001). In oligodendrocytes, Olig1 and Olig2 are two important bHLH transcription factors whose activity is regulated by ID2 and ID4 (Chen et al. 2009; Gokhan et al. 2005; Samanta and Kessler 2004). Olig2 is central in the specification of oligodendrocytes (Lu et al. 2002; Zhou and Anderson 2002), whereas the loss of Olig1 results in a delay in oligodendrocyte maturation (Lu et al. 2002; Xin et al. 2005) and impaired remyelination (Arnett et al. 2004).

However, if Olig2 is removed from dorsal progenitors in the telencephalon, there is no impairment in OPC production but rather arrested myelination, indicating that Olig2 is also required for oligodendrocyte maturation and myelination (Yue et al. 2006). Similar to the impaired production of immature oligodendrocyte and mature oligodendrocyte markers on myelin, if OPCs develop *in vitro* with overexpression of ID2 (Wang et al. 2001) and ID4 (Kondo and Raff 2000; Marin-Husstege et al. 2006) their expression of galactocebroside is inhibited, which is a marker for a slightly later stage of oligodendrocyte development than the onset of O4 (Jessen 2004). During oligodendrocyte development, ID2 and ID4 act as transcriptional inhibitors by antagonizing Olig1 and Olig2 function (Chen et al. 2009; Gokhan et al. 2005; Samanta and Kessler 2004). In mice, both Olig1 and Olig2 bind to Sox10 (Li et al. 2007; Wissmuller et al. 2006). Olig1 and Sox10 can act directly on a conserved DNA motif in the promoter region to increase MBP transcription, synergistically (Li et al. 2007). Olig1 also regulates the gene expression of numerous myelin genes such as MBP, PLP and MAG (Chen et al. 2009) as well as the expression of important transcription factors MRF and Sox10 (Chen et al. 2009), which are themselves also key regulators of myelin gene expression (Emery et al. 2009; Li et al. 2007; Stolt et al. 2002). Thus, increased ID2 and ID4 expression in oligodendrocyte lineage cells in contact with myelin is predicted to decrease myelin proteins such as MOG and MBP as well as MRF and Sox10, which is what we observed in the present study.

ID2-deficient mice are available, but due to early postnatal death it is not possible to assess its role during myelination *in vivo*. However, the loss of ID2 results in a small increase in the amount of premature OPC differentiation *in vitro* (Wang et al. 2001). By contrast, loss of ID4 results in impaired OPC differentiation as well as maturation (Marin-Husstege et al. 2006). We

found increased cell death when ID4 was knocked down in OPCs, which is consistent with the increase in cell death of cells adjacent to the subventricular zone in ID4 KO animals (Marin-Husstege et al. 2006). Due to the redundant roles of ID2/ID4 binding to Olig1/Olig2 (Samanta and Kessler 2004), it may not be surprising that knockdown of ID2 alone did not increase oligodendrocyte maturation on myelin.

ID2 and ID4 are regulated during oligodendrocyte development by multiple factors. BMP4 increases ID family members expression in neural precursor cells resulting in increased astrogenesis (Feigenson et al. 2009; Samanta and Kessler 2004). Likewise, both Wnt3a and its downstream effector β-catentin block myelin gene expression as well as stimulate ID2 and ID4 in OPCs (Feigenson et al. 2009; Ye et al. 2009). G protein-coupled receptor 17 (GPR17) also increases ID2 and ID4 expression and is a negative regulator of CNS myelination (Chen et al. 2009). One transcription factor that interacts with the ID4 promoter and decreases its expression is the transcription factor Ying Yang 1 (YY1) (He et al. 2007). YY1 is thought to act by recruiting histone deacetylase 1/2 (HDAC) to the ID4 promoter region, which decreases ID4 expression. HDAC1/2 is a chromatin-modifying enzyme that is required for the myelin development (Shen et al. 2005) and acts, in part, by regulating numerous transcription factors that are negative regulators of oligodendrocyte development such as Sox2, Sox11, Egr1 and Hes5 (Shen et al. 2008; Swiss et al. 2011; Ye et al. 2009). As myelin did not result in changes to Hes1, Hes5, or YY1, it is unlikely that the maturation of OPCs by myelin is mediated via HDAC 1/2 or YY1-regulating ID2/ID4. It remains to be determined whether myelin stimulates the BMP, Wnt or GPR17 signalling pathways.

3.4.2 Myelin inhibits the maturation of oligodendrocyte lineage cells without affecting proliferation

Oligodendrocyte development requires OPCs to exit the cell cycle in order to differentiate into immature oligodendrocytes (Raff et al. 1988; Temple and Raff 1986). Oligodendrocyte differentiation can be inhibited by maintaining cells in a proliferative state, such as by adding the mitogen PDGF (Barres and Raff 1999; Noble et al. 1988; Raff et al. 1988). At elevated concentrations of PDGF, there is a higher proportion of cells that remain OPCs and fewer that become oligodendrocytes (Barres and Raff 1999). When the concentration of PDGF is lowered, the percentage of OPCs decreases. Likewise, cell cycle inhibitors such as p21, p27 and p57 regulate oligodendrocyte development (Casaccia-Bonnefil et al. 1999; Dugas et al. 2007; Durand and Raff 2000). For example, p27 inhibits OPC proliferation (Casaccia-Bonnefil et al. 1999; Tokumoto et al. 2002) and p57 levels can regulate how many times an OPC divides before differentiating (Dugas et al. 2007). One explanation as to how myelin inhibits oligodendrocyte development was the possibility that myelin maintained cells in a proliferative state. However, there was no change in the percentage of proliferating OPCs in the presence of mitogens, indicating no inability to proliferate on myelin. We also found no increase in proliferating OPCs in the absence of mitogens, indicating that OPCs were not being held in a proliferative state. Likewise, in the absence of mitogens there was no increase in the percentage of precursors, meaning that OPCs were able to downregulate their early markers and become post-mitotic cells. Hence, contact with myelin severely impaired several aspects of oligodendrocyte maturation with no effect on the cell cycle regulation, indicating that separate mechanisms likely regulate

these processes. As a corollary, exit from the cell cycle, in itself, is not sufficient to affect the later stages of oligodendrocyte development.

3.4.3 Myelin inhibition of remyelination after SCI

Myelin clearance after focal demyelination in animal models normally occurs relatively rapidly, the majority of clearance occurring in the first 96 hours after LPC mediated demyelination (Ousman and David 2000). Aging drastically impairs this process, with extracellular myelin debris present at 4 weeks after ethidium bromide (EB) (Shields et al. 1999) and lysolecithin (LPC) (Gilson and Blakemore 1993) mediated demyelination. By contrast, after spinal cord injury, myelin debris is present in the extracellular space for at least 8 weeks in rodents (Buss and Schwab 2003) and 3 years in humans (Buss et al. 2005). Extracellular myelin debris after SCI is regarded as an inhibitor of axonal regeneration, containing several inhibitors of axonal outgrowth (Filbin 2003; Liu et al. 2006b; Schwab 2004; Yiu and He 2006). However, given the slow Wallerian degeneration, myelin is a potential inhibitor of remyelination after trauma. Demyelination is a prominent feature following SCI (Basso et al. 1996; Beattie et al. 1997; Blight 1985; Gledhill et al. 1973a; Harrison and McDonald 1977; Smith and Jeffery 2006; Totoiu and Keirstead 2005), likely because of acute oligodendrocyte necrosis or apoptosis occurring mostly over the first 2-3 weeks after injury (Casha et al. 2001; Crowe et al. 1997; Li et al. 1999; Liu et al. 1997). The extent to which this demyelination persists and the extent to which it is amenable to remyelinating strategies in chronic settings is intensively debated (Basso et al. 1996; Eftekharpour et al. 2008; Karimi-Abdolrezaee et al. 2010; Lasiene et al. 2008; Totoiu and Keirstead 2005). A persistent demyelination would indicate that endogenous OPCs are not sufficient to remyelinate axons after SCI. This has clinical relevance as remyelination was the

basis of the phase 1 Geron Corporation clinical trial that transplanted OPCs derived from embryonic stem cells (now on hold for financial reasons). However, the finding that transplantation of PDGF-responsive neural precursors, cells that are able to remyelinate axons after SCI, did not increase the extent of myelination or functional recovery argues that, at least in the rodent, endogenous cells within the spinal cord might be relatively efficient (Plemel et al. 2011). Remyelination by exogenous and endogenous cells is also potentially regulated by environmental factors surrounding the injury site, such as myelin-associated inhibitory molecules or limited amounts of pro-myelinating cues. Thus, remyelination after SCI might be best improved by promoting a remyelinating niche by adding factors that increase OPC proliferation or differentiation such as by blocking remyelination inhibitors like myelin and its debris. In this context it is of note that after experimental demyelination, new remyelinated sheaths emerge at the lesion boundary, behind areas where phagocytosis of myelin debris has occurred (Kotter et al. 2011). Similarly, in MS remyelination is most common on the lesion boarder (Patrikios et al. 2006). The prominence of oligodendrogenesis at the injured lesion border (Tripathi and McTigue 2007), an area also rich in phagocytotic cells (Okada et al. 2006), gives credence to the argument that myelin phagocytosis is important for remyelination after SCI. The extent of remyelination—and by proxy demyelination—of spared axons after SCI is high, with 53% of spared rubrospinal axons remyelinated in a chronic setting (Powers et al. 2012). Thus, remyelination, and hence demyelination, occurs extensively after SCI in rodents and might be an important source of intervention to promote recovery after SCI in humans, where myelin clearance is notoriously slow. Blocking the inhibition of myelin on oligodendrocyte cells could accelerate remyelination after SCI, potentially sparing more fibres and ultimately improving function.

2.4.4 Conclusions

Myelin contact does not affect the proliferation of OPCs or the normal downregulation of OPC markers *in vitro.* Instead, these cells were halted in a pre-immature oligodendrocyte stage that is characterized by a simple branched morphology without the typical phenotypic markers of that stage such as O4 or CNP. This inhibition of oligodendroglial maturation by myelin was associated with increased ID2 and ID4 expression, which are known potent inhibitors of oligodendrocyte development. Thus, upregulation of ID2 and ID4 provides a mechanistic explanation how myelin blocks oligodendrocyte lineage cell maturation. Blocking the inhibitory nature of myelin debris on remyelination is one promising strategy to find new therapies in white matter disorders.

