BRAIN LIPID BINDING PROTEIN EXPRESSION IN REMYELINATING SCHWANN
CELLS OF THE SPINAL CORD IN MULTIPLE SCLEROSIS

by

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Abstract

Myelin is important for axon maintenance and survival, as well as for saltatory conduction of nerve impulses. Consequently, loss of myelin after spinal cord injury or in demyelinating diseases such as Multiple Sclerosis (MS) results in dysfunction of nerve impulse propagation and progressive axonal damage and cell death.

Endogenous remyelination can occur in response to MS and is mainly mediated by oligodendrocyte precursor cell-derived oligodendrocytes, but Schwann cells (SCs) can also participate. SC remyelination has been documented in spinal cord lesions following traumatic spinal cord injury in humans, and in animal models of demyelination such as lysolecithin-induced demyelination, but endogenous remyelination by SCs in the context of MS has not been as well studied.

In the present study we used immuno-fluorescent detection to analyze the expression of brain lipid binding protein (BLBP) and peripheral myelin protein zero (P0) in MS and non-MS human spinal cord as well as in the lysolecithin-demyelinated mouse spinal cord. BLBP (also known as Fatty Acid Binding Protein 7) is a nervous system-specific fatty acid binding protein. In the context of the present study, BLBP is important because it has been previously used to identify spinal cord radial glia (RG) in the developing and adult mouse spinal cord. BLBP is also expressed by SC precursors, and by immature SCs before they differentiate into myelinating SCs. We investigated whether, like the mouse spinal cord, the aged human spinal cord preserves a population of BLBP+ spinal cord RG. We found that (1) in contrast to the mouse spinal cord RG, human spinal cord RG do not express BLBP; (2) unlike the mouse, some subpopulations
human myelinating SCs express BLBP in the PNS of MS (and some non-MS) cases; (3) in the MS spinal cord, BLBP-positive SCs extensively myelinate axons in large GFAP-rich areas; (4) and that BLBP is more readily detected in uncompacted myelin sheaths.

Collectively these data provide evidence of robust SC remyelination in the human spinal cord beyond what has been previously reported, and highlight BLBP as a developmentally regulated protein whose expression is significantly different between mouse and human spinal cord RG and SCs.
Preface

This work was conducted at UBC Life Sciences Institute by Raymundo Aguas-Hernandez under the supervision of Dr. Roskams. All human tissue from MS patients was obtained from Dr. Wayne Moore in collaboration with his lab. I was responsible for all aspects of the study with the exception of the LPC stereotaxic injections, which were performed by Dr. Jie Liu in collaboration with Dr. Wolfram Tetzlaff lab, I supported with animal care and surgery preparation. This work was performed in accordance with animal ethics protocols approved by the UBC Animal Care Committee, specifically this study was done under protocol A09-0741. The human tissue used in the Western blot was obtained from the Cashman laboratory, and the Western blot was performed by Megan O’Neill, in collaboration with the Cashman lab. The immunostaining of SMI312/P0 in MS tissue used in figure 2.1 was performed by Kathryn Douglas, I produced the image. Audrey Petit produced images A and B from figure 3.1, the Roskams lab has published a version of these images in the following report: Petit A, Sanders AD, Kennedy TE, Tetzlaff W, Glattfelder KJ, Dalley RA, et al. (2011) Adult Spinal Cord Radial Glia Display a Unique Progenitor Phenotype. PLoS ONE 6(9): e24538. doi:10.1371/journal.pone.0024538.
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<th>Description</th>
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<tr>
<td>ADAM-17</td>
<td>ADAM metallopeptidase domain 17</td>
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<tr>
<td>BACE1</td>
<td>Beta-secretase 1</td>
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<tr>
<td>BLBP</td>
<td>Brain lipid binding protein</td>
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<tr>
<td>bmp2-4</td>
<td>Bone morphogenetic protein 2</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CST</td>
<td>Corticospinal tract</td>
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<tr>
<td>Cx 32</td>
<td>Connexin 32</td>
</tr>
<tr>
<td>DREZ</td>
<td>Dorsal root entry zone</td>
</tr>
<tr>
<td>EAE</td>
<td>Experimental autoimmune encephalomyelitis</td>
</tr>
<tr>
<td>erb3</td>
<td>Human epidermal growth factor receptor 3</td>
</tr>
<tr>
<td>FABP7</td>
<td>Fatty acid binding protein 7</td>
</tr>
<tr>
<td>GFAP</td>
<td>Glial fibrillary acidic protein</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>HDAC</td>
<td>Histone deacetylase</td>
</tr>
<tr>
<td>IGF2</td>
<td>Insulin-like growth factor 2</td>
</tr>
<tr>
<td>LPC</td>
<td>Lysophosphatidylcholines</td>
</tr>
<tr>
<td>MAG</td>
<td>Myelin-associated glycoprotein</td>
</tr>
<tr>
<td>MBP</td>
<td>Myelin basic protein</td>
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<tr>
<td>MRF</td>
<td>Myelin-gene Regulatory Factor</td>
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<tr>
<td>MS</td>
<td>Multiple sclerosis</td>
</tr>
<tr>
<td>NCC</td>
<td>Neural crest cell</td>
</tr>
<tr>
<td>NFATc4</td>
<td>Nuclear factor of activated T-cells, cytoplasmic 4</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
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<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>notch</td>
<td>Signaling pathway involved in a variety of outputs such as neurogenesis.</td>
</tr>
<tr>
<td>nrg1</td>
<td>Neuregulin 1</td>
</tr>
<tr>
<td>NT3</td>
<td>Neurotrophin-3</td>
</tr>
<tr>
<td>OPC</td>
<td>Oligodendrocyte precursor cell</td>
</tr>
<tr>
<td>P0</td>
<td>Myelin protein zero</td>
</tr>
<tr>
<td>PDGF-BB</td>
<td>Platelet-derived growth factor beta polypeptide</td>
</tr>
<tr>
<td>PLP</td>
<td>Myelin proteolipid protein</td>
</tr>
<tr>
<td>PNS</td>
<td>Peripheral nervous system</td>
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<tr>
<td>PPMS</td>
<td>Primary progressive multiple sclerosis</td>
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<tr>
<td>PRMS</td>
<td>Primary relapsing multiple sclerosis</td>
</tr>
<tr>
<td>PrPc</td>
<td>Cellular prion protein</td>
</tr>
<tr>
<td>RRMS</td>
<td>Relapsing-remitting multiple sclerosis</td>
</tr>
<tr>
<td>SCd</td>
<td>Spinal Cord</td>
</tr>
<tr>
<td>SCs</td>
<td>Schwann cells</td>
</tr>
<tr>
<td>SCAP</td>
<td>SREBP cleavage-activating protein</td>
</tr>
<tr>
<td>SCI</td>
<td>Spinal cord injury</td>
</tr>
<tr>
<td>SCP</td>
<td>Schwann cell precursor</td>
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<tr>
<td>SCRG</td>
<td>Spinal cord radial glia</td>
</tr>
<tr>
<td>SM4</td>
<td>Seminolipid and sulfatide</td>
</tr>
<tr>
<td>SPMS</td>
<td>Secondary progressive multiple sclerosis</td>
</tr>
<tr>
<td>SREBPs</td>
<td>Sterol Regulatory Element-Binding Proteins</td>
</tr>
<tr>
<td>SVZ</td>
<td>Subventricular zone</td>
</tr>
<tr>
<td>WPL</td>
<td>Weeks post lesion</td>
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<td>------</td>
<td>-------------------</td>
</tr>
<tr>
<td>Yy1</td>
<td>Transcriptional repressor Yin Yang 1</td>
</tr>
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</table>
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First I owe great gratitude to Dr. Roskams, for her support, great patience, academic advice and offering me the opportunity to enter into graduate studies. I would also like to thank the past and current members of the Roskams Lab, for their great companionship, valuable technical and intellectual consultations during my training and the performance of this study. Very special thanks to Dr. Catherin Cowan for her meticulous proof-reading and innumerable suggestions to improve my thesis draft.

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And last but not least many thanks to my family, specially my parents for all the support and encouragement, I am very grateful to have you.
Dedication

To my Parents Raymundo and Paula.
Chapter 1: Introduction

1.1 Myelin

Myelin is an important component of both the central and peripheral nervous systems, essential for proper development and function of the brain and spinal cord (SCd). Myelin is produced by myelinating glial cells: the Schwann cells (SCs) of the peripheral nervous system (PNS), and the oligodendrocytes of the central nervous system (CNS). In both places the myelinating glia establishes contact with axons through plasma membrane extensions that wrap axons, forming a multiple-layer myelin sheath that acts as an electrical insulator enabling rapid conduction of nerve signals (Fields 2014).

1.1.1 The role of myelin

The myelin sheath has multiple roles; it regulates the axon caliber, is involved in axon maintenance and survival and is also responsible for the saltatory conduction of nerve impulses (Nave 2010). The latter function has profound physiological implications enabling myelinated axons to transmit nerve impulses 100 times faster than non-myelinated axons. This property has been instrumental in allowing vertebrates to develop complex motor, sensory and cognitive neural systems and behaviors.