Figure 3.1 Details of the myelin spot assay. Myelin (1µl) was placed on a coverslip in 3-4 spot roughly equidistant from one another (A). Markers of immature (O4; red) or mature oligodendrocytes, but not OPC markers (NG2; green) diffusely label the myelin spot. These markers were used to determine if the cells were located "On Myelin" or "Off Myelin" (B). Due to the granular appearance of myelin, it was possible to distinguish it from cellular structures. Thus, myelin was not a source of false positives during the quantification of cellular markers. Nuclei are stained with Hoechst (blue) and the scale bar is 200µm (B)

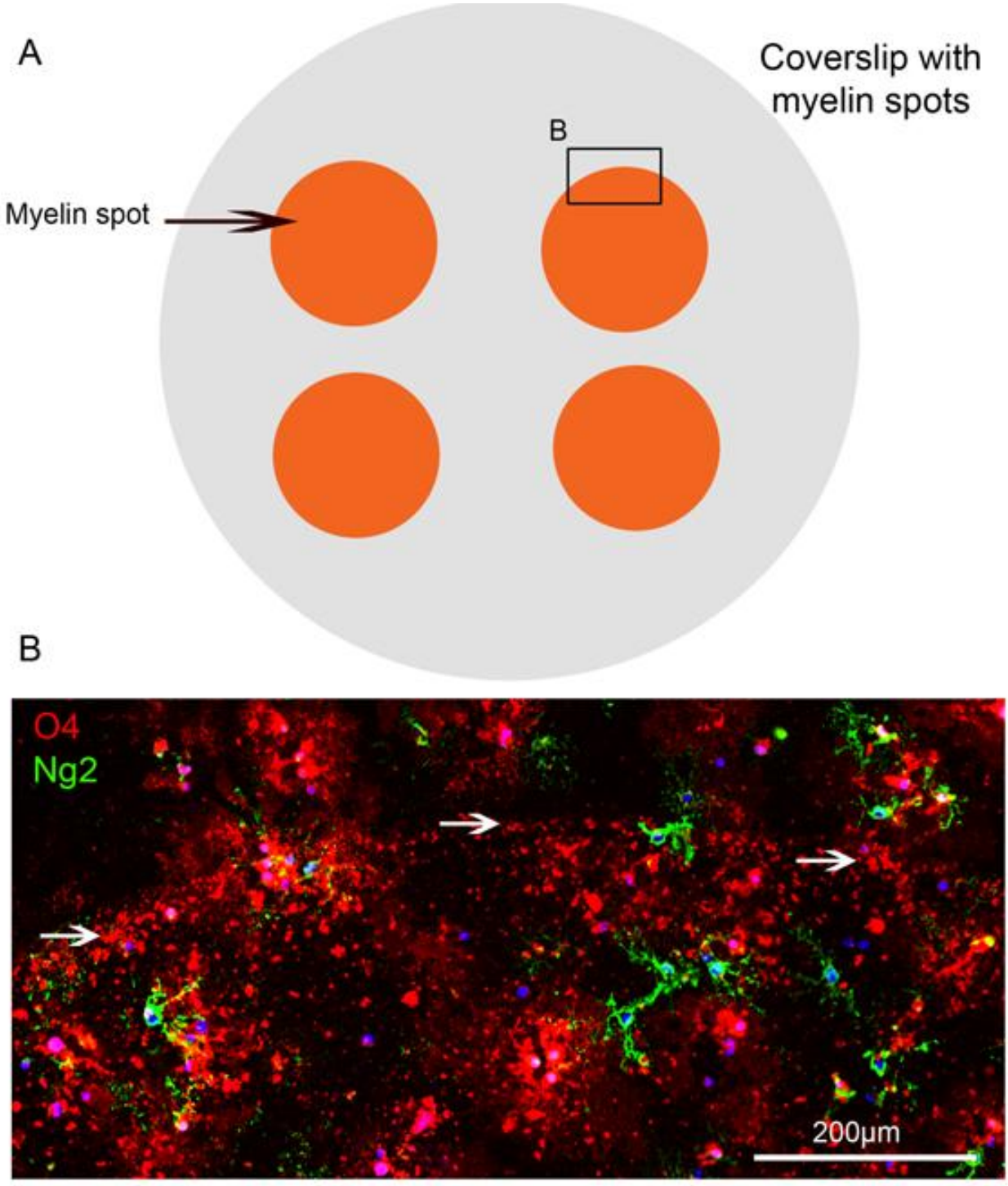

A
Coverslip with
myelin spots
B
Myelin spot
B
O4
Ng2
200µm

Figure 3.2 Typical purity of oligodendrocyte culture. OPCs were expanded in mitogens for 1 week, before being plated onto coverslips and fed with media lacking mitogens to induce differentiation. After 2 days *in vitro*, the majority of OPCs differentiated and express the immature oligodendrocyte marker, O4 (red; A). A small proportion of cells are maintained as OPCs as indicated by the marker NG2 (green). NG2 expression and O4 expression are typically mutually exclusive (example with white arrow). Those cells expressing both OPC markers (NG2 or PDGFR-α) and O4, were considered to be O4-positive. There was a small percentage of contaminating astrocytes (GFAP; green) (C). Here, 66% of cells label with O4, 23% of cells label with NG2 and 2% of cells label with GFAP, indicating that the vast majority of cells (89%) express oligodendrocyte lineage markers (D). We did not find evidence of neuronal (βIII-tubulin) or microglial (IBA1) contamination. Nuclei are stained with Hoechst (blue) and the scale bar is 200μm (B). At least 600 cells were counted for each group.

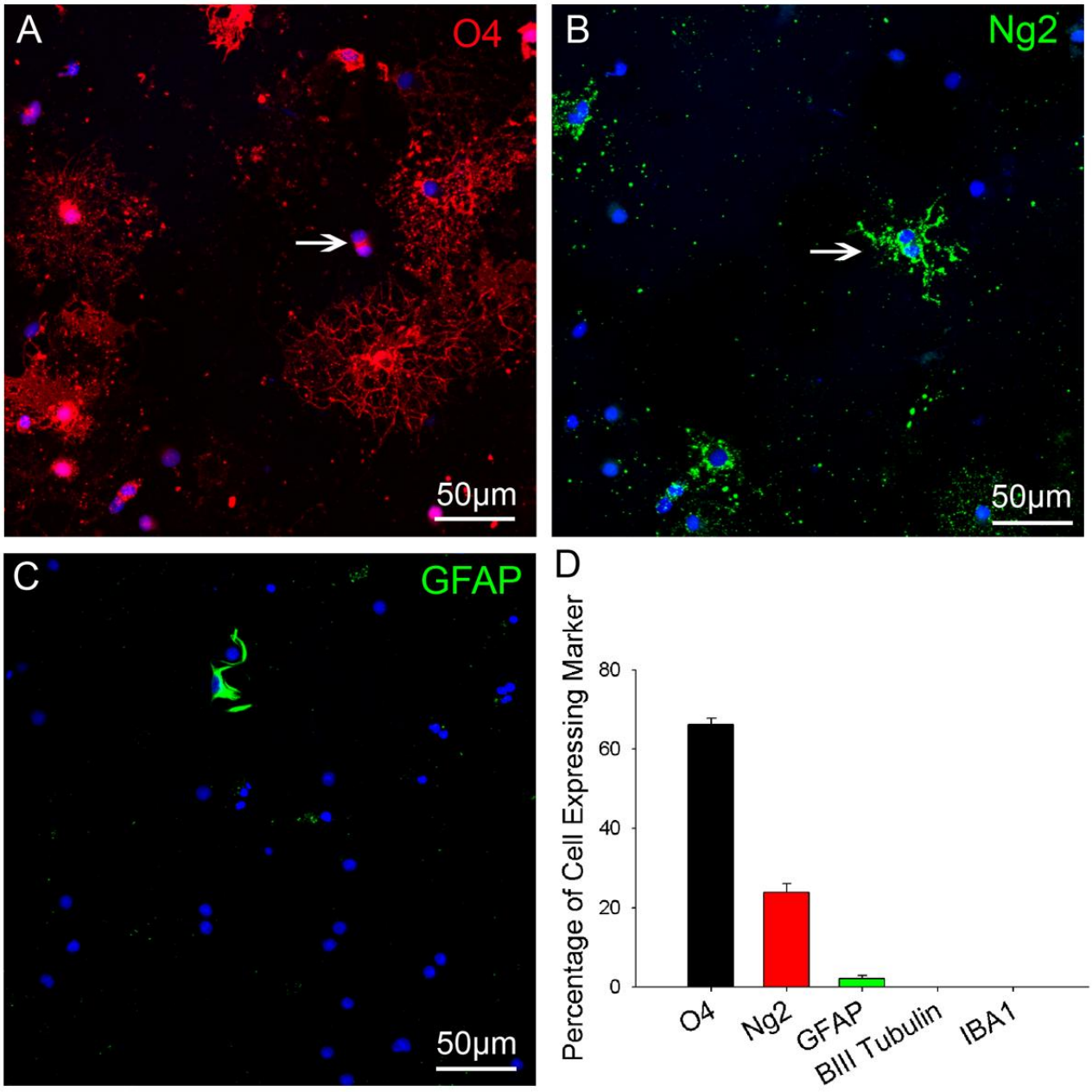

A
O4
50μm
B
Ng2
50μm
C
GFAP
50μm
D
Percentage of Cell Expressing Marker
80
60
40
20
0
O4
Ng2
GFAP
BIII Tubulin
IBA1

Figure 3.3 Contact with myelin supressed the ability to express immature and mature oligodendrocyte markers as well as myelin gene products. OPCs were cultured on coverslips containing isolated myelin spots for 2 days *in vitro* under differentiating conditions. The percentage of cells with healthy nuclei (Hoechst; blue) expressing CNP (A, B), O4 (C, D) and MBP (E, F) was measured. There was a significant reduction in the percentage of cells expressing immature oligodendrocyte markers CNP and O4 as well as the mature oligodendrocyte markers MBP (G). RNA was isolated from OPCs cultured for days 2 *in vitro* on myelin (50/100μg/cm^2) or PDL and the extent of *mbp* or *mog* mRNA was measure (H). When cultured on myelin, oligodendrocyte lineage cells have significantly impaired production of *mbp* and *mog*. There was no difference in the percentage of cells expressing O4 when OPCs were cultured for 2 days *in vitro* on liver membrane (I-K). Scale bar is 50μm (A-F, I, J) and error bars are standard error of mean (G, H, K). At least 1200 cells were counted for each group.

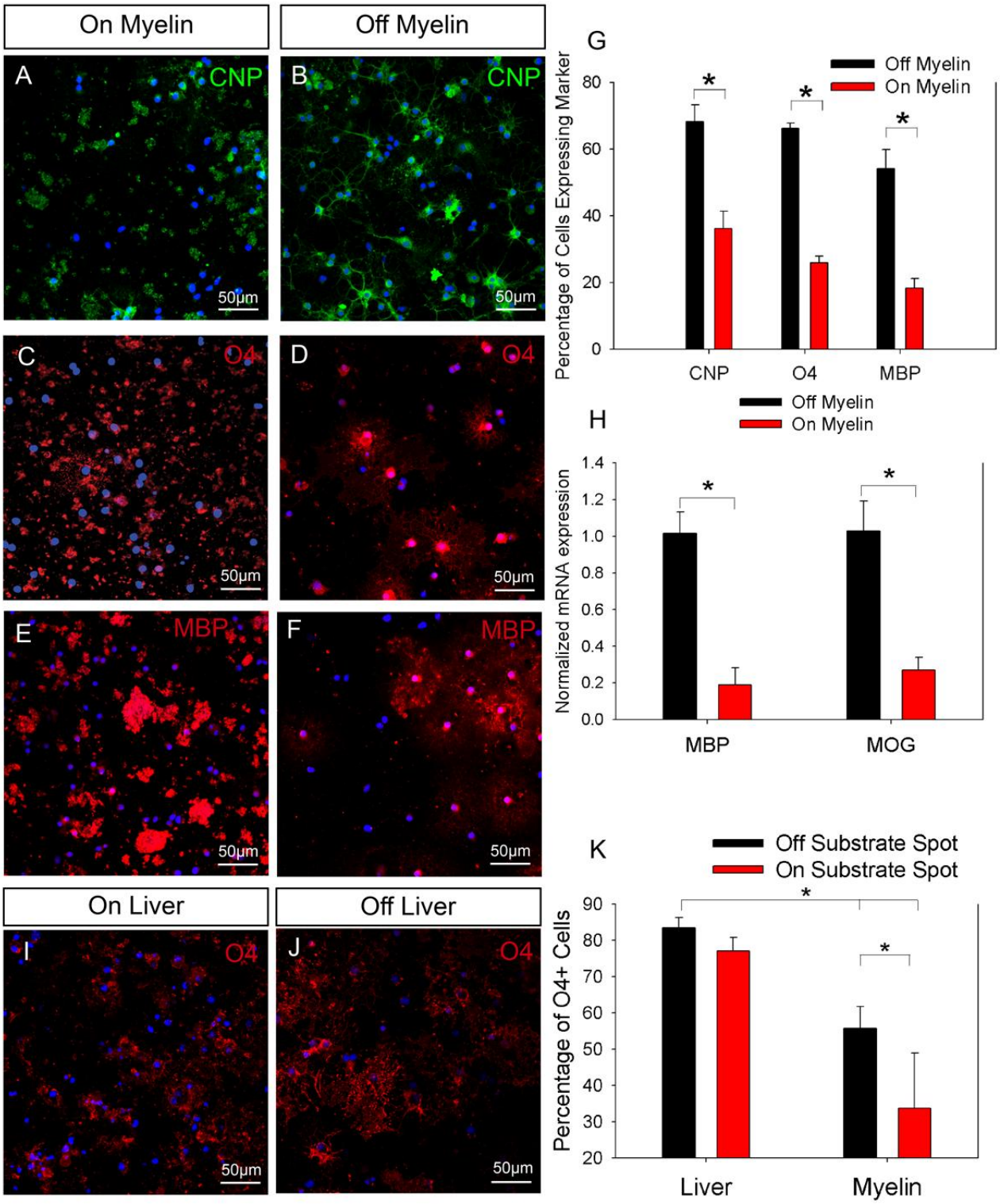

On Myelin
Off Myelin
A
CNP
B
CNP
50μm
50μm
C
O4
D
O4
50μm
50μm
E
MBP
F
MBP
50μm
50μm
On Liver
Off Liver
I
O4
J
O4
50μm
50μm
G
Off Myelin
On Myelin
Percentage of Cells Expressing Marker
0
20
40
60
80
*
*
*
CNP
O4
MBP
H
Off Myelin
On Myelin
Normalized mRNA expression
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
*
*
MBP
MOG
K
Off Substrate Spot
On Substrate Spot
Percentage of O4+ Cells
20
30
40
50
60
70
80
90
*
*
Liver
Myelin

Figure 3.4 Contact with myelin inhibited the morphological complexity of oligodendroglia. OPCs were cultured for 2 days *in vitro* in differentiating conditions and were visualized with the antibodies directed to both O4 (red) and MBP (green). Oligodendroglia were measured according to the following morphological criteria: simple, complex, or membrane containing morphologies (A, B). Contact with myelin significantly increased the percentage of O4-negative cells and significantly decreased the percentage of cells that have a complex morphology (C). There were a significantly higher proportion of O4-positive cells with a simple morphology and a significantly lower proportion of O4-positive cells with a complex morphology when in contact with myelin (D). Living cells, as visualized by Calcein AM, were measured on morphological criteria after 1 day *in vitro* (E). When in contact with myelin, there was a greater proportion of cells with a simple morphology and a lower proportion of cells with complex morphology (F). Nuclei are stained with Hoechst (blue) and scale bars are 50μm (A, D) and 12μm (B). Error bars are standard error of mean (C, D, F). At least 2000 cells were counted for each group.

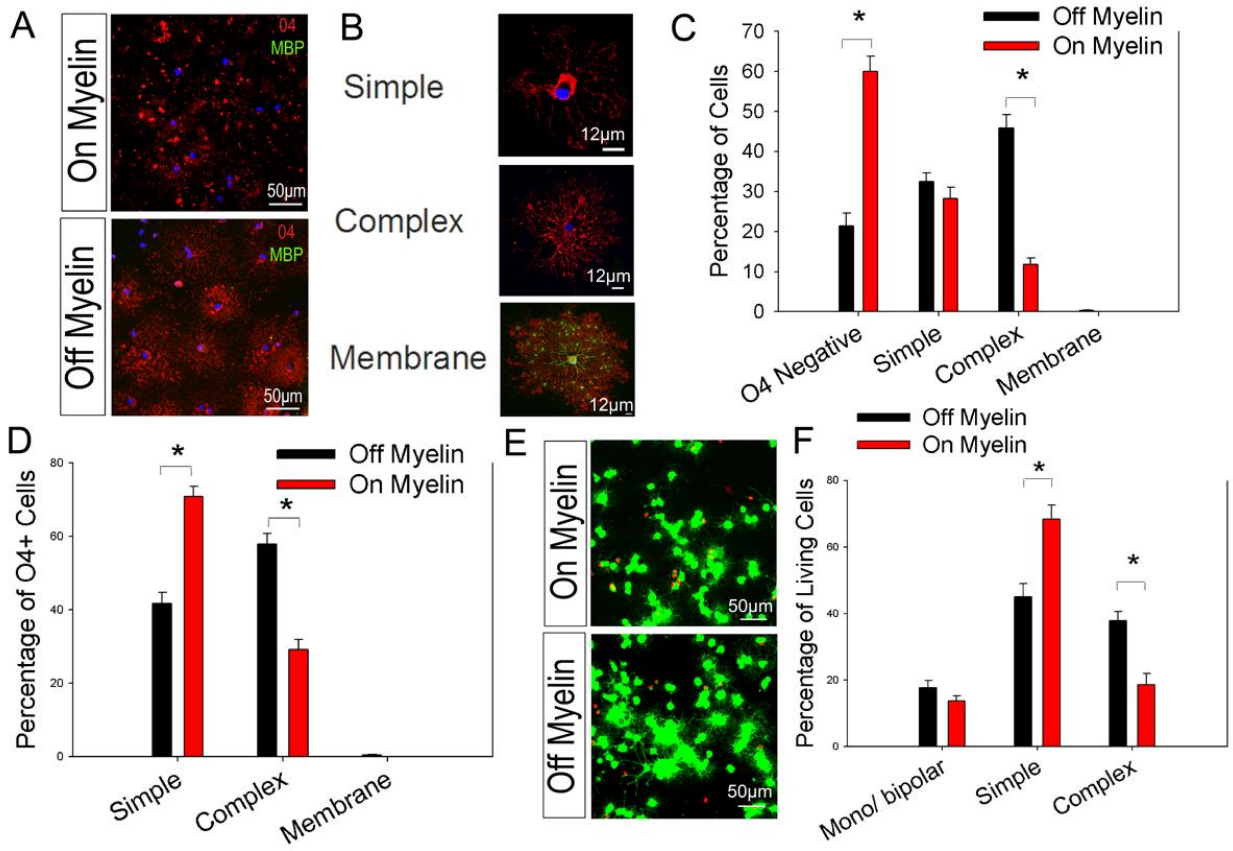

A
On Myelin
Off Myelin
04
MBP
50μm
B
Simple
Complex
Membrane
12μm
C
Off Myelin
On Myelin
Percentage of Cells
O4 Negative
Simple
Complex
Membrane
D
Percentage of O4+ Cells
E
F
Percentage of Living Cells
Mono/ bipolar

Figure 3.5 Contact with myelin did not maintain oligodendrocyte lineage cells as precursors. OPCs were cultured for 2 days *in vitro* and visualized with 2 OPC antibodies (NG2; green and PDGFR-α; red) (A-F). The vast majority of cells express both NG2 (A, B, D, E) and PDGFR-α (A, C, D, F). There was no difference in the percentage of OPCs (NG2 positive cells or PDGFR-α positive cells) that were in contact with myelin (A-C, I) compared to cells on a control substrate (D-F, I). Contact with myelin did not change the percentage of OPCs (NG2 positive cells and/ or PDGFR-α positive cells) expressing PDGFR-α (H). There was a small (3-4%), but significant increase in the percentage of OPCs expressing NG2 and decrease in the percentage of OPCs expressing NG2/ PDGFR-α when in contact with myelin (H). Contact with myelin did not affect the expression of PDGFR-α mRNA (I). Nuclei are stained with Hoechst (blue) and scale bars are 50μm (A-F). Error bars are standard error of mean (G, I). At least 2400 cells were counted for each group.

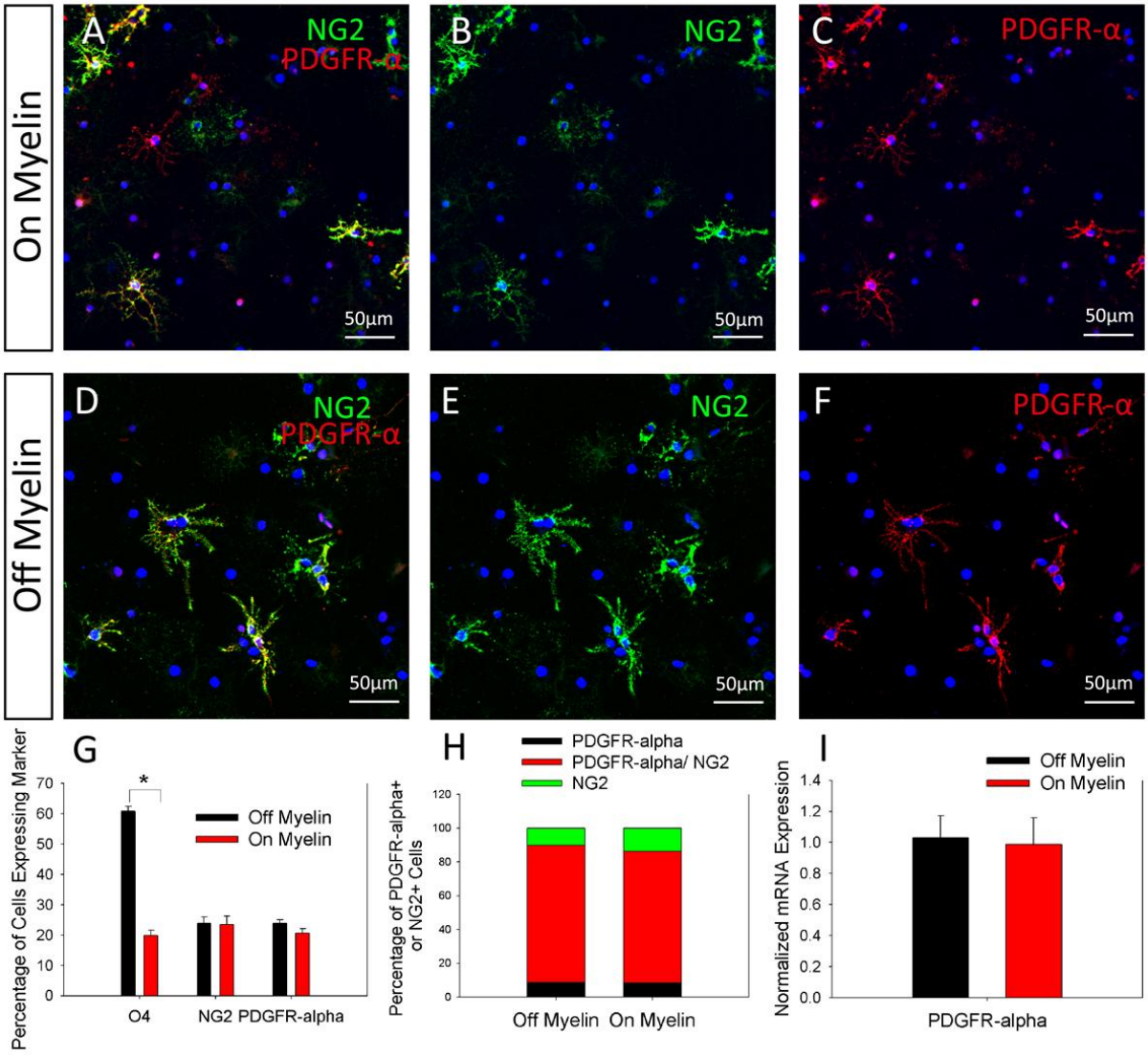

On Myelin
Off Myelin
A
NG2
PDGFR-α
B
NG2
C
PDGFR-α
D
NG2
PDGFR-α
E
NG2
F
PDGFR-α
50µm
G
Percentage of Cells Expressing Marker
Off Myelin
On Myelin
*
O4
NG2
PDGFR-alpha
H
PDGFR-alpha
PDGFR-alpha/ NG2
NG2
Percentage of PDGFR-alpha+ or NG2+ Cells
Off Myelin
On Myelin
I
Off Myelin
On Myelin
Normalized mRNA Expression
PDGFR-alpha

Figure 3.6 Contact with myelin did not alter the extent of proliferation. OPCs were cultured for 2 days *in vitro* in the absence of mitogens (A,B) and the presence of mitogens (C,D). OPCs expressing a cell cycle antigen (Ki-67; red) and an OPC marker (NG2; green) were measured. Relative to a control substrate, contact with myelin did not alter the proportion of proliferating (Ki-67+) OPCs (NG2+) in the presence or absence of mitogens compared to cells on a control substrate Nuclei are stained with Hoechst (blue) and scale bars are 50μm (A-D). Error bars are standard error of mean (E). At least 3000 cells were counted for each group.