Much of our knowledge about how myelin has shaped the vertebrate nervous system comes from studying the structure of the myelin sheath. The axons are myelinated in a segmental way: this means that along the axon specialized glial processes wrap at multiple points to form an internode of myelin, with each internode separated by short gaps of unmyelinated axon known as nodes of Ranvier. Both components of the myelinated axon (internodes and nodes of Ranvier)
are important for the saltatory conduction of nerve impulses. The nodes of Ranvier are rich in voltage-gated sodium channels, which are involved in propagating action potentials. The internodal segments contain two functionally specialized regions, the paranode and the juxta-paranode. The juxta-paranode contains voltage-gated potassium channels that modulate the excitability of the node, and the paranode contains adhesion molecules important for axon-glia junctions that help to spatially separate the sodium and potassium channels. The high electrical resistance of the myelin internodes forces action potentials to jump between nodes of Ranvier and in consequence significantly accelerates the speed of neural impulses (Siegel 1999, Baumann and Pham-Dinh 2001).

1.1.2  Myelin-producing cells

Myelinating cells of the central nervous system:

Oligodendrocytes are the myelinating glia of the CNS, originating post-natally from oligodendrocyte precursor cells (OPCs) that emerge and migrate from various niches of the brain and spinal cord. Brain OPCs emerge in three waves: the first originates in the medial ganglionic eminence and anterior entopeduncular area of the ventral forebrain; the second wave derives from the lateral and caudal ganglion eminences; and the third wave of OPCs originates from the postnatal cortex. Spinal cord oligodendrocytes come from two sources of OPCs, those originating in the ventral ventricular zone and those from the dorsal spinal cord. (Mitew, Hay et al. 2014)

OPCs must then differentiate into pro-myelinating oligodendrocytes. The differentiation process is coordinated by intrinsic and extrinsic signals such as extracellular ligands, chromatin remodeling, microRNAs and neuronal activity. At late stages of oligodendrocyte differentiation,
transcription factors such as myelin gene regulatory factor (MRF) seem to be very important to start the myelination program (Mitew, Hay et al. 2014).

As oligodendrocytes mature they undergo polarization and project their membrane towards adjacent axons. One oligodendrocyte establishes contact with multiple axons and myelinates them. The same oligodendrocyte can myelinate an axon at multiple points but adjacent myelin segments are never from the same oligodendrocyte (Baumann and Pham-Dinh 2001).

**Myelinating cells of the peripheral nervous system:**

Schwann cells (SCs) are the specialized glia of the PNS. They are the predominant cell type in the peripheral nerves, and their lineage can be tracked to neural crest cells (NCCs) (Dupin, Creuzet et al. 2006, Campana 2007), which have their origin on the neuroepithelium at the dorsal edges of the closing neural folds (Lobsiger, Taylor et al. 2002). In brief, NCCs mature into SC precursors, and SCs precursors to immature SCs, which can then mature into myelinating or non-myelinating SCs. This work focuses only on myelinating SCs. In contrast to oligodendrocytes in the CNS, each myelinating SC associates only with one axon in a 1:1 relationship, in a process that will be reviewed in section 2.1.

**1.1.3 How is myelin maintained**

The mechanisms that control myelin maintenance in the CNS and PNS are complex, and involve many elements of the glia-axon association. The expression of transcription factors such as myelin gene regulatory factor (MRF) and Krox-20 is involved in maintaining the proper gene expression required for myelin maintenance. Ablation of MRF causes delayed but severe
demyelination in the CNS, while Krox20 inactivation causes rapid demyelination in the PNS and SC dedifferentiation (Decker, Desmarquet-Trin-Dinh et al. 2006, Koenning, Jackson et al. 2012). Both transcription factors are not only important for starting the myelination program, but are also needed to maintain the myelin sheath. Other components are important because of their structural roles: for example, mutations of myelin-associated glycoprotein (MAG) or CNPase lead to adult-onset axonal degeneration of the PNS and CNS respectively (Schachner and Bartsch 2000, Rasband, Tayler et al. 2005). Another protein that appears to have a role in myelin maintenance is the SC protein connexin Cx32, mutations in the gene for which lead to Charcot-Marie-Tooth disease, a demyelinating neuropathy of the PNS (Sango, Yamauchi et al. 2014). PrPc, a glycoprotein protein mainly found in lipid rafts of neurons and glia, is found at high levels in neurons of adult brain and spinal cord and at lower levels in the myelinating glia, as well as in the axons and SCs in the PNS. Expression of PrPc is required in axons for myelin maintenance, as ablation of PrPc in mouse leads to chronic demyelination (Bremer, Baumann et al. 2010).

In addition to transcription factors and structural proteins, lipids have been shown to be important for myelin maintenance. Myelin lipids make up ~70% of myelin dry mass. There are no lipids specific to myelin, but there are myelin-enriched lipids (Baumann and Pham-Dinh 2001). The mayor lipid types in myelin are cholesterol, phospholipids and glycosphingolipids, in a composition that is very conserved among mammals (Baumann and Pham-Dinh 2001). Cholesterol makes up ~27% of the myelin dry mass, and contributes to myelin maintenance and stability by influencing the membrane biophysical properties, e.g. membrane thickness and membrane fluidity (Fewou, Jackman et al. 2010). Cholesterol participates in the formation and
stabilization of lipid microdomains, and limits ion leakage through the membrane. Loss of SCAP, an activator of the sterol regulatory element-binding proteins (SREBPs) causes a loss of gene expression of a group of genes involved in fatty acid and cholesterol synthesis, resulting in an alteration in myelin synthesis (Pereira, Lebrun-Julien et al. 2012). Another example comes from galactolipids enriched in myelin such as seminolipid and sulfatide (SM4). CST (cerebroside sulfotransferase)-null mutant mice do not synthesize several myelin-enriched galactolipids, resulting in structural myelin alterations (reviewed in Fewou, Jackman et al. 2010).

1.2 Schwann Cell Development

1.2.1 Schwann cell development and fate determination

Schwann cells (SCs) are the specialized glia of the PNS, originating from neural crest cells (NCCs) in the developing neuroepithelium. Their development can be divided into three main events or three cell stage transitions: (a) from neural crest cells to SC precursors; (b) from SC precursor to immature SCs; and (c) from immature SCs to differentiated myelinating SCs or non-myelinating SCs (Armati 2007).

NCCs have their origin on the neuroepithelium at the dorsal edges of the closing neural folds (Lobsiger, Taylor et al. 2002), from where they delaminate and migrate through the periphery to different regions of the vertebrate embryo. This population sets out as multipotent NCCs; however, as they migrate their cellular environment restricts their fate to differentiate into specific cell types (Le Douarin and Dupin 2003).
a) From NCCs to SC precursors

The extracellular signals and pathways that drive NCCs fate into SC precursors have not been fully elucidated (Woodhoo and Sommer 2008), but it has been proposed that SC fate determination might emerge when other NCC fates -like neurogenesis -are suppressed (Jessen and Mirsky 2005). In addition, studies using Sox-10 mutant mice show a lack of peripheral glia, evidencing the importance of Sox-10 in SC fate determination (Britsch, Goerich et al. 2001). Sox-10 is absent in neurons and other derivatives of the neural crest, but is not limited to the peripheral glia lineage: it is also expressed by the other NCCs lineages such as differentiating melanocytes. It appears that Sox-10 could be regulating SCs fate in a context-dependent manner by altering NCCs’ responsiveness to environmental cues (Woodhoo and Sommer 2008), or by maintaining the levels of expression of molecules that are necessary to maintain SC precursor survival and proliferation like NRG1 receptor Erb3 (Britsch, Goerich et al. 2001).

Other molecules that have been studied for their effects in NCCs and a potential role in SC fate determination are Notch, BMP2 -4, and NGR-1. In the case of Notch, several in vitro studies using neural crest cultures have shown that Notch activation results in inhibition of neurogenesis and increases the number GFAP positive SCs (Armati 2007). Whether Notch, BMP2- 4 or NGFR-1 are determining SC fate directly by instructing the cells to commit to SC fate or indirectly by inhibiting neurogenesis still needs to be clarified (Armati 2007). No model has explained yet whether NCCs’ default mode is to become peripheral glia or if this needs to be induced through specific signals.
b) From SC precursors to immature SCs:

The transition between SC precursors and immature SCs correlates with an important step in the organization of the PNS. At E12-14 in rodents, the peripheral nerves are composed of axons and SC precursors that are normally localized at the surface and within the developing nerves, which together form a network and divide the nerves into bundles longitudinally (Armati 2007). As the transition from SC precursor to immature SCs happens and the nerves are populated by the new immature SCs the bundles of axons become smaller, and they begin to associate with endoneurial fibroblasts, blood vessels and other cells of connective tissue that are found in the adult PNS (Armati 2007). This developmental transition seems to be regulated in large part by axonal signals such as Notch, NRG1 and endothelins, the effects of which have been faithfully reproduced in in vitro studies (Woodhoo and Sommer 2008) showing evidence of the importance of axon-SC communication during the development of the PNS.