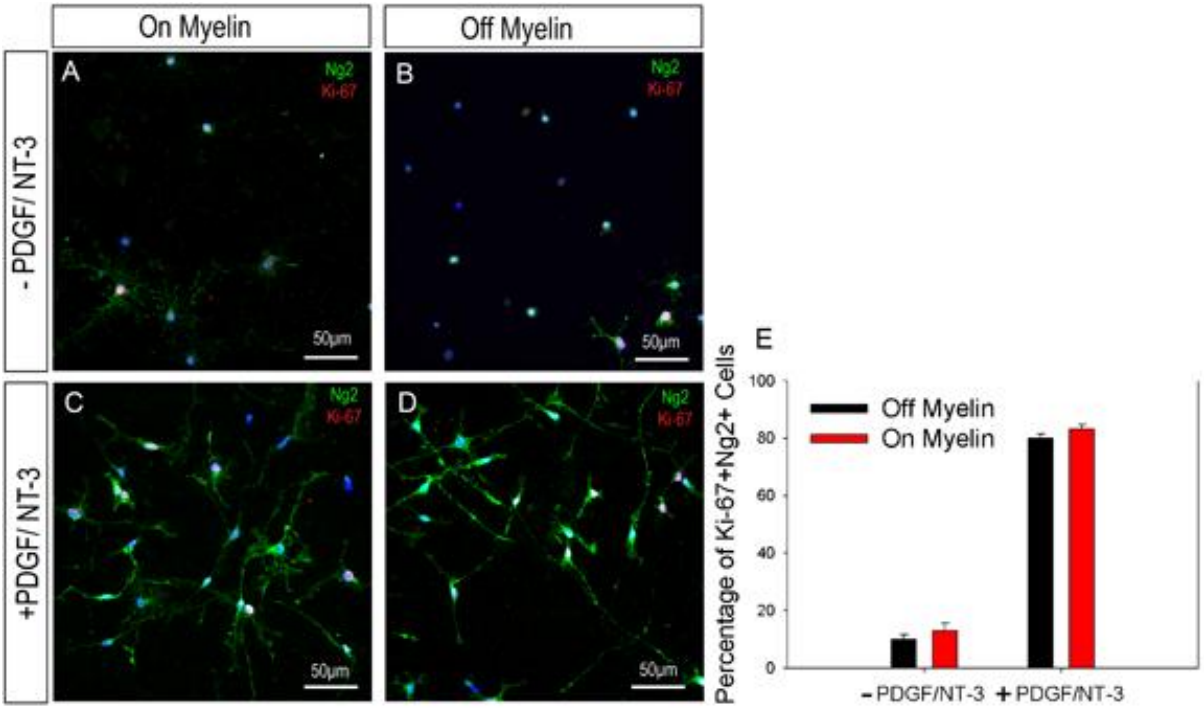


Figure 3.7 Myelin did not induce astrogenesis of OPCs or increase cell death. Contact with myelin significantly decreased the percentage of cells expressing either the immature oligodendrocyte marker O4 (red) or the OPC marker NG2 (green) (A, B). The percentage of astrocytes in contact with myelin (GFAP; green) was no different after 2 days *in vitro* when compared to astrocytes located on control substrates (C, D). At 4 days *in vitro*, there were significantly fewer astrocytes in contact with myelin (D). Live cells (AM; green) and dead cells (PI; red) were visualized at 1 day *in vitro* (E, F) and 3 days *in vitro* (G, H) when in contact with myelin (E, G) or a control substrate (F, H). Relative to a control substrate there was no difference in the percentage of living cells (I) or dead cells (J) that were in contact with myelin after 1 day *in vitro* suggesting that myelin was not toxic to oligodendroglia. When located on myelin, there was a significant increase in the percentage of living cells and a corresponding decrease in the percentage of dead cells when cells compared to cells located on a control substrate (I, J). Nuclei are stained with Hoechst (blue) (A, C) and scale bars are 50μm (A, C, E-H). Error bars are standard error of mean (B, D, I, J). At least 2400 cells were counted for each group.

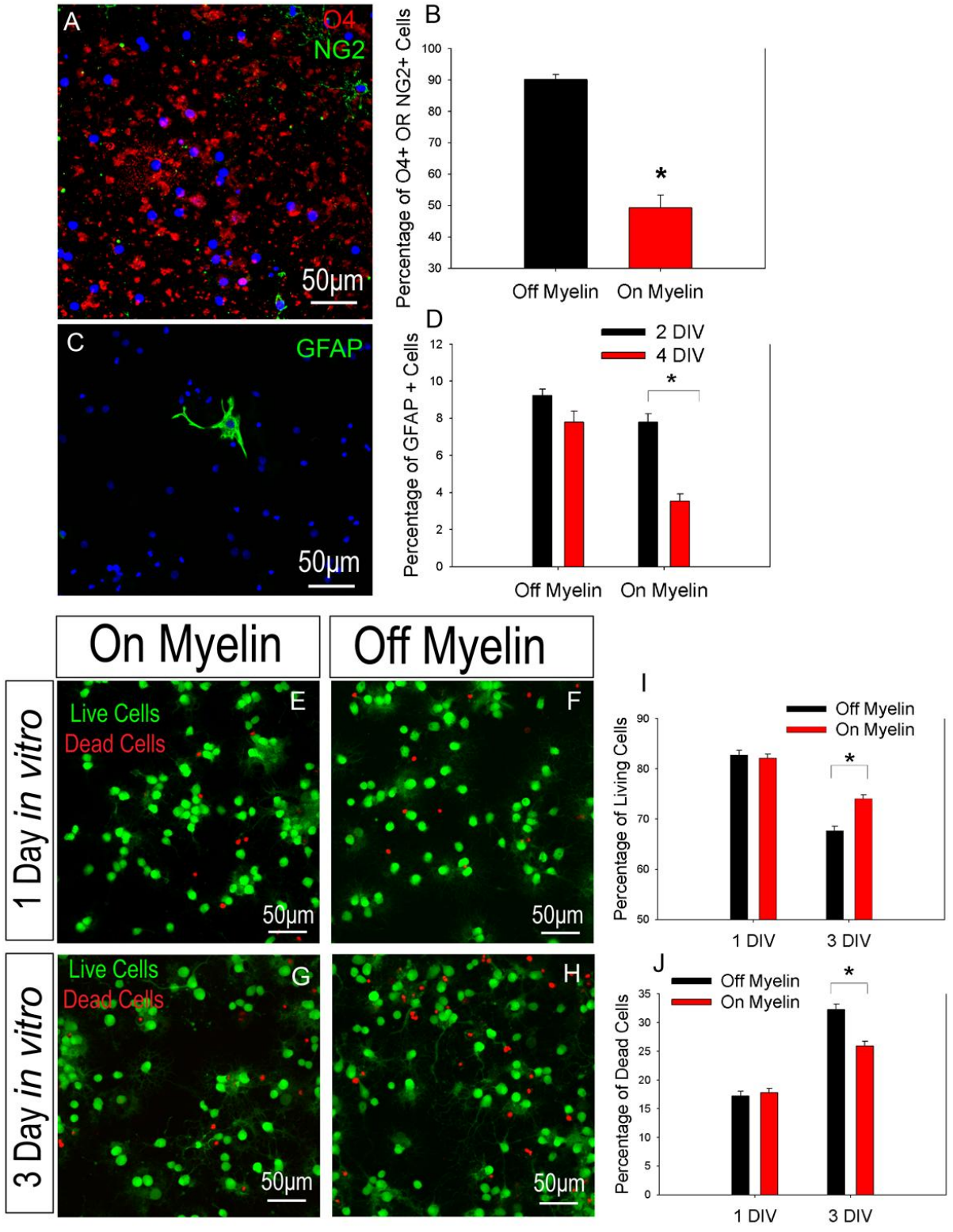

A
O4
NG2
50μm
B
Percentage of O4+ OR NG2+ Cells
Off Myelin
On Myelin
C
GFAP
50μm
D
Percentage of GFAP + Cells
2 DIV
4 DIV
Off Myelin
On Myelin
On Myelin
Off Myelin
1 Day in vitro
3 Day in vitro
Live Cells
Dead Cells
E
F
G
H
I
Percentage of Living Cells
1 DIV
3 DIV
J
Percentage of Dead Cells

Figure 3.8 Contact with myelin inhibited maturation in a dose-dependent manner. OPCs were cultured for 2 days *in vitro* on coverslips containing different concentrations of myelin spots: 50µg/ml (A, B), 250µg/ml (C, D), 500µg/ml (E, F) and 1000µg/ml (G, H). At higher concentrations of myelin (500µg/ml, 1000µg/ml) there was more suppression of O4 expression compared to lower concentrations of myelin (250µg/ml 50µg/ml) (I). Likewise, there was more suppression of O4 expression at 250µg/ml than 50µg/ml. The percentage difference of NG2 was comparable at all concentrations of myelin (J). Nuclei are stained with Hoechst (blue) and scale bars are 50µm (A-H). Error bars are standard error of mean (I, J). At least 1000 cells were counted for each group

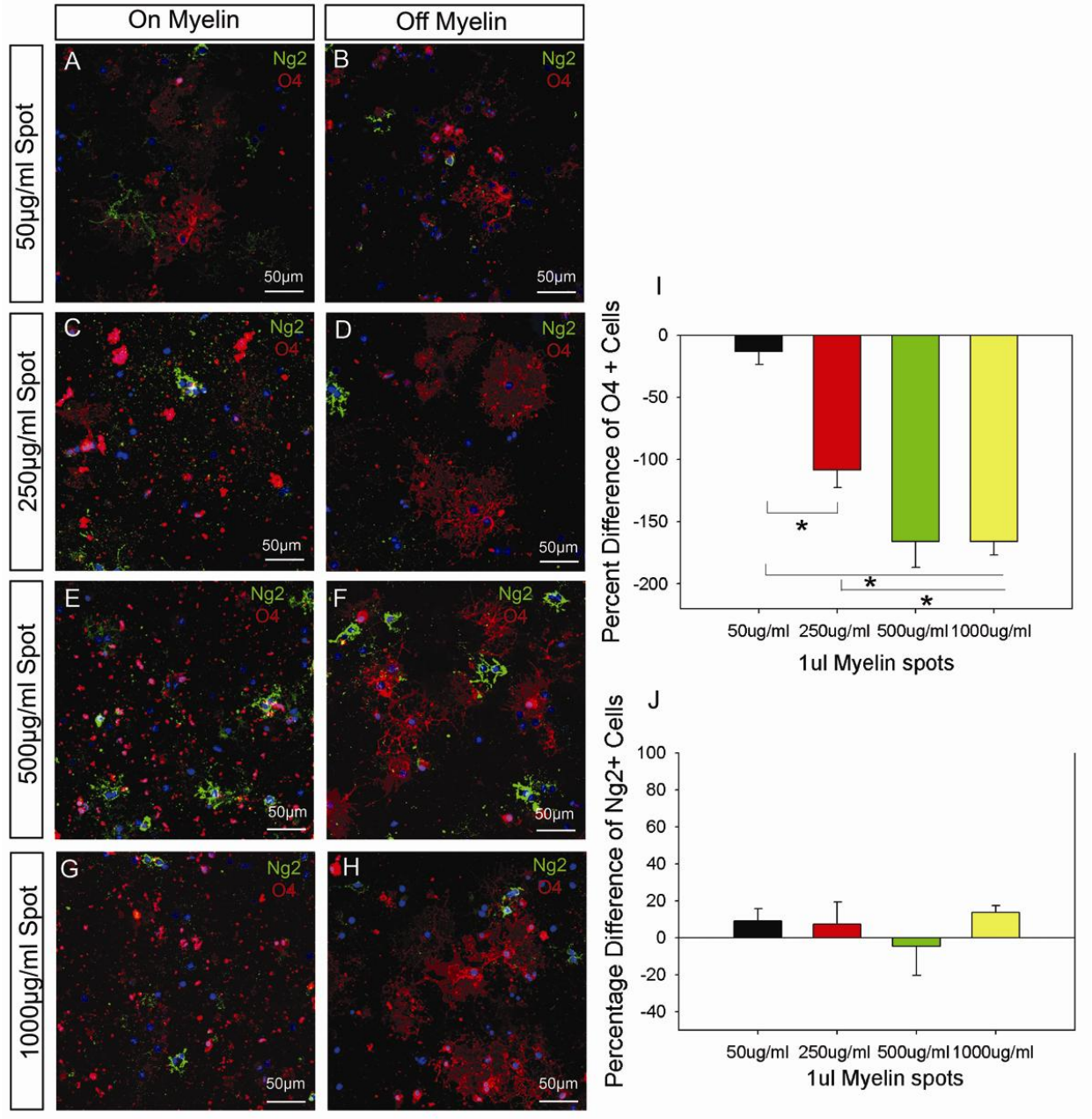

On Myelin
Off Myelin
50µg/ml Spot
250µg/ml Spot
500µg/ml Spot
1000µg/ml Spot
A
B
C
D
E
F
G
H
Ng2
O4
50µm
I
Percent Difference of O4 + Cells
0
-50
-100
-150
-200
*
*
*
50ug/ml 250ug/ml 500ug/ml 1000ug/ml
1ul Myelin spots
J
Percentage Difference of Ng2+ Cells
100
80
60
40
20
0
-20
-40
50ug/ml 250ug/ml 500ug/ml 1000ug/ml
1ul Myelin spots

Figure 3.9 Contact with myelin increased the production *id2* and *id4* mRNA. To understand better how myelin inhibited oligodendroglial maturation, the mRNA expression was measured for transcription factors that are known to inhibit oligodendrocyte development (A). When cultured on myelin, the expression of *hes1, hes5* and *sox6* is unchanged in oligodendrocyte lineage. Oligodendroglia located on myelin have a significant increase in *id2* mRNA expression and an increase in *id4* mRNA in 4/5 samples (p= 0.1). Contact with myelin was also associated with decreased expression of s*ox10* and *mrf* mRNA. To determine the timing of *id2* and *id4* regulation during development, OPCs were expanded in mitogens for 1 week before being switched into media lacking mitogens to induce differentiation (C, D). After 2 days *in vitro,* there was no difference in the expression of *id2* and *id4* mRNA compared to OPC (C). After 4 days *in vitro,* there was a significant decrease in the production of *id2* mRNA (C). At 4 days *in vitro* there was also a significant increase in the production of *mbp* mRNA compared to OPCs in differentiating conditions for 2 days *in vitro* (D). Error bars are standard error of mean (A, B).

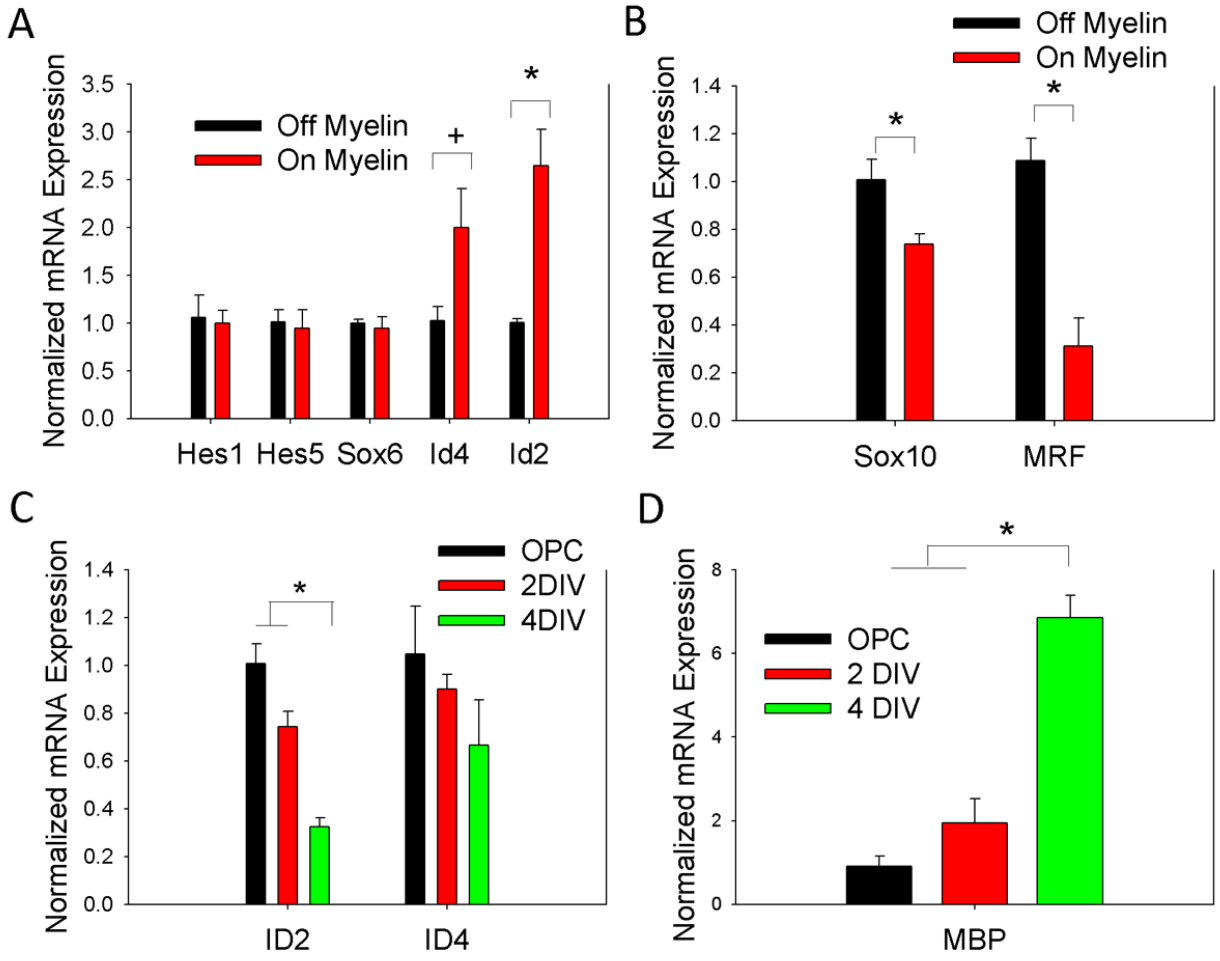
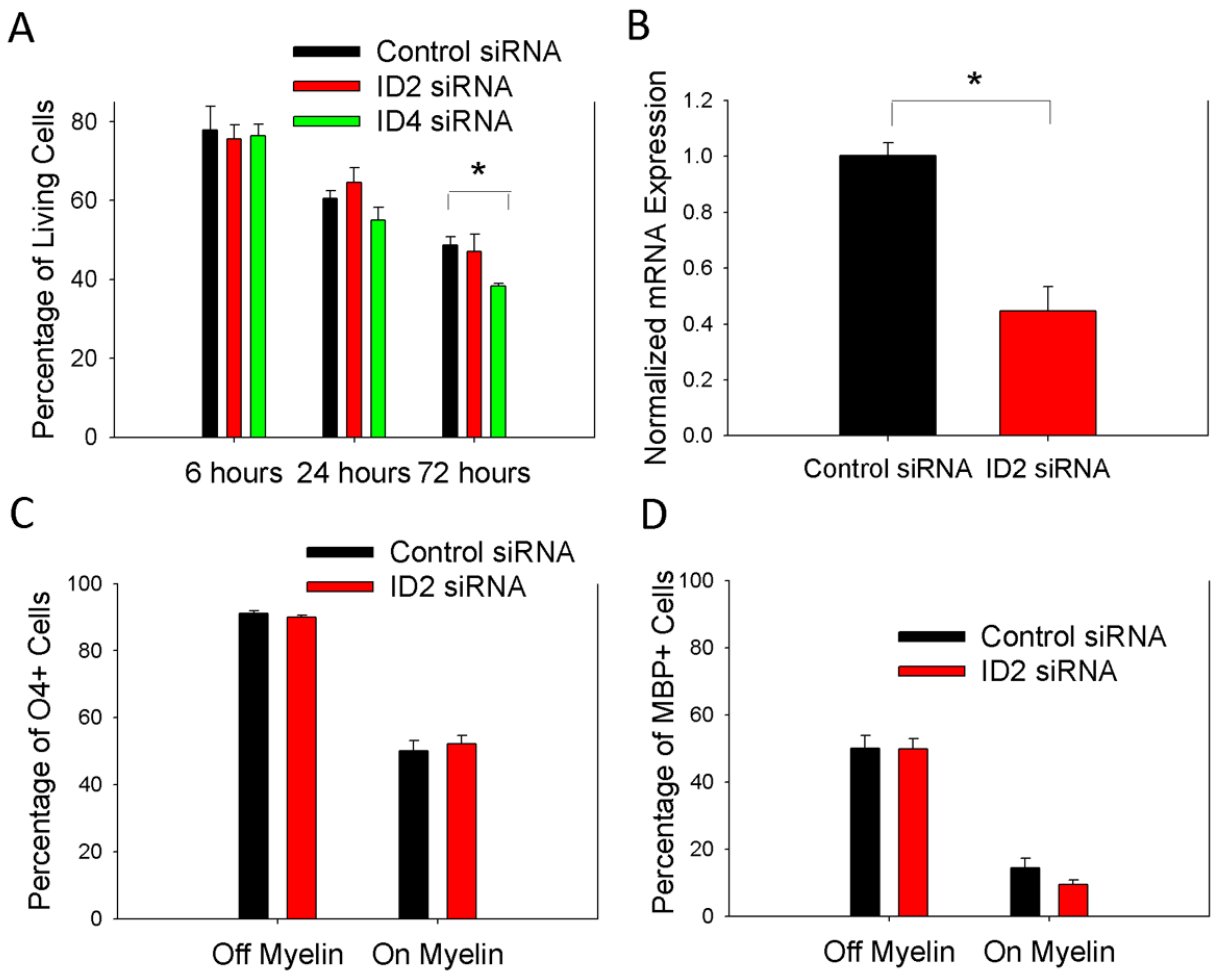