Another key change in the differentiating SCs when they pass from precursors to immature SCs is their ability to support their own survival. Unlike SC precursors, the immature SCs do not rely on the axonal signals to survive; immature SCs can also use extracellular factors signaling like NT3, IGF2, PDGF-BB in the absence of axonal signals to sustain their own existence. These cues first sent by axons and later on by SC signals are responsible for phenotypic changes that increase the survival and proliferation of SCs (Armati 2007). SC proliferation reaches its maximum level at their immature phenotype stage, which seems to be important for generating the required amount of SCs needed for the reorganization and maturation of the peripheral nerves (Armati 2007).
c) From immature SCs to myelinating or non-myelinating SCs:

The molecular mechanism behind immature SCs fate determination is not fully understood. All immature SCs have the same differentiation potential, but their axonal association, which happens around the time of birth, appears to be instructive in determining the fate of the immature SCs. Around E18 in rodents, immature SCs can be found ensheathing large groups of axons, and after birth immature SCs start to selectively associate with axons in a 1:1 relationship by a process called “radial sorting”. SCs that engage with large diameter axons will mature into a myelinating SC, and groups of small diameter axons will be ensheathed by SCs that differentiate into non-myelinating SCs (Jessen and Mirsky 2005).

1.2.2 Brain lipid binding protein (BLBP) during SC development and sciatic nerve lesion

BLBP (also known as FABP7) is a fatty acid binding protein specific to the nervous system. Other members of the fatty acid binding protein family found in other tissues are known for carrying signaling molecules within the cells. No ligand has yet been identified for BLBP, but it has been suggested that BLBP could be involved in the transport of hydrophobic ligands with potential morphogenic activity during CNS development (Feng, Hatten et al. 1994, Anton, Marchionni et al. 1997, Miller, Li et al. 2003). In the CNS, BLBP is robustly expressed during development, with a peak of expression around E12 marking the radial glial stage at which most CNS neurogenesis occurs (Anthony, Klein et al. 2004). Later in the postnatal nervous system, BLBP is highly expressed transiently by astrocytes (during their massive postnatal expansion) in the brain, spinal cord and olfactory system. However, in the adult, BLBP expression is retained only by few specialized glial cell types (referred to as aldynoglia) (Gudino-Cabrera and Nieto-Sampedro 1999) such as olfactory ensheathing glia in the olfactory bulb and olfactory...
epithelium; spinal cord radial glia; and neural stem cells in neurogenic niches of the brain such as the SVZ (Au and Roskams 2003, Cheng, Pastrana et al. 2009, Petit, Sanders et al. 2011).

In the PNS, BLBP expression is detected in the SC lineage at E12.5, after neural crest cells have transitioned into SCP. These BLBP + SCP have been shown to give rise to SCs and fibroblasts in vivo (Joseph, Mukouyama et al. 2004). Immature SCs retain BLBP expression but they stop expressing it after they stop dividing (like astrocytes), and mature into myelinating or non-myelinating SC (Jessen and Mirsky 2002, Jessen and Mirsky 2005). However, BLBP can be up-regulated by adult SCs after sciatic nerve crush (Miller, Li et al. 2003). Myelinating SCs dedifferentiate when they lose axonal contact; they transit into a “less mature” and proliferative phenotype that resembles immature SCs (Mirsky, Woodhoo et al. 2008, Jessen and Mirsky 2010). This has been observed in vivo (after inducing sciatic nerve injury) and in vitro (culture of dissociated SCs without neurons). When myelinating SCs lose contact with their axons, they digest their myelin in a process in which macrophages participate, and they change their gene expression up-regulating genes expressed by immature SC such as P75, GFAP and BLBP. BLBP expression is observed 14-30 days after sciatic nerve crush (Miller et al. 2003).

1.2.3 Schwann cell myelination

SCs have been widely studied for their capacity to myelinate and provide axonal protection in several neurodegenerative environments like spinal cord injury and Multiple Sclerosis lesions. Their participation in the injured CNS can be spontaneous, but endogenous SC myelination is often limited and insufficient. In order to learn how to potentiate SC participation and promote CNS regeneration, it is important to understand the regulators of myelination in the PNS.
SC myelination is highly regulated by positive and negative transcriptional controls. A key positive control is Sox10. Sox10 activates Oct 6, and then both synergistically induce the expression of Krox20. Activation of Krox20 is a critical turning point in SC myelination because Krox20 suppresses myelination inhibitors and activates many myelin genes that are important for maintaining SC in a myelinating state. Other transcription factors that positively regulate myelination include NFATc4, Yy1 and NFkappaB (Pereira, Lebrun-Julien et al. 2012). Other mechanisms involve negative regulators of myelination. Identifying them is important because negative regulation of myelination is necessary to produce dedifferentiation of myelinating SC into a SC phenotype that promotes a favorable environment for axonal regrowth. Additionally, negative regulators might help us to understand dysregulated pathways in diseases like demyelinating neuropathies. Examples of negative regulators of myelination are Notch 1 and c-Jun. After peripheral nerve injury, Notch signalling is up-regulated in SC and drives demyelination. Furthermore, demyelination can be induced when Notch is ectopically activated in uninjured PNS (Woodhoo, Alonso et al. 2009). Others factors such as c-Jun similarly regulate myelination by promoting SC dedifferentiation after injury (Pereira, Lebrun-Julien et al. 2012).

In addition, SC myelination is regulated by epigenetic and posttranslational mechanisms. Protein acetylation driven by HDACs has been shown to be a critical factor that regulates myelination. HDAC 1 and HDAC 2 can control SC differentiation at least partially by modifying NFkappaB acetylation state, and both seem to act coordinately to epigenetically affect several myelination genes (Chen, Wang et al. 2011). Accordingly, SC with deficient HDAC1 and HDAC2 fail to myelinate (Jacob, Christen et al. 2011). Post-translational controls can positively or negatively regulate myelination with beta and alpha secretases, like members of the ADAM family and beta
secretases such as BACE1 (beta-secretase 1). These myelination controls act through NRG1-III (neuregulin 1 type 3). Both BACE1 and ADAM17 (ADAM metallopeptidase domain 17) can cleave NRG1-III but with opposite effects: ADAM-17 cleavage of NRG1-III negatively regulates the amount of functional NRG1-III, but BACE1 cleavage of NRG1-111 acts as a positive regulator of myelination (Pereira, Lebrun-Julien et al. 2012).

1.3 Multiple Sclerosis

MS is an inflammatory demyelination disease that affects the CNS. It is mediated by aberrant T cell subsets in conjunction with other immune infiltrates like B cells and macrophages that attack CNS myelin, oligodendrocytes and axons, causing demyelinating lesions in brain and spinal cord. This aberrant autoimmune activity may either be localized in focal lesions or may be diffusely spread throughout the brain and spinal cord white matter (Lassmann 2013, Sánchez-Gómez, Pérez-Cerdá et al. 2014).

1.3.1 Demyelination in MS and how it presents in patients

The lesions start with the attack and removal of myelin and oligodendrocytes; therefore at the early stages, the lesions consist of demyelinated axons, myelin debris, and immune infiltrates. At this stage the lesions can be partially repaired by re-myelinating oligodendrocytes or SCs. However, with the progression of the disease, the demyelination progresses and the denuded axons degenerate, resulting in irreversible tissue loss and CNS atrophy (Sánchez-Gómez, Pérez-Cerdá et al. 2014). Typically, MS presents with a variety of neurological symptoms and signs referable to virtually anywhere in the CNS. MS diagnosis is based on the identification of white matter lesions and assessment of their dissemination in space and time, by clinical criteria and/or
scanning the CNS with MRI (magnetic resonance imaging) (Polman and Rudick 2010). Additionally, cerebrospinal fluid analysis can inform about the immunological reaction in the CNS lesions (Hurwitz 2009, Sánchez-Gómez, Pérez-Cerdá et al. 2014). The course of MS is generally unpredictable, but the patterns of progression of the disease can be used to classify it into four main subtypes, which are diagnosed based on the pattern of lesion dissemination and neurological deterioration (Sánchez-Gómez, Pérez-Cerdá et al. 2014).

1.3.2 Types of MS

The most common type of MS is relapsing-remitting multiple sclerosis (RRMS), affecting 85-90% of patients diagnosed. This type of MS is characterized by intermittent attacks of neurological dysfunction (relapses) followed by clinical improvement (remissions)(Hurwitz 2009). These two events alternate over time, with a gradual tendency towards increased disability. About 85% of patients diagnosed with RRMS experience a change in the pattern of progression of the disease, whereby the pattern switches into a more steadily progressive disease. This is now known as secondary progressive multiple sclerosis (SPMS), which involves fewer relapses but a more pronounced increment of disability over time. A less common type of MS is primary progressive multiple sclerosis (PPMS). Documented in around 15% of patients (Hurwitz 2009), this type of MS is characterized by a progressive evolution of the disease from the beginning, without relapsing or remitting events. Finally the rarest type is progressive relapsing multiple sclerosis (PRMS), which is characterized by a steadily progression of the disability from the beginning with occasional relapses that increases the progression of the disease, but without remissions (Hurwitz 2009). Although MS has been traditionally subdivided in these four subtypes that broadly represent all MS phenotypes, there has been an increasing
understanding of the pathology and clinic aspects MS in the recent years, and a recent revision of the MS course descriptions suggests that we should refine the MS course descriptions to better represent all known aspects associated with the disease (Lublin, Reingold et al. 2014).

1.3.3 Endogenous remyelination by SC in the MS spinal cord

The loss of myelin in MS is usually followed by spontaneous remyelination, which in MS can be mediated by oligodendrocytes (Bradl and Lassmann 2010). The CNS endogenous response to demyelination is to recruit OPCs to the lesions. The OPCs differentiate and remyelinate denuded axons, but their contribution is insufficient and the remyelinated axons are not exempt from the subsequent immune attacks (Bradl and Lassmann 2010). In addition to oligodendrocytes, SC myelin has been found in MS lesions of the spinal cord (Ghatak, Hirano et al. 1973, Itoyama, Ohnishi et al. 1985, Yamamoto, Kawamura et al. 1991). The abnormal invasion of SC within the CNS is known as schwannosis, which is known to occur spontaneously in the human spinal cord after spinal cord injury (SCI), and in demyelination diseases such as MS and neuromyelitis optica (Itoyama, Webster et al. 1983, Guest, Hiester et al. 2005, Ikota, Iwasaki et al. 2010). Similarly, schwannosis has also been reported in rodent models of SCI, and spinal cord demyelination (Zawadzka, Rivers et al. 2010, Nagoshi, Shibata et al. 2011). Different patterns of schwannosis have been described, SC often occur nearby PNS sources such as spinal nerve roots, and around perivascular spaces (Itoyama, Webster et al. 1983), in these cases it appears that SC associated with axons invade the CNS from the periphery, and it has been speculated these SC have limited integration into the CNS. Additionally a different pattern of schwannosis involves SC myelinating axons in the CNS parenchyma in and around lesions, in this case schwannosis can presumably contribute to CNS regeneration. Furthermore it has been suggested that SC
Remyelination is favoured in areas that lack glial fibrillary acidic protein (GFAP) immunoreactivity (Itoyama, Ohnishi et al. 1985, Franklin and Blakemore 1993).

Schwannosis has not been extensively studied in the MS spinal cord, but SC spontaneous remyelination in CNS lesions suggest a good therapeutic potential for the use of SC in cell-mediated therapies for MS repair, the clinical relevance of spontaneous SC remyelination in the MS spinal cord is not known.

Nevertheless, endogenous SC-mediated remyelination has been implicated in functional recovery of paraplegia in the rat demyelinated spinal cord (Jasmin, Janni et al. 2000) and, furthermore, SC transplants in various animal lesion models have resulted in functional recovery (Blight and Young 1989, Biernaskie, Sparling et al. 2007, Papastefanaki, Chen et al. 2007, Zujovic, Doucerain et al. 2012, Zaminy, Shokrgozar et al. 2013).

1.4 Project summary and objectives

In the present study I investigated the expression of BLBP in the adult human spinal cord of cases with MS and non-MS (control). Due to the high evolutionary conservation between mouse and human FABPs (Smathers and Petersen 2011), and a similar pattern of BLBP expression between the mouse and human developing brains (Feng, Hatten et al. 1994, Howard, Zhicheng et al. 2008), I hypothesized that similarly to the aged mouse spinal cord (Petit, Sanders et al. 2011), the human spinal cord retains a population of BLBP-positive spinal cord RG, anchored at the margin of the spinal cord. In addition, I asked if re-myelinating SCs in the mouse LPC-demyelinated spinal cord express BLBP, as do the re-myelinating SC in the MS cases spinal cord
I found (1) that in contrast to the mouse spinal cord RG, human spinal cord RG do not express BLBP, (2) that unlike the mouse, human myelinating SCs express BLBP in the PNS of MS and non-MS cases, (3) that BLBP is more highly expressed in uncompacted myelin in SCs with an abnormal concentric appearance, and (4) that in the MS spinal cord, BLBP-positive SCs extensively myelinate axons in GFAP-rich areas.

Collectively, these data provide evidence of extensive peripheral-type myelin beyond what has been previously reported, and highlight significant differences of BLBP expression between mouse and human spinal cord RG and SCs.
Chapter 2: Materials and Methods

2.1 Human tissue

Human spinal cord and brain sections from MS cases were obtained through collaboration with Dr. Wayne Moore at International Collaboration on Repair Discoveries (ICORD). Tissue from 17 cases (11 MS cases and 3 non-MS cases) was fixed in 10% formalin upon post-mortem resection, and embedded in paraffin according to standard protocols, and sectioned at 5 µm thickness.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Region</th>
<th>Age</th>
<th>Gender</th>
<th>Duration</th>
<th>Diagnosis</th>
<th>PM</th>
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<tr>
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<td>NA</td>
<td>Control</td>
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<td>2</td>
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<td>PPMS</td>
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<td>6 days</td>
</tr>
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<td>Region</td>
<td>Age</td>
<td>Gender</td>
<td>Duration</td>
<td>Diagnosis</td>
<td>PM</td>
</tr>
<tr>
<td>---------</td>
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<tr>
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<td>*</td>
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<tr>
<td>12</td>
<td>T2 (above lesion)</td>
<td>77yr</td>
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<td>62yr</td>
<td>chronic SCd trauma</td>
<td>1 day</td>
</tr>
<tr>
<td>12</td>
<td>Lesion</td>
<td>77yr</td>
<td>Female</td>
<td>62yr</td>
<td>chronic SCd trauma</td>
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<tr>
<td>13</td>
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<td>31yr</td>
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<td>NA</td>
<td>&lt;1 day</td>
</tr>
<tr>
<td>14</td>
<td></td>
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<td>Male</td>
<td>16yr</td>
<td>PPMS</td>
<td>3 days</td>
</tr>
</tbody>
</table>

Table 2.1 Human cases used in the study.

NA= MS, type not available (not recorded in clinical records)

PPMS = Primary Progressive MS

Duration= Disease duration

PM = Post-mortem time
2.2 Animals

Six month-old mice, five males and four females, of the Fabp7-EGFP reporter line were used in this study. All procedures were approved by UBC animal care committee in accordance with guidelines of the Canadian Council on Animal Care.

2.2.1 Lysolecithin-induced demyelination

The demyelination procedure was carried out in collaboration with the Tetzlaff lab. Mice received buprenorphine (30 µg per kg of body weight) as an analgesic treatment and were anesthetised with isoflurane. Surgery was performed to expose the vertebral column at the cervical level, and laminectomy (removal of vertebral bone) was carried out to expose Dorsal C4 spinal cord. Demyelination was induced with an injection of 0.5ul of 1% Lα-lysophosphatidylcholine (Lysolecithin, Sigma) in the dorsal column of C4 with a stereotaxic pump over 15 minutes, before closure. Tissue was collected at 1 or 3 weeks post lesion (WPL).

2.2.2 Tissue processing

Mice were perfused transcardially with PBS, followed by 4% PFA, pH 7.4, under terminal anaesthesia. Spinal cord s were dissected and equilibrated in successive concentrations of 10 and 30% sucrose in PBS for 24 hours each. The cervical portion of the spinal cord was then embedded in optimum cutting temperature (O.C.T.) media and frozen. Spinal cord s were sectioned at 14 um using a HM 500 cryostat (Micron), mounted onto charged Superfrost glass slides (Fisher, Edmonton AB), and stored at -20 °C.
2.3 Immunofluorescence

Fluorescence immuno-chemistry was performed on human tissue (obtained through collaboration with Dr. Wayne Moore) and mouse tissue (prepared as given above). The human tissue sections had been embedded in paraffin, therefore a deparaffinization procedure was applied before the immunostaining. To deparaffinize the sections, the slides were warmed on a slide warmer at approximately 42 °C for 10 min, followed by a sequence of three 3 minute immersions in each of xylene, 100%, 95%, and 75% ethanol; followed by two 5 minute washes in Phosphate-buffered saline (PBS). Following deparaffinization, sections were permeabilized in 0.1% triton X-100 for 30 minutes, followed by two 5 minutes washes in PBS; non-specific binding sites were blocked with 10% normal donkey or goat serum/2% Bovine serum albumin (BSA) at room temperature for 30 minutes. For mouse tissue the same steps were followed except that no deparaffinization was performed and the blocking solution was 4% normal donkey or goat serum in PBS. For immunodetection of proteins the sections were incubated in primary antibody (see Table 2.2) in 5% normal donkey or goat serum serum/1% BSA (2% normal serum for mouse sections) in PBS at 4 °C for 12-14 hours. Sections were washed twice for 5 minutes in PBS and then incubated for one hour in fluorescent-conjugated secondary antibodies at 1:200. Secondary antibodies were Molecular Probes Alexa 488, Alexa 594 and Alexa 647 raised in donkey or goat. The sections were washed twice for 5 minutes in PBS at room temperature, and then nuclei were counter-stained with Diaminopyridine imidazole (DAPI; 1:10 000, Sigma) for 10 minutes at room temperature, followed by two 5 minute washes in PBS and then coverslips were mounted with antifade mountant Prolong gold (Life Technologies).
<table>
<thead>
<tr>
<th>Antibody</th>
<th>Host</th>
<th>Supplier</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>BLBP</td>
<td>Brain lipid binding protein</td>
<td>Mouse</td>
<td>My Biosource</td>
</tr>
<tr>
<td>BLBP</td>
<td>Brain lipid binding protein</td>
<td>Rabbit</td>
<td>Millipore</td>
</tr>
<tr>
<td>Col IV</td>
<td>Collagen IV</td>
<td>Rabbit</td>
<td>Abcam</td>
</tr>
<tr>
<td>GFAP</td>
<td>Glial fibrillary acidic protein</td>
<td>Chicken</td>
<td>Thermo</td>
</tr>
<tr>
<td>GFAP</td>
<td>Glial fibrillary acidic protein</td>
<td>Rabbit</td>
<td>Dako</td>
</tr>
<tr>
<td>GFAPδ</td>
<td>Glial fibrillary acidic protein- δ</td>
<td>Rabbit</td>
<td>Millipore</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
<td>Chicken</td>
<td>Aves</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
<td>Rabbit</td>
<td>Millipore</td>
</tr>
<tr>
<td>H-NF</td>
<td>Heavy chain neurofilament</td>
<td>Rabbit</td>
<td>Sigma</td>
</tr>
<tr>
<td>MBP</td>
<td>Myelin basic protein</td>
<td>Rat</td>
<td>Millipore</td>
</tr>
<tr>
<td>pNF</td>
<td>Phosphorylated neurofilament</td>
<td>Mouse</td>
<td>Private lab</td>
</tr>
<tr>
<td>PLP</td>
<td>Myelin proteolipid protein</td>
<td>Chicken</td>
<td>Aves</td>
</tr>
<tr>
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<td>Myelin proteolipid protein</td>
<td>Mouse</td>
<td>Serotec</td>
</tr>
<tr>
<td>P0</td>
<td>Myelin protein zero</td>
<td>Chicken</td>
<td>Aves</td>
</tr>
<tr>
<td>P75</td>
<td>p75 neurotrophin receptor</td>
<td>Rabbit</td>
<td>Millipore</td>
</tr>
<tr>
<td>P75</td>
<td>p75 neurotrophin receptor</td>
<td>Mouse</td>
<td>Biosensis</td>
</tr>
<tr>
<td>SMI312</td>
<td>Pan axonal cocktail antibody</td>
<td>Mouse</td>
<td>Covance</td>
</tr>
<tr>
<td>S100β</td>
<td>S100 calcium binding protein B</td>
<td>Mouse</td>
<td>Sigma</td>
</tr>
</tbody>
</table>