A
Normalized mRNA Expression
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Off Myelin
On Myelin
+
*
Hes1
Hes5
Sox6
Id4
Id2
B
Off Myelin
On Myelin
Normalized mRNA Expression
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
*
Sox10
MRF
C
Normalized mRNA Expression
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
OPC
2DIV
4DIV
*
ID2
ID4
D
Normalized mRNA Expression
8
6
4
2
0
*
OPC
2 DIV
4 DIV
MBP

Figure 3.10 ID2 expression is sufficient, but was not necessary, to block oligodendrocyte development in response to myelin. OPCs were transfected with either a pool of non-targeting siRNA or a pool of siRNA targeting ID*2* or ID4 and cultured for 6, 24 and 72 hours. The percentage of living and dead cells was measured (A). At 72 hours after transfection with a pool of siRNA targeting ID4, there was a significant decrease in the percentage of living cells compared to cells transfected with a pool of non-targeting siRNA or a pool of siRNA targeting ID2 (A). Transfecting OPCs with a pool of siRNA targeting ID2, significantly decreased the expression of *id2* mRNA compared to cells transfected with a pool of non-targeting siRNA after 24 hours *in vitro* (B). Transfection with a pool of siRNA targeting ID2 did not increase the percentage of cells labelled with immature oligodendrocyte markers (C) or mature oligodendrocyte markers (D) compared to cells transfected with a pool of non-targeting siRNA when cells were located on myelin or on a control substrate. Error bars are standard error of mean (A-D). At least 2200 cells were counted for each group.


A
Control siRNA
ID2 siRNA
ID4 siRNA
Percentage of Living Cells
0
20
40
60
80
*
6 hours
24 hours
72 hours
B
Normalized mRNA Expression
0.0
0.2
0.4
0.6
0.8
1.0
1.2
*
Control siRNA
ID2 siRNA
C
Control siRNA
ID2 siRNA
Percentage of O4+ Cells
0
20
40
60
80
100
Off Myelin
On Myelin
D
Control siRNA
ID2 siRNA
Percentage of MBP+ Cells
0
20
40
60
80
100
Off Myelin
On Myelin

Chapter 4

General Discussion

4.1 Summary of thesis

In this thesis I examined two strategies to promote remyelination: endogenous and exogenous repair. SCI results in substantial oligodendrocyte death (Abe et al. 1999; Casha et al. 2001; Crowe et al. 1997; Li et al. 1999; Liu et al. 1997) and demyelination (Blight 1983; Bresnahan et al. 1976; Guest et al. 2005; Kakulas 1999; Norenberg et al. 2004; Powers et al. 2012; Smith and Jeffery 2006; Totoiu and Keirstead 2005). Remyelination is hypothesized as a means to improve conduction and rescue vulnerable axons. Cell replacement strategies have been widely investigated following SCI (Tetzlaff et al. 2011), where numerous types of neural precursor or progenitor cells have been transplanted after SCI. However, the optimal cell type for transplantation remains to be determined. Fetal and adult neural tissues have substantially lower risk of tumour formation relative to cells derived from embryonic stem cells, and thus appear to be an attractive source for oligodendrocyte precursors. It must be noted, however, that these sources (particularly those of fetal origin) are not without logistical, ethical and legal concerns that may hinder their clinical translation. PRPs are multipotent, capable of producing neurons, oligodendrocytes and astroctyes *in vitro* (Chojnacki and Weiss 2004; Gregg et al. 2007). In Chapter 2, it was investigated whether murine PRPs differentiated into remyelinating oligodendrocytes and improve functional recovery after SCI.

Transplanted PRPs integrated into host tissue, differentiated into extensively-branched mature oligodendrocytes that ensheathed multiple axons and produced mature myelin. PRP-derived oligodendrocytes also produced compact myelin with normal periodicity following transplantation into dysmyelinated Shiverer mice, a well-characterized model used to test the myelinating potential of CNS transplant candidate cells (Gansmuller et al. 1986; Lachapelle et al.

1983; Liu et al. 2000; Maire et al. 2009; Vitry et al. 2001; Warrington et al. 1993; Windrem et al. 2004). Thus, PRP-derived oligodendrocytes were capable of generating mature myelin sheaths on nude/denuded CNS axons. To my surprise, although transplanted PRPs efficiently produced oligodendrocytes in the injured spinal cord, there was no significant increase in the total number of myelinated axons in PRP-transplanted animals versus media-injected control animals. Similarly, there was no improvement in functional recovery following transplantation in two separate behavioural experiments.

The fact that the transplantation of myelinating cells failed to provide functional recovery in the present study may carry interesting implications for the nature of chronic demyelination in our model of SCI. If chronically demyelinated axons exist and the remyelination of those axons fails due to a deficit in the endogenous OPCs, then the addition of exogenous precursors that myelinate would be expected to improve total myelin content and, presumably, locomotor recovery following SCI. Given that I did not find significant increases in total myelin or behavioural recovery after PRP treatment, one may speculate that the endogenous precursors provide a sufficient level of spontaneous remyelination in our injury model. Support for this notion comes from the finding that traced axons, which bypass the lesion site in a chronic SCI, are not demyelinated but likely undergo remyelination (Lasiene et al. 2008; Powers et al. 2012). An alternative possibility is that the same extrinsic factors that block remyelination by endogenous precursors may also block the remyelination by transplanted PRPs or their progeny. There are many known factors that impair myelination: LINGO (Mi et al. 2007; Mi et al. 2004; Mi et al. 2005), BMPs (Cheng et al. 2007; Gao et al. 2006), Notch ligands (Givogri et al. 2002;

Wang et al. 1998), and myelin (Baer et al. 2009; Kotter et al. 2006). Since some of these factors are present following SCI, remyelination might be improved by targeting these factors directly.

Remyelination is deemed critical because denuded axons not only lack the myelin necessary to achieve normal conduction velocity, but are also at increased risk of degeneration, which leads to permanent functional loss (Irvine and Blakemore 2008). A more rapid remyelination after SCI is thus hypothesized to spare more axons from axonal degeneration, ultimately sparing neurological circuitry from the secondary damage that continues in the days and weeks following SCI. Blocking known inhibitors of oligodendrocyte differentiation or maturation in the face of remyelination inhibitors following SCI should promote myelin repair. Promoting improved endogenous remyelination also circumvents the ethical and logistical concerns surrounding transplantation.

In Chapter 3, I adapted a common strategy to promote tissue or cellular regeneration by attempting to block the influence of an inhibitory molecule. Myelin inhibits expression of immature oligodendrocyte markers *in vitro,* and infusion of myelin inhibits remyelination (Kotter et al. 2006; Robinson and Miller 1999). Given the presence of myelin debris following demyelination and trauma, even years following the initial trauma in humans (Buss et al. 2005), I hypothesized that myelin debris inhibits remyelination after SCI. I developed an approach to block the inhibitory influence of myelin on OPC maturation: a spot assay where OPCs are grown in culture with spots of myelin, allowing me to measure OPC maturation both on and off this inhibitory substance. Using this approach, I found that on myelin there is a near 3-fold decrease in the percentage of cells expressing markers characteristic of immature and mature oligodendrocytes, as well as a 5-fold decrease in the gene expression of myelin markers.

Associated with this decrease in the percentage of cells expressing markers of oligodendrocyte maturation, there was also a higher proportion of cells with simple morphologies and relatively fewer cells with the characteristic complex oligodendrocyte morphologies. This decrease in cells expressing mature markers when in contact with myelin was not due to increased cell death as there was actually a small, albeit significant, increase in the percentage of living cells after 3 days *in vitro* on myelin. Conversely, there was no change in the percentage of cells expressing the OPC marker NG2, and no difference in OPC proliferation on-versus-off myelin. Myelin, therefore, does not extend OPC proliferation in the absence of mitogens, which is one major mechanism to block the differentiation of an OPC into an immature oligodendrocyte. Instead, myelin inhibited process complexity and the production of immature and mature oligodendrocyte markers.

To understand how myelin inhibits maturation, I measured the gene expression of a number of well-characterized OPC-specific transcription factors that negatively regulate oligodendrocyte development. The expression of two transcription factors known to prevent OPC differentiation and maturation were increased in oligodendroglia in contact with myelin: ID2 and ID4. Since overexpression of ID2 and ID4 in OPCs severely decreases the percentage of cells expressing mature oligodendrocyte markers (Kondo and Raff 2000; Wang et al. 2001), increased ID2 and ID4 expression due to myelin is sufficient to block oligodendrocyte lineage cell maturation. This provides a mechanistic understanding of how myelin inhibits oligodendrocyte maturation.

4.2 Transplantation vs promoting endogenous repair

Despite the findings in this thesis, transplantation has been demonstrated to be an effective strategy to improve functional recovery after SCI. Of the 28 studies reviewed by Tetzlaff and colleagues that assessed behavioural outcomes following transplantation of neural progenitor cells or glial precursor cells in animal models of SCI, 23 demonstrated improvements in functional recovery (2011). Although it is still unclear as to the mechanism(s) by which transplantation improves function, one important proposed mechanism is via accelerating remyelination.

In contrast to transplantation, it might be possible to accelerate remyelination by enhancing the ability of endogenous OPCs to be recruited and differentiated into oligodendrocytes. This particular approach has several benefits in comparison to cellular transplantation. First, cellular transplantation of neural progenitors or glial precursors would require individuals receiving the transplantation to be immunosuppressed for indefinite periods. Second, the cellular source for transplantation can also be problematic. Cells derived from embryonic stem cells, which expand relatively easily, have the potential to produce embryonic tumours when transplanted (Reubinoff et al. 2000; Thomson et al. 1998). Alternatively, primary human fetal or adult neural tissue are difficult to obtain, and human neural tissue expands more slowly in culture relative to their murine counterparts (Chojnacki et al. 2008), making it difficult to generate enough cells for clinical treatment. These logistic, and perhaps even ethical, considerations surrounding transplantation hinder its ready translation to clinical practice. Transplantation is, after all, an experimental approach that has been tested for over two decades

but has yet to see meaningful clinical translation. Improving remyelination by harnessing the endogenous regenerative process avoids these concerns.