Table 2.2 Antibodies used for fluorescence immunodetection
2.4 Image analysis and histological quantification

All images were captured using Axioplan 2 microscope (Zeiss, Jena, Germany) using a Retiga 1350EX camera (Quantitative Imaging Corporation) with Northern Eclipse software (Empiz Imaging Inc., Mississauga, Ontario), except image 3.1 (F), 3.4 (F) and 3.8 (E) that were captured with a Fluoview FV1000 laser scanning confocal microscope (Olympus) using a 60x oil immersion objective (NA 1.35). All images and compiled using Adobe Photoshop CS6 and Adobe Illustrator CS6 (Adobe Systems, San Jose, California). Computational analysis of the images was done with Image J (Wayne Rasband; NIH USA) or CellProfiler 2.0 (Anne Carpenter; Broad Institute, USA). A Heat-map of P0 expression in spinal cord of MS case was generated by using the Heat map histogram plugin of Image J.

Uncompacted myelin quantification

Sections of human spinal cord /dorsal root were immunostained for peripheral myelin with chicken anti-P0 and mouse anti-BLBP. Three 20X random fields in the dorsal roots were imaged from every MS case and 3 controls (non-MS cases). The fluorescent images were analyzed with Image J in RGB with the ‘Cell counter’ plugin, by manually counting all the morphologically distinct ‘onion bulb-like myelin rings’, these are defined as of uncompacted myelin rings with an abnormal concentric pattern, the counting was done in three images. The data from each image was used to calculate the mean for each individual patient, and the mean and raw quantifications from each image were plotted in a column scatterplot using Prism GraphPad.

BLBP-positive myelin quantification

In the images described above, we separately quantified the number of myelin (P0+) rings that
were positive for BLBP and the number of myelin rings (P0+) that were not positive for BLBP. Quantification of BLBP positive and negative myelin was carried out in CellProfiler2.0. The mean was calculated for each MS case and raw quantifications at each field were plotted in a column scatterplot using Prism GraphPad.

**Axonal immunodetection**

We used a number of antibodies against proteins enriched in axons, examples of this are shown in the figure below.

![Axonal detection](image)

**Figure 2.1 Axonal detection.** (A) In the posterior rootlet of MS pt 7 axons are stained with pan-axonal antibody SMI312 (green), P0+ myelin (red). (B) In the anterior rootlet of MS patient 3, axons are stained with anti-heavy chain neurofilament (green), P0+ myelin (red). (C) In the posterior rootlet of MS patient 6, axons are stained with anti-phosphorylated neurofilament (green), P0+ myelin (red). Scale bar 25um.
2.5 Tissue homogenization and western blotting

Normal human spinal cord tissue was obtained from the Cashman laboratory, and western blotting was performed with another lab member, Megan O’Neill. Slices of post-mortem human spinal cord were taken from previously flash-frozen sections, then the grey matter was dissected out and separated from the remainder of the tissue, (white matter and peripheral roots), termed “white matter”. Mouse and human tissues were homogenized at 10% w/v homogenates in cold phosphate buffered saline (PBS) with 1X complete, EDTA-free protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany), using an Omni TH115 tissue homogenizer (Omni International, Marietta, GA). Tissues were homogenized with 3X 30 second pulses, with 30-60 seconds on ice between pulses. Homogenates were centrifuged at 3000 rpm for 5 minutes at 4°C to remove debris, and the supernatant was removed, aliquoted and immediately frozen at -80°C.

Total protein concentration of each homogenate was determined using BCA analysis (Sigma; BCA1-1KT) standardized with BSA (Sigma; P0914). Equal amounts of total protein were boiled in SDS sample buffer with 1% b-mercaptoethanol for 5 minutes and loaded onto SDS-PAGE gels (2.55mg total protein loaded per lane). Samples were run on Novex® 4-20% tris-glycine acrylamide gels (Invitrogen; EC60285BOX), then transferred to PVDF membrane (GE Healthcare, Amersham Hybond-P; RPN303F). After transfer, membranes were blocked with 5% milk in tris-buffered saline, 0.1% Tween-20 (TBST) for 1 hour. Membranes were incubated with primary antibodies in 5% milk in TBST with 0.02% azide, overnight at 4°C on orbital shaker; Ra-b-actin (1:1000; Cell Signaling Technology, 4967), Ra-BLBP (1:1000; Millipore, A13N14), and Ma-BLBP (1:1000, MyBioSource, MBS200130). The following day, membranes were washed 3X 10 minutes in TBST, incubated in horseradish peroxidase (HRP)-linked secondary antibodies in 5% milk in TBST for 1 hour at room temperature on orbital shaker;
DaR-HRP or SaM-HRP (both 1:5000; GE Healthcare Life Sciences, NA934 and RPN4201, respectively), and washed 3X 10 minutes in TBST. Chemiluminescent detection was performed with SuperSignal West Femto (Thermo Scientific; 34095), and visualized using a VersaDoc Imager (BioRad Laboratories).
Chapter 3: Results

3.1 Adult human spinal cord radial glia (SCRG) do not express Brain Lipid Binding protein (BLBP).

We have previously shown that BLBP, a classic neural stem and radial glia protein, is expressed by a novel population of adult spinal cord RG in the mouse (Petit, Sanders et al. 2011). This population of spinal cord RG is anchored at the margin of the spinal cord and extends long processes towards the center of the spinal cord (Fig 3.1 A). These cells also express glial fibrillary acidic protein (GFAP), and can co-express neural progenitor proteins such as SOX2 and nestin (Fig 3.1 A-B). To investigate if the human spinal cord also retains a population of spinal cord RG that similarly express BLBP we tested human spinal cord sections (at cervical and lumbar levels) from multiple sclerosis (MS) patients and controls using fluorescence immunochemistry. We tested human spinal cord (age range 51-79 years old, See Table 2.1) for proteins normally enriched in radial glia such as BLBP, GFAP and GFAPδ (the isoform of GFAP found in human radial glia). We found that, as in the mouse, the human spinal cord contains a sparse population of radially-arranged cells that are anchored at the margin of the spinal cord, which inwardly extend processes to the center of the spinal cord. These cells share the same morphology as mouse spinal cord RG and also express RG markers such as GFAP (Fig 3.1 C) and human radial glia marker GFAPδ (Fig 3.1 D); however, they do not express BLBP.

Although BLBP was not found in spinal cord RG, it was found instead in numerous ring structures detected in the white matter of the spinal cord of MS patients (Fig 3.1 E, F). These structures are illustrated in the lateral funiculus of MS patient #3, in between the white matter – grey matter interface and the edge of the spinal cord. We also observed the same ring structures
in the anterior and posterior funiculus of the spinal cord white matter of other MS patients. The BLBP+ rings did not co-label with any radial glial proteins and were found in areas rich in GFAP+ astrocytes (Fig 3.1).
BLBP is expressed by mouse but not human spinal cord radial glia. In the FABP7-GFP mouse SCd (A, B published in Petit, Sanders et al. 2011) FABP7-GFP (green) is expressed in Nestin + (red) radial glia (RG) processes. These cells have SOX2 +/ Vimentin (cyan) nuclei with cell bodies (arrows) located in the SCd periphery. (C) The human SCd contains bipolar cells found at the periphery of the SCd that are GFAP + (green, arrow heads). (C’) Boxed area in C, shows a cell body found at the edge of the SCd with GFAP+ processes extending towards the center of the SCd. (D) Human radial glia processes are detected with GFAPδ at the edge of an adult human SCd. (E-F) BLBP + (green) is detected in ring structures (arrows) in the SCd WM of an MS patient, in the lateral funiculus of the SCd (red). Scale bar 50 μm (A-B, E). 25um (F).
Since the lack of BLBP in human radial glia and its presence in human spinal cord white matter were somewhat unexpected findings, we confirmed the fidelity of our BLBP antibodies (see Table 2.2). Using the Fabp7-EGFP reporter mouse, we confirmed that both rabbit polyclonal and mouse monoclonal anti-BLBP co-localized with GFP-positive radial cells at the margin of the spinal cord of the adult mouse (Fig 3.2 B). At postnatal day 5 (P5) the mouse brain is still undergoing neurogenesis and neuronal migration, and at this stage BLBP is robustly expressed by radial glia, developing astrocytes and Bergmann glia in the cerebellum. Accordingly, we detected both mouse and rabbit anti-BLBP immuno-reactivity in GFP-positive Bergmann glia in the cerebellum of the P5 Fabp7-EGFP reporter mouse (Fig 3.2 A), and throughout the brain (data not shown). We next confirmed that the BLBP antibodies used indeed recognize human BLBP. On western blots of human spinal cord white matter homogenate as well as mouse P5 brain, both the monoclonal and polyclonal anti-BLBP antibodies used detect a strong band corresponding to the expected size of BLBP-approx 15-17 kDa (Fig 3.2 C,D). These data confirm that the methods used accurately and specifically recognized both mouse and human BLBP.
Figure 3.2: BLBP is found in P5 brain in the mouse and colocalizes with GFP in the transgenic GFP-FABP7 mouse. (A) Mouse SCRGs are co-labeled with GFP, mouse anti BLBP, and rabbit anti BLBP (arrows). (B) In the postnatal day 5 (P5) mouse brain, Bergmann glia processes co-label with GFP, mouse anti BLBP and rabbit anti BLBP (arrows). GFP is found in the cell bodies and processes and BLBP is mainly found in the processes of the Bergman glial cells. C and D show western detection of BLBP with rabbit (C) and mouse (D) BLBP antibodies recognize BLBP in adult mouse SCd, P6 brain, and adult human SCd (white matter). Scale bar 100um (A), 500um (B).
3.2 BLBP is found in P0-positive myelin of the spinal cord of MS patients:

Based on the observation that BLBP was found in the spinal cord white matter of MS patients, and had a ring structure that resembled a myelin sheath, we asked whether BLBP is found in human myelin, by testing its co-localization with specific myelin markers. We examined the MS spinal cord by double immunofluorescence with BLBP in combination with Myelin basic protein (MBP), used to identify both peripheral and central myelin; proteolipid protein (PLP) to identify central myelin; or myelin protein zero (P0) to identify SC myelin. Although BLBP has not previously been associated with myelin, we know from rodent studies that, in the PNS, BLBP is expressed by immature dividing SCs before they mature into myelinating or non-myelinating SCs (Mirsky, Woodhoo et al. 2008). Analysis revealed that BLBP is found in MBP+ myelin sheaths (Fig 3.3 A-C). These BLBP rings were negative for PLP, suggesting that the BLBP was not produced by oligodendrocytes (Fig 3.3 D-F). Surprisingly, the BLBP+ rings were almost all positive for P0 (Fig 3.3 G-H). In rare cases, however, we observed BLBP not associated with myelin, in cells that morphologically resemble reactive astrocytes (Fig 3.3 I). Although BLBP was almost entirely produced by P0+ cells, not all P0+ cells expressed BLBP. These data indicate that BLBP can be enriched in PNS-type but not CNS-type myelin of the human spinal cord.
**Figure 3.3: BLBP is detected in P0-positive myelin in the spinal cord of multiple MS patients.** (A) Diagram shows SCd region from which each image was taken. (B-E) In the SCd white matter of MS patients, BLBP (green) is found in MBP+ (red) myelin rings. (F-I) These BLBP rings did not co-label with PLP+ (red) CNS myelin. (G-I) BLBP (red) is found in P0+ (green) myelin. Most P0 myelin in the SCd of MS patients is positive for BLBP (yellow where co-localized): arrows point at P0 myelin that is partially BLBP+. (I) In the MS SCd BLBP is almost exclusively found in SCs but can also occasionally be detected in cells that morphologically resemble astrocytes (arrowheads). Scale bar 50μm (B, D, E, F, G, I) 25μm (C, H).
3.3 **BLBP is expressed by myelinating Schwann cells of MS and non-MS cases:**

After identifying P0+ myelin as the source of BLBP in the MS spinal cord, we investigated if P0+ SCs in the rootlets around the spinal cord also express BLBP. We analyzed posterior and anterior rootlets around the spinal cord of two MS cases (cervical level) and non-MS cases (controls with non-demyelinating diseases, lumbar level: see Table 2.1) by fluorescence immunochemistry. We found that BLBP is produced by myelinating SCs of adult human PNS in both MS and non-MS peripheral nerve roots (Fig 3.4). We noticed that the amount of BLBP detected in SCs appears higher in rootlets of MS cases in comparison with the non-MS cases (Fig 3.4 A, D), and that highest levels of BLBP detected were in more loosely wrapped SCs that resembled onion bulb myelin. These SCs show a concentric appearance with multiple visible layers ensheathing large or medium size axons the axons that are negative for heavy chain neurofilament (H-NF), we refer to these myelin profiles as uncompacted myelin. Such structures are present in both MS and non-MS cases but are more numerous in MS cases (Fig 3.4 C, F). SCs myelinating small caliber axons do not express BLBP. In addition, disruption of the dorsal root entry zone (DREZ) was observed in both MS patients: at the DREZ many SCs had transgressed the CNS boundary and many expressed BLBP (Fig 3.4 G).
Figure 3.4: BLBP is expressed by myelinating SCs in the normal and MS human PNS. (A, D) BLBP (red) partially overlaps with P0+ (green) myelin in control (Pt 1) and MS patient (Pt 3) dorsal root, with higher frequency of BLBP+ rings in MS (Pt 3). (B) P0+ (green) and MBP+ (red) “normal” myelin indicates an intact dorsal root entry zone (DREZ) at the CNS/PNS boundary. (C) BLBP (red) appears to be more highly expressed in uncompacted myelin rings (arrow) than in myelin rings with more typical morphology. (E) PNS myelin is recognized by two different BLBP antibodies, monoclonal mBLBP (red) and polyclonal rBLBP (green). (F) Uncompacted myelin rings in MS patient #3 (arrows) co-labels with rBLBP (red) and P0 (green). (G) DREZ of MS patient 3 is disrupted: P0+ and mBLBP+ (yellow) SC (arrow) are found in lower part of the dorsal root and also in the periphery of the SCd white matter. (G’) Box in G. Scale bar 50µm.
Further examination of the rootlets in longitudinal and transverse sections confirmed that BLBP is highly enriched in uncompacted myelin (Fig 5 A-C).

To test the variation in number of BLBP + SCs across MS and non-MS cases we quantified the amount of BLBP positive and negative myelin rings found in three sampled rootlets of each patient.

The amounts of BLBP+ myelin found in MS and in non-MS cases were within the same range. The data did not suggest a relationship between the number of BLBP+ myelin rings and number of uncompacted myelin rings, and the ratio of BLBP+ to BLBP-negative myelin rings was highly variable between patients (Fig 3.5 D). The number of BLBP-negative myelin rings was higher than BLBP+ myelin rings in most cases, but other cases had similar amounts of BLBP + and negative myelin (Fig 3.5 D).

Since BLBP appeared to be enriched in uncompacted myelinating SCs, and these were more numerous in the two MS cases than the non-MS cases, we investigated whether this was significant in a larger sample of MS cases. For this we analyzed rootlets and spinal cord of 11 MS patients and 3 non-MS cases (Post mortem intervals 3-6 days, Table 2.1) and quantified the amount of uncompacted myelin found in three sampled rootlets of each patient. The amount of uncompacted myelin is highly variable across MS cases, ranging from 0 to 318 in total uncompact myelin rings. Variation within an individual’s spinal cord was also observed. Within and across the same sections, several MS cases displayed a mix of intact rootlets comprising compact and normal-looking myelin, and rootlets where half of the myelin was uncompacted
(Fig 3.5 E). No relationship between the number of uncompacted myelin rings and post mortem interval was observed (Table 2.1).