4.3 Future directions

4.3.1 Cellular transplantation after SCI

In Chapter 2, PRPs from mice were transplanted following SCI in rats. While no functional benefits were observed, this may have been due to species differences, which could be tested by autologous or allogeneic transplantation of mouse PRPs. If transplantation of PRPs were ever to be translated into a clinical therapy for SCI patients, it would be absolutely critical to determine the efficacy of transplanting fetal or adult human PRPs (Chojnacki et al. 2008) after SCI. Human-derived PRPs might provide benefits that mouse PRPs do not, thus it is important to also evaluate the efficacy of transplanting human PRPs in rat models of SCI. EGF- and/or FGF-responsive precursors are the source of many transplantation studies as they consistently generate oligodendrocytes from rodent tissue. However, human EGF- and/or FGF-responsive precursors have a much lower capacity for generating oligodendrocytes *in vitro* compared to human PRPs (Chojnacki et al. 2008; Horiguchi et al. 2004; Ostenfeld et al. 2002). Human PRPs are therefore a more appropriate source of oligodendrocytes than the classical EGF- and/or FGF-responsive precursors.

Remyelination is likely one of several benefits associated with cellular transplantation. For example, hESC-derived OPCs, which promote functional recovery following SCI (Keirstead 2005; Sharp et al. 2010), can secrete trophic factors *in vitro* and presumably also *in vivo* (Zhang

et al. 2006b). Transplanted cells can also potentially modulate the immune system (Pluchino et al. 2003; Pluchino et al. 2005; Ziv et al. 2006) or improve endogenous remyelination (Einstein et al. 2009). Given that astrocytes are often produced by transplanted cells (Tetzlaff et al. 2011) and BMP-treated astrocytes improve function after SCI (Davies et al. 2006; Davies et al. 2008; Davies et al. 2011), astrocyte production is also a potential general mechanism of improved function by cell transplantation. Transplant-derived astrocytes potentially improve glutamate uptake, provide metabolites, participate in synaptic plasticity, regulate ion balance (Seth and Koul 2008; Sofroniew 2005), or participate in production of the glial scar, which is thought to be important in restricting damaging-immune cells early after injury (Okada et al. 2006). The mechanisms of cellular transplantation need to be more adequately defined. Transplantation of neural progenitor cells (NPC) isolated from Shiverer mice (which do not form compact myelin) did not confer the same benefits as the NPC cells from wild-type animals (Yasuda et al. 2011). This suggests that remyelination may be one benefit of transplanting NPCs. How cellular transplantation improves function could be investigated by transplanting cells that are deficient in candidate neurotrophins, glutamate transporters, cytokines or chemokines. If defined mechanisms can be better elucidated, it might be possible to target these mechanisms with pharmaceuticals or by infusion of key molecules.

4.3.2 Myelin inhibition of remyelination after SCI: an unproven hypothesis

In Chapter 3, I hypothesized that myelin inhibits remyelination after SCI, yet more work remains to completely test this hypothesis. The failure of pooled siRNA directed towards ID2 to improve the maturation of oligodendrocyte lineage cells in contact with myelin suggests that ID2 is not an ideal target to disinhibit OPCs in contact with myelin. From Chapter 3, as well as the

work of Syed and colleagues, it appears that unknown proteins within myelin make contact with receptors on OPCs or immature oligodendrocytes to block their maturation (2008). The myelin components that inhibit oligodendrodroglial maturation are different from the major myelin components that inhibit axonal regeneration (Syed et al. 2008), indicating that the specific myelin-derived protein(s) that inhibit oligodendroglial maturation have yet to be determined. By determining such protein(s) inhibitors in myelin, their function(s) or their interaction(s) with potential receptors, their function can be antagonized or blocked. To this end, proteomic analysis has been conducted to develop a short-list of potential myelin-associated inhibitors of oligodendroglial maturation (Baer et al. 2009). More work is required to identify candidates from this short-list. Given the presence of known receptors/co-receptors mediating myelin inhibitors of axonal regeneration on oligodendroglia such as LINGO-1 and p75, one may speculate that such factors participate in myelin inhibition of oligodendroglia maturation. Upon determining either the protein(s) within myelin or their receptors, it will be important to antagonizing these signals after injury and determine their role in remyelination after SCI.

4.4 Endogenous repair after SCI

The primary and secondary damage following SCI presents unique conditions for remyelination and should be investigated more thoroughly. While much is known about oligodendrocyte development, less is known about remyelination and far less regarding remyelination following SCI.

4.4.1 Recruitment after SCI and potential therapies

To enhance recruitment of OPCs after SCI, a greater understanding of the sources of OPCs, as well as the factors controlling their proliferation and migration is required. After SCI there are separate pools of proliferating cells that potentially contribute to remyelination. Ependymal cells proliferate rapidly after injury (Barnabe-Heider et al. 2010; Horner et al. 2000; Johansson et al. 1999; Meletis et al. 2008) and when isolated *in vitro* are multipotential, capable of producing astrocytes, oligodendrocytes, and neurons. They are also able to self-renew in response to the growth factors EGF and FGF (Barnabe-Heider et al. 2010; Johansson et al. 1999; Meletis et al. 2008). After a stab injury, ependymal cells proliferate/migrate into regions of injury; however, the vast majority of these cells express markers indicative of ependymal cells or astrocytes and only a small number of ependymal cells differentiate into oligodendroglial cells (Barnabe-Heider et al. 2010; Meletis et al. 2008). The stab injury does not, however, model the demyelination observed after a contusive SCI (Siegenthaler et al. 2007), suggesting the importance of ependymal cells during demyelinating injuries might be understated. The majority of oligodendrocytes formed after SCI most likely arise from proliferating NG2-positive OPCs that are present throughout the parenchyma and readily proliferate after SCI (Horky et al. 2006; Lytle et al. 2009; McTigue et al. 2001; Zai and Wrathall 2005). The capacity and extent of OPC-derived remyelination still requires genetic mapping of OPCs prior to SCI. It remains to be determined the extent to which OPCs, ependymal cells or other potential spinal cord precursors, such as radial glia remyelinated after SCI.

To test the importance of OPC recruitment after SCI, a better understanding of the mitogens driving OPC proliferation is required. PDGF is a an essential mitogen of OPCs in

development (Barres and Raff 1993; Barres et al. 1993; Calver et al. 1998; Fruttiger et al. 1999) and remyelination (Hinks and Franklin 1999; Woodruff et al. 2004) and would be expected to serve a similar role after SCI. However, to my knowledge, PDGF expression early after injury has not been measured. PDGF overexpressing mice increase OPC proliferation in development (Calver et al. 1998) and following demyelination (Woodruff et al. 2004), suggesting that PDGF may also increase OPC proliferation after SCI. PDGF-overexpressing mice have elevated OPC numbers during demyelination (Woodruff et al. 2004) and are therefore a useful animal model to determine the potential benefits of increased OPC density and PDGF overexpression following SCI. This experiment would also provide a useful proof-of-principal study as to the relative importance of OPC proliferation after SCI.

Other OPC mitogens are known to increase after SCI and might account for OPC proliferation. For example, bFGF levels increase early after SCI (Koshinaga et al. 1993; Mocchetti et al. 1996; Tripathi and McTigue 2008) and bFGF is known to induce proliferation in OPC cultures (Barres et al. 1993; Bogler et al. 1990; Eccleston and Silberberg 1985). Likewise, IGF1 can also promote proliferation (Barres et al. 1993; McMorris and Dubois-Dalcq 1988) when in combination with another OPC mitogen (Barres and Raff 1994). IGF1 is expressed at 3 weeks post injury (Hawryluk et al. 2012). However, OPC proliferation occurs rapidly after SCI and it is unclear how early IGF1 expression is increased. CNTF may play a role in OPC proliferation after SCI given that it induces OPC proliferation *in vitro* and *in vivo* during development (Barres et al. 1996), and is increased early after SCI (Tripathi and McTigue 2008; Zai et al. 2005). The neuregulin family member glial growth factor (GGF) also promotes OPC proliferation *in vitro* (Canoll et al. 1996; Shi et al. 1998) and its expression is elevated early after

injury (Zai et al. 2005). Conducting gain and loss of function experiments with these mitogens will help determine their role in the recruitment of OPCs after SCI and could help determine if recruitment after SCI is a rate-limiting step in remyelination. OPCs are responsive to a plethora of factors and adding a combination of mitogens such NT-3/BDNF (McTigue et al. 1998) or GGF/FGF (Lytle et al. 2009) results in increased proliferation after SCI, as well as increased oligodendrogenesis (Lytle et al. 2009; McTigue et al. 1998). A combination of mitogens is thus likely to drive OPC proliferation after SCI. Increasing OPC proliferation is potentially therapeutically beneficial after SCI considering that systemic GGF or GGF/FGF increases OPC proliferation and oligodendrogenesis, resulting in improved functional recovery after SCI (Whittaker et al. 2012).

4.4.2 Oligodendrocyte differentiation after SCI and potential therapies

The factors controlling remyelination after SCI are currently unknown. Several factors are known to inhibit remyelination after a focal demyelination, yet their specific functions after SCI remain unclear. For instance, the expression of molecules capable of inhibiting myelination such as PSA-NCAM, hyaluronan, and notch ligands delta or jagged has not, to my knowledge, been measured after SCI. Anti-LINGO-1 treatments increase myelination in culture (Mi et al. 2009) and increase remyelination after focal demyelination (Mi et al. 2007; Mi et al. 2009). Anti-LINGO-1 treatment also improves functional recovery following SCI, while this is largely attributed to the role of LINGO-1 in mediating the inhibition of axons due to myelin proteins (Ji et al. 2006). Semaphorin 3A is another potential remyelination inhibitor relevant to SCI considering that it inhibits differentiation *in vitro* (Syed et al. 2011). The role of semaphorin 3A *in vivo* is less clear as semaphorin 3A infusion inhibits remyelination (Syed et al. 2011).

However, semaphorin 3A overexpression with a lentivirus does not inhibit remyelination (Piaton et al. 2011). Semaphorin 3A expression peaks one week after SCI in regions associated with gliosis, such as areas within or adjacent to the lesion epicenter (Kaneko et al. 2006). Treatment with a semaphorin 3A inhibitor increased regeneration of serotenerigic fibres following a transection, suggesting that semaphorin 3A impedes axonal regeneration (Kaneko et al. 2006). In view of the lack of demyelination of spared axons after a transection injury (Siegenthaler et al. 2007), semaphorin 3A inhibition in a contusion model of SCI would be needed to determine the role of semaphorin 3A in remyelination.

Overactive Wnt signalling inhibits developmental myelination (Fancy et al. 2009; Fancy et al. 2011b; Ye et al. 2009) and remyelination (Fancy et al. 2009; Fancy et al. 2011b). After focal demyelination, attempts to attenuate Wnt signalling in OPCs by the stabilization of axin2 with a tankyrase inhibitor (Huang et al. 2009) increased oligodendrocyte differentiation in culture and improves remyelination (Fancy et al. 2011a). What remains to be determined is why dampening Wnt signalling improves remyelination. The heightened Wnt signalling after demyelination suggests that a Wnt agonist(s) is (are) present following focal demyelination and is(are) actively inhibiting remyelination. Yet if this is true, what is (are) the Wnt agonist(s)? It stands to reason that similar cues might be present following SCI. Thus, it would be advantageous to determine the extent of Wnt signalling in OPCs after SCI, as well as to determine if attenuating Wnt signalling improves functional recovery or remyelination after SCI.