To confirm that the P0 + staining found in the spinal cord of MS cases was not an artifact, we imaged the sections at high magnification (60X) and successfully identified Schwann cell somas with DAPI-stained nuclei (F). Additionally we visualized basal lamina using an antibody to collagen IV, since basal lamina is produced by SCs but not by oligodendrocytes. SC basal lamina -identified with Collagen IV - was found to be present around both compact and uncompacted myelin rings (Fig 3.5 G-H). Immunofluorescence detection of P0, BLBP and collagen IV shows that basal lamina is produced around some P0+/BLBP+ cells (Fig 3.5 H-I), supporting the idea that the CNS P0+/BLBP+ myelin is produced by SCs.
Figure 3.5: Quantification of uncompacted myelin and BLBP-positive SCs. (A) Longitudinal section of dorsal rootlet from MS patient 6, and (B) transverse section of dorsal rootlet from MS patient 3, labelled for P0 myelin (green) and BLBP (red). Both panels illustrate that BLBP is more highly expressed in uncompacted myelin (arrows). (C) Schwann cell soma (arrow) in PNS of MS patient, nucleus (blue), P0 (green), BLBP (red). (D) Quantification of BLBP-positive and -negative myelin rings from same rootlets used for onion bulb-like myelin quantification. Circles represent the number of BLBP+ myelin rings quantified at each rootlet, inverted triangles represent the number of BLBP− myelin rings at each rootlet, horizontal bars represent the mean. (E) Quantification of onion bulb-like myelin at three rootlets in each human case. Each point represents the number of onion bulb-like myelin rings quantified, the horizontal bars represent the mean from the three counts. (F) BLBP+ myelinating Schwann cell in the SCd WM of MS patient3, arrow indicates cell soma. (G) MS Patient 8 rootlet, myelin (green), BLBP (red), basal lamina labeled with Collagen IV (cyan), showing that basal lamina is found around each individual BLBP+ myelin ring. (H) CNS P0+ (green) myelinating cells produce basal lamina (collagen IV, cyan) in the SCd of MS patients, BLBP (red). (I) Collagen IV within the same the SCd region showed in panel H. Scale bar 50um (A,B,G,H,I) 12um (C,F).
3.4 Schwann cell myelin in the spinal cord of MS cases

Although sparse sub-populations of axons with SC myelin has been previously reported in the spinal cord of sporadic MS cases (Ghatak, Hirano et al. 1973, Itoyama, Webster et al. 1983, Yamamoto, Kawamura et al. 1991), there is little information about how much these cells can contribute to endogenous remyelination in MS. To investigate this, we focused on MS cases with documented spinal cord Schwannosis, and inspected lumbar and cervical spinal cord sections for Schwann cell myelin using immunofluorescence detection of P0. We found that in the MS spinal cord Schwann cell remyelination can occur at any location of the white matter (not only next to root entry zones), is highly variable within and across patients, and can be very abundant in the spinal cord (Fig 3.6 A, D). As MS case 8 at the cervical level shows (Fig 3.6 A), approximately 2/3 of the spinal cord myelin is produced by SCs, mainly in the posterior portion of the spinal cord but also in anterior and lateral regions of the white matter. Several other patients exhibited P0 in posterior (Fig 3.6 B) and lateral regions of the spinal cord (examples in Fig 3.6 C-D). At no time did we detect overlap between P0 and PLP immunoreactivity, indicating that P0-positive myelinating cells ensheath axons that are not myelinated by oligodendrocytes. In patients with Schwannosis (presence of SCs in the spinal cord (Treacy, Redmond et al. 2009), not all the demyelinated lesions contained SCs (Fig 3.6 C, E). Additionally, we looked in the brain white matter of 4 MS patients that had SCs in the spinal cord to investigate if endogenous SCs remyelination occurs in the brain. No P0+ myelin was found in the brain, although demyelination lesions were present, identified by the lack of PLP and Iba1+ microglia (6 F-G).
Figure 3.6: SCs extensively re-myelinate the spinal cord but not brain of MS patients. (A,B,C) Sections from three different MS patients were labelled for SC myelin (P0, green) and oligodendrocyte myelin (PLP, red). In the SCd of Pt 8 (A) SCs had myelinated at dorsal, ventral and lateral locations extensively: there was more P0+ myelin than PLP+ myelin. (B) In Pt 8 extensive Schwann cell myelination was evident in the posterior column and DREZ (right side of image). SC myelination extended far into the posterior horn; CNS myelin was mostly preserved except for the P0 myelinated areas and the left posterior part of the SCd. In Pt 3 (C) we observed a large loss of CNS myelin (red), the right side of the SCd had very little PLP, and some axons mainly on the lateral funiculus and close to the DREZ had been myelinated by SC. (D) Heat map of P0 in MS patient 3 aids visualization of the amount and location of SC in the SCd. (E) In the SCd of MS pt 7, we observed a disruption of the DREZ and a small number of SC in the CNS (arrows). (F) In the brain white matter we observed a lack of P0 myelin (green). A section from Pt 7 is shown as a representative case: this phenomenon was observed in all four MS cases analyzed. (G) Shows Brain WM of MS pt7, GFAP (red) shows astrocytes in the brain, especially around lesion areas, Iba1 (green) shows microglia (arrow head) in a region of the brain affected by loss of myelin. Scale bar 5mm (A-D), 50um (E-G).
spinal cord SCs have been suggested to infiltrate from nerve fibers associated with blood vessels and DREZ (Franklin and Blakemore 1993, Duncan and Hoffman 1997). To understand better the location and potential source of CNS SC we analyzed the spinal cord of 11 MS cases and documented the location where SC myelin, detected by P0 immuno-reactivity, was found. We observed that SC myelination occurs in three distinct places: in proximity to dorsal or ventral root entry zones (8 cases Fig 3.7 A -B), occasionally disrupting the structure of the root entry zone; in perivascular spaces (9 cases, Fig 3.7 C-D); or in parenchymal white matter at locations distal from roots and perivascular spaces (7 cases, Fig 3.7 E-F). Although the presence of SC myelin in the analyzed MS cases is common (9 out of 11) the amount of SC myelin rings in the MS spinal cord was variable: in most cases levels of SC myelination was low with approximately 20 myelin rings quantified per spinal cord section, while 2 cases had robust SC remyelination (greater than 200 rings per spinal cord section) (Fig 3.6 A-D).
Figure 3.7: In the MS spinal cord SCs are evident at three main locations. (A-B) In the MS SCd CNS SCs are often found in proximity to a disrupted DREZ. (A) In MS patient 6, Schwann cells (P0, green, arrow) have transgressed the CNS-PNS boundary at the DREZ, and are surrounded by PLP+ (red) myelin. (B) In MS patient 3, several axons at the periphery of the posterior horn have been myelinated by Schwann cells (P0, green), while that part of the SCd has lost all CNS-type myelin (PLP, red). (C-D) In the MS SCd SCs are also found encapsulated in perivascular spaces. (C) In MS patient 5, a cluster of SCs (P0, green, arrow) are located in the perivascular space of the anterior spinal artery. (D) In MS patient 276, a cluster of SCs (P0, green, arrow) is shown encapsulated in the perivascular space of an anterior branch of the anterior spinal artery. (E-F) In MS, SCs also myelinate axons in white matter at distal locations from arteries and root entry zones. (E) In MS patient 3 SCs (arrows) have myelinated axons in the lateral funiculus in an area that has lost all CNS-type myelin (PLP, red). (F) MS patient 3 shows SCs (green) in SCd white matter myelinating axons in a GFAP-rich area (red). (G) Shows GFAP detected with rabbit anti-GFAP (green) and chicken anti-GFAP (red) in the dorsal spinal cord WM of MS pt 5. Scale bar 50 um (A-E) 25 um (F, G).
3.5 Spontaneous SC re-myelination in the mouse demyelinated spinal cord

To investigate if mouse remyelinating SCs express BLBP in the mouse as they do in the spinal cord of MS patients, we tested a chemical demyelinating model that may mimic some of the demyelinating pathology of MS. We used L-α-Lysophosphatidylcholine-induced (LPC) demyelination lesions in the dorsal column of the spinal cord (Cervical, C4) of 6 month old FABP7-EGFP mice. At 1 and 3 weeks post lesion (WPL), we identified the demyelination lesion by immunofluorescence detection of MBP (myelin basic protein) and Iba1 (ionized calcium-binding adapter molecule 1, found in macrophages and microglia). At 1 WPL, loss of myelin (MBP) was evident at the site of injection, and a large number of apparently activated microglia were observed both within and outside of the lesion (Fig 3.8 A). There were also many reactive astrocytes highly expressing BLBP in the lesion, and some GFAP reactive astrocytes up-regulated BLBP at the spinal cord dorsal margin (Fig 3.8 B). No P0+ cells could be detected at 1 WPL (8 C). At 3WPL, MBP+ cells were abundant throughout the lesion site: some debris is observed at the center of the lesion but the lesion site appeared to have been repopulated by oligodendrocytes (Fig. 8D). Activated microglia still persist at the lesion site (Fig 3.8 D) but are less abundant than at 1WPL. In 3 mice out of 6 the lesion also appeared to be re-myelinated by some P0-expressing SCs (Fig 3.8 E, G). However, unlike in the spinal cord of MS patients, mouse spinal cord SCs that express P0 after demyelination do not appear to up-regulate BLBP, by 3 WPL. Rather BLBP at this time is mostly found at the injection site and around the margin of the spinal cord and expressed by reactive GFAP+ astrocytes (Fig 3.8 F). Most spinal cord SCs were clustered at the center of the lesion, but some clusters of SCs where also found in proximity to a disrupted DREZ (Fig 3.8 G) and blood vessels (Fig 3.8 H). In one animal the demyelination
extended beyond the dorsal column into a ventral horn, where rare SCs could be detected (data not shown).

In summary, LPC-induced demyelination in the dorsal column of the spinal cord resulted in loss of CNS myelin in the dorsal column by 1WPL, with no oligodendrocytes or SCs detected at the lesion site. At 3 WPL, oligodendrocytes largely restored myelin at the lesion site. Additionally, SCs remyelinated the centre of lesion and in proximity to DREZ and in blood vessels, but mouse remyelinating SCs did not express BLBP.
Figure 3.8: BLBP is not expressed by myelinating SCs in the LPC demyelinated SCd. (A-C) LPC demyelinated SCd 1 week post lesion (WPL). (A) At 1 WPL myelin is absent at the injection site (dorsal column) of the mouse SCd. Activated microglia (green) are present around the lesion site. (B) At 1WPL GFAP (red) and BLBP (green) are upregulated by astrocytes around the lesion site. (C) No P0+ (green) myelin is found anywhere in the SCd at 1WPL. (D-H) LPC demyelinated SCd at 3WPL. (D) At 3WPL the microglia (green) remain at and around the lesion site; CNS myelin (red) has been replaced at the lesion. (E) At 3 WPL SC (green) myelination can be observed at the epicenter of the lesion. BLBP (red) is highly expressed at the lesion site but is not expressed by SCs. (F) At 3 WPL BLBP (green) is highly expressed by astrocytes (red) that moved in to the lesion site. (G) At 3 WPL SC myelin (green) can be observed at the periphery of the lesion where the demyelination extended to the dorsal horn, next to the DREZ. BLBP+ astrocytes (red) are abundant around SCs. (H) At 3 WPL SCs can be observed in a blood vessel at the periphery of a dorsal lesion. The blood vessel is surrounded by endfeet of BLBP+ astrocytes (red). Arrow indicates an astrocyte in contact with a blood vessel. Scale bar 50 um (A-G) 12 um (H).
Chapter 4: Discussion

The expression of BLBP has proven to be extremely useful for tracking the development of CNS progenitors. BLBP was first established as a classic radial glia protein in the rodent CNS; however, it has subsequently been found in spinal cord and brain progenitor cells of non-mammal vertebrates such as zebrafish, turtle, and frog (Adolf, Chapouton et al. 2006, D'Amico, Boujard et al. 2011, García, Libisch et al. 2012). The Roskams lab has used the expression pattern of BLBP to demarcate the distribution of spinal cord RG in the adult mouse spinal cord (Petit, Sanders et al. 2011) and olfactory ensheathing cells in the olfactory system (Westendorf, 2008). In the present study we tested the dynamics of BLBP-expressing glia in the human spinal cord of MS and non-MS cases. We show that unlike the mouse spinal cord, BLBP is not found in aged human spinal cord RG, although a morphologically similar population of spinal cord RG is preserved, identified by the expression of the RG proteins GFAP-δ and GFAP (Fig 3.1). More strikingly, the expression of BLBP was localized in white matter myelin sheaths of MS cases (Fig 3.1, 2). The progenitors of the human spinal cord have not been fully characterized, and the pattern of BLBP expression in the adult human spinal cord has not been thoroughly tested, although analysis of RG in the human telencephalon shows that the antigens used to identify RG in rodents such as vimentin, GLAST, GFAP and BLBP also label RG in the developing human CNS (Howard, Zhicheng et al. 2008). In the mouse, BLBP is restricted to RG populations in the central canal and spinal cord margin; these cells co-express other classic neural stem cell genes such as Sox2, Vimentin and Nestin (Petit, Sanders et al. 2011). Changes in spinal cord BLBP expression have been reported after spinal cord compression injury (SCI) and EAE (experimental autoimmune encephalomyelitis) demyelination (White, McTigue et al. 2010). In both lesion models, up-regulation of BLBP is mostly associated with astrogliosis. After spinal cord injury
BLBP is up-regulated in astrocytes associated with the glial scar around the border of the lesion, and also in astrocytes with hypertrophied morphology in spared white and grey matter of the injured spinal cord (White, McTigue et al. 2010). Following EAE, reactive astrocytes show high BLBP expression around and within demyelinating lesions (Bannerman, Hahn et al. 2007), and are more numerous during the remission phase in spinal cord white matter lesions (Kipp, Gingele et al. 2011). Additionally, BLBP+ reactive astrocytes are present in human MS brain lesions, and are more numerous in early MS lesions than in lesions from patients with long MS duration (Kipp, Gingele et al. 2011). Our study reports the presence of BLBP+ astrocytes in the spinal cord white matter of one MS case, but it was not observed in the other MS cases studied (Fig 3.3). In addition to astrocytes, BLBP is up-regulated in the spinal cord after EAE demyelination and SCI by spinal cord RG and central canal progenitors (Roskams lab, unpublished observations). After SCI, spinal cord RG divide, and up-regulate BLBP and other developmental markers around the lesion core at the margin of the white matter (Petit, Sanders et al. 2011).

Here, we report the presence of PNS-type myelin in the spinal cord white matter of MS cases, as evidenced by the observation of abnormal BLBP expression coincident with P0 in the MS spinal cord (Fig 3.3). Unexpectedly, BLBP was also expressed by myelinating SC in the PNS in anterior and posterior rootlets of the spinal cord of both MS and non-MS cases (Fig 3.3). Although BLBP is normally expressed by SC precursors and immature SC (Mirsky, Woodhoo et al. 2008) it has not previously been associated with re-myelinating SC. One study in mouse has reported abnormal BLBP expression in the adult PNS 14-30 days after sciatic nerve crush Miller, Li et al. 2003). However, in contrast to our present findings in human SCs, BLBP in the crushed mouse nerve does not overlap with the myelin sheath; rather, it was only detected outside the re-
myelinated axon rings. Although BLBP was likely produced by SC, no co-labeling of BLBP with any cell-type marker was shown to confirm the cell producing BLBP after nerve crush (Miller, Li et al. 2003). Here we show that BLBP is in P0-positive axon ensheathments of SCs, most of which are myelinating medium-large caliber axons (Fig 3.4). We show a type of myelin pathology of uncompacted myelin rings, and that varies widely in abundance across MS cases. We demonstrate that uncompacted myelin is associated with higher BLBP expression in P0-positive ensheathments (Fig 3.4). It is possible that the presence of structures resembling onion bulbs could be an artifact of the post mortem time, and myelin breakdown. However, we find that the number of onion-bulb like myelin rings per patient is not correlated with the post-mortem time or duration of disease (Fig 3.4 and Table 2.1), and that the variability of detection within the same section each patient (with obviously the same post-mortem time and tissue treatment) makes this an unlikely explanation. The causes and significance of PNS pathology in MS are not well understood, but onion-bulb-like formations and other peripheral nerve damage has been reported in several MS cases before (Poser 1987).

The actual role of BLBP during SC development and disease is not understood; however, one study suggests that BLBP could be involved in controlling SC morphology, as well as SC-axon interaction. SCs which lack NF1 display a defective interaction with axons when co-cultured with neurons in vitro. However, when these cells are treated with BLBP blocking antibodies, SC morphology shifts from process-free to stellate, concomitant with an improvement in SC-axon interactions (Miller, Li et al 2003). Although our analysis of peripheral myelin shows that BLBP is most highly detected in myelinating SC with less-compact morphology, the relationship between abnormal PNS myelin and BLBP should be further investigated. Alternatively, BLBP
may be more readily detected in uncompacted myelin, because its antigen is more accessible to antibody in the less compact myelin. Since BLBP is normally expressed by immature-like glial phenotypes with migratory and proliferative characteristics, such as olfactory ensheathing glia, immature astrocytes, and immature SC, it is worth using a more extensive battery of reagents to ask if in the human patients BLBP expression could be an indicator of SC with characteristics of a more “immature” phenotype.

Peripheral-type myelin has been reported in lesions of injured spinal cord, and animal models of spinal cord injury and demyelination (Jasmin, Janni et al. 2000, Jasmin and Ohara 2002, Guest, Hiester et al. 2005, Ikota, Iwasaki et al. 2010, Zawadzka, Rivers et al. 2010), but only few studies have reported this in the spinal cord of MS cases (Ghatak, Hirano et al. 1973, Itoyama, Webster et al. 1983, Itoyama, Ohnishi et al. 1985, Yamamoto, Kawamura et al. 1991) and two of these four were from a single case study (Ghatak, Hirano et al. 1973, Yamamoto, Kawamura et al. 1991), highlighting the importance of analyzing more MS cases to learn about peripheral-type re-myelination in the context of MS. Our study reports the presence of peripheral myelin in the MS spinal cord of 9 cases: we found that the amount and location of peripheral-type myelin varies significantly across patients, and that the incidence of peripheral myelin that we have detected can be far more extensive than previously reported (Fig 3.5, 3.6). In accordance with previous studies of peripheral myelin in the MS spinal cord, we observed that peripheral-type myelin mainly occurs near potential PNS sources, such as spinal roots and perivascular spaces. However, in the MS cases with more robust peripheral-type myelin, it was also found throughout white matter lesions distal from PNS sources. It is thought that the peripheral-type myelin found in the MS spinal cord is likely a result of SCs transgressing the CNS-PNS boundary. This
assumption is based on the observations that spinal cord SC are normally found in close proximity with PNS sources, and that very often the CNS-PNS glial boundary in the dorsal roots is disrupted in MS cases and animal demyelination models with spinal cord SC. Our data corroborates these observations, and thus supports this hypothesis in some of the cases reported here. An alternative origin of CNS peripheral-type myelin, however, could be from CNS precursor cells. At least in the LPC-induced demyelinated spinal cord, PDGFRA/NG2 expressing precursors can give rise to re-myelinating SC in the mouse spinal cord (Zawadzka, Rivers et al. 2010). Abnormal uncompacted myelin is common in the analyzed MS cases (Fig 3.4), and that disrupted DREZ was also common in MS patients with spinal cord peripheral-type myelin (Fig 3.6) and also contained unusual P0+/BLBP+ myelin