The immune response is important to promote remyelination after focal demyelination (see Chapter 1). For instance, tumour necrosis factor-α (TNF- α) (Arnett et al. 2001; Arnett et al. 2003) and interleukin 1β (IL-1β) (Mason et al. 2001) are necessary for remyelination after focal

demyelination. TNF- α and IL-1β are proinflammatory cytokines that are important in initiating a cascade of other cytokines to stimulate an immune response and are generally thought to be detrimental after SCI (Esposito and Cuzzocrea 2011). After SCI, TNF-α and IL-1β levels peak early after injury in animal models (Stammers et al. 2012), but are not detectable in human CSF as late as 36 hours after injury (Kwon et al. 2010). TNF-α might be dispensable after SCI, as TNF-α-deficient mice have no improvement or worsening of functional recovery compared to wild type control animals after SCI (Farooque et al. 2001). Whether there is altered remyelination after SCI in TNF-α-deficient mice remains to be determine.

Hormones can promote remyelination. Pregnancy, increases remyelination *in vivo*, in part by upregulation of the peptide hormone prolactin (Gregg et al. 2007). Likewise, remyelination is improved after cuprizone-mediated demyelination by administration of the thyroid hormone triiodothyronine (T3) (Harsan et al. 2008). Other hormone receptors are involved in remyelination. The retinoid x receptor (RXR), a nuclear receptor family member, is increased in oligodendroglia during remyelination (Huang et al. 2011). RXRs heterodimerize with vitamin D receptors, thyroid hormone receptors, peroxisome proliferation activator receptors and liver X receptors to regulate numerous cellular processes (Germain et al. 2006). RXR-γ-deficient oligodendrocyte demonstrate impaired maturation, while the RXR agonist 9-cis retinoic acid improve oligodendrocyte maturation in culture (Huang et al. 2011). In addition, 9-cis retinoic acid treatment after focal demyelination is also sufficient to improve remyelination (Huang et al. 2011). It will be of great interest to determine if other agonists of nuclear hormone receptors also increase remyelination, considering that RXR heterodimerizes with many other nuclear receptors, which can then increase the promiscuity of its binding partners (Dawson and Xia

2012). To my knowledge, the efficacy of RXR agonists or prolactin following SCI has also yet to be determined. T3 treatment is beneficial after SCI, but it is unclear if this is due to improvements in remyelination or axonal plasticity (Tator et al. 1983; Tator and van der Jagt 1980).

4.5 Is remyelination a target after SCI?

One general hypothesis that remains central to this thesis is that improving remyelination after SCI is beneficial to the recovery of function. This is largely based on two principal findings: demyelination occurs after SCI and transplantation of neural precursor/progenitor cells can improve the extent of remyelination and functional recovery. The notion that improving remyelination can serve as a therapeutic strategy after SCI is attractive because it provides several targets for therapeutic intervention. It should still be mentioned, however, that this hypothesis is based on core assumptions that are not sufficiently tested.

4.5.1 Does remyelination spares axons from axonal degeneration?

Despite the many potential benefits of remyelination, the link between remyelination and the enhanced sparing of axons is indirect. Demyelination places axons in a vulnerable state (Trapp and Stys 2009; Waxman 2006), but does this continued vulnerability result in increasing axonal damage and eventual degeneration? Surprisingly, there is very little evidence to demonstrate a direct link between long-term demyelination and increased axonal degeneration. There is a correlation between remyelination and behavioural recovery in demyelinating models (Jeffery and Blakemore 1997; Liebetanz and Merkler 2006), as well as a correlation between

remyelination efficiency and axonal degeneration as older animals remyelinate at a slower rate (Hinks and Franklin 2000; Shields et al. 1999) and have increased 'demyelination-induced' axonal degeneration (Irvine and Blakemore 2006). Conversely, the increase in axonal degeneration in older animals is potentially explained by increased gliosis and/or an altered immune response.

Facilitating remyelination by transplanting myelinating cells is another approach that has proven to be neuroprotective in animal models of demyelination (Totoiu et al. 2004). However, those cells also secrete neurotrophins (Zhang et al. 2006b) and modulate the immune system (Pluchino et al. 2005; Ziv et al. 2006). Perhaps the best evidence that remyelination protects axons from demyelination-mediated axonal degeneration comes from Irvine and colleagues (2008) who blocked remyelination by X-irradiating animals to kill all dividing cells, including the OPCs. These authors found that after injury, the X-irradiated animals had more axonal loss and axonal degeneration compared to the non-irradiated control animals (Irvine and Blakemore 2008). This enhanced axonal loss and degeneration was rescued if animals were transplanted with neural progenitor cells prior to demyelination. This supports the role of remyelination in axonal preservation, but the results are confounded by X-irradiation which disrupts the blood brain barrier (Diserbo et al. 2002; Li et al. 2004b), induces astrogliosis (Wilson et al. 2009) and cell death (Ben Abdallah et al. 2007), and impairs the immune response, all of which are important for remyelination (Bieber et al. 2003; Kotter et al. 2001; Kotter et al. 2005).

These findings highlight the need to determine the fate of persistently demyelinated axons by means that will allow more specific impairment of remyelination. Such an experiment could be conducted by assessing axonal degeneration in mice that have genetic defects that delay

remyelination, such as in Olig1-deficient mice (Arnett et al. 2004). These experiments are, on the other hand, confounded by developmental defects. Alternatively it is possible to induce a conditional removal of factors that are necessary for oligodendrocyte differentiation or maturation prior to demyelination. Two laboratories have already developed mice that express a tamoxifen inducible cre-recombinase specifically within OPCs to allow conditional removal of genes that are flanked by loxP sites (so called "floxed" mice) (Kang et al. 2010; Rivers et al. 2008). By breeding mice containing the inducible cre-recombinase in OPCs with mice 'floxed' for important transcription factors in oligodendrocyte development such as Olig2 (Chen et al. 2008; Yue et al. 2006) or MRF (Emery et al. 2009), it will be possible to specifically block or delay remyelination. This approach, in addition to lacking developmental confounds, may also help determine the time course of degeneration and axonal loss. In principle, mice given tamoxifen ablate the 'floxed' gene, suggesting the possibility of controlling the length of remyelination by adjusting the tamoxifen dosage. A high tamoxifen dosage will remove the 'floxed' transcription factor in as many as 80-90% of OPCs prior to demyelination, but the remaining unaffected OPCs are predicted to proliferate and eventually remyelinate. By giving a smaller dose of tamoxifen, fewer OPCs will remove the 'floxed' transcription factor. With fewer OPCs impaired, remyelination is predicted to occur more rapidly. This strategy might be instrumental in demonstrating the degree of axonal degeneration associated with impaired or aborted remyelination. It might also give insight into the timeframe of axonal degeneration with prolonged demyelination. Conditionally removing a 'floxed' transcription factor from OPCs during remyelination after SCI would also prove instrumental in determining the fate of demyelinated axons after injury.

4.5.2 Can endogenous remyelination can be improved after SCI?

There is evidence from numerous sources that demyelination occurs after SCI in both animals (Blight 1983; Blight 1985; Bresnahan et al. 1976; Gledhill et al. 1973a; James et al. 2011; Lasiene et al. 2008; Powers et al. 2012; Siegenthaler et al. 2007; Totoiu and Keirstead 2005) and humans (Bunge et al. 1993; Guest et al. 2005; Kakulas 1999; Norenberg et al. 2004). Yet, intense debate still exists as to whether, in fact, demyelinated axons remain. Only two studies have currently assessed the myelin status of spared axons: one measured the rubrospinal tract in mice (Lasiene et al. 2008) and the other measured the corticospinal and rubrospinal tracts in rats (Powers et al. 2012). Both studies found no evidence of chronic demyelination in the two spinal cord tracts examined. Therefore, it remains possible that other spinal cord tracts remain demyelinated. The timeframe in these studies was not assessed, and thus based on these studies, axons might remain demyelinated for as long as 2.5-3 months. Remyelination can be observed as early as 1-2 weeks by measuring axons that have a thin compact myelin sheath, a very late marker of myelination. It will be important to measure how rapidly axons are ensheathed by oligodendrocytes after SCI as some benefits from oligodendrocytes do not require mature myelin (Griffiths et al. 1998; Nguyen et al. 2009; Uschkureit et al. 2000). This is now possible by genetically labelling OPCs with a membrane bound GFP prior to demyelination.

When measuring the myelin status of spared rubrospinal axons adjacent to the lesion site, Powers and colleagues also determined that a majority of axons were remyelinated, suggesting that at some point these axons were demyelinated (Powers et al. 2012). However, even with such robust demyelination, it is not clear whether increasing the rate of remyelination would ultimately spare more axons. Perhaps after SCI endogenous remyelination is as efficient as

possible. Demyelination is thought to place axons in a vulnerable state (Trapp and Stys 2009; Waxman 2006), but will further improvements on endogenous remyelination truly spare more of these vulnerable axons? Improving endogenous remyelination by days, weeks or even months might not spare enough axons to make a difference in improving function after SCI. Addressing these important concerns requires more studies that specifically improve remyelination. Cell transplantation has demonstrated a correlation between remyelination and functional recovery (Cao et al. 2005; Cummings et al. 2005; Hofstetter et al. 2005; Karimi-Abdolrezaee et al. 2006; Keirstead et al. 2005; Lee et al. 2005; Mitsui et al. 2005), but cell transplantation likely has multiple mechanisms of action. Promoting endogenous remyelination after SCI would help address whether increasing the rate of remyelination will improve function after SCI. Addressing the benefits of increasing remyelination requires a more targeted approach. One such approach is to remove the receptors to putative myelin-associated inhibitors or antagonizing Wnt signalling specifically in oligodendroglia during remyelination after SCI. Only with specific oligodendroglial gain of function studies will there be a solid proof-of-principal for therapeutically targeting remyelination after SCI.

4.5.3 Does remyelination after SCI also inhibit axonal plasticity?

Myelin, in addition to increasing the rate of axonal conduction and providing trophic support to axons, is also thought to decrease axonal plasticity in development (McGee et al. 2005) and more importantly after injury (Bregman et al. 1995; Chen et al. 2000; Li et al. 2004a; McKerracher et al. 1994; Mukhopadhyay et al. 1994; Schnell and Schwab 1990; Wang et al. 2002). Remyelination may also inhibit axonal regenerative sprouting/plasticity (Filbin 2003), which is thought to be partly responsible for functional recovery after SCI (Courtine et al. 2008).

The interrelationship between remyelination and plasticity has yet to be explored, however remyelination might not be as inhibitory to axonal plasticity as myelin debris. DRG cells transplanted into white matter tracts are capable of growth (Davies et al. 1997; Davies et al. 1999), suggesting that growth can occur on myelinated fibres. Still, the degree that remyelination inhibits axonal plasticity warrants further investigation and could be assessed following specific remyelination gain of function and loss of function experiments addressed above.

4.6 Final thoughts

The work in this thesis demonstrated that (a) remyelination by endogenous cells might be more efficient than previously thought and (b) that myelin stimulates increased expression of ID transcription factors in oligodendroglia, which inhibits their maturation. Further experimentation needs to be conducted to fully test the general hypothesis that myelin inhibits remyelination after SCI. In truth, much remains to be determined on whether improving remyelination after SCI poses any great benefit with regards to restoration of function in rodents and humans. If a benefit or benefits do become known, then the many factors that are likely to regulate remyelination after SCI and other white matter disorders will subsequently need to be determined. Too long the benefits of remyelination after SCI have been assumed, but with little evidence. Currently there is a plethora of new tools available to study remyelination *in vivo*. The ability to trace cell lineage and specifically remove genes of interest within a given cell type are powerful technologies to describe mechanisms *in vivo*. Additionally, with expansions in the field of optogenetics, as well as the increasing availability of two-photon microscopes, there is an ever-

growing availability of tools to study pathology in living animals. With many core assumptions left unexplored, there has never been a better time to study remyelination.

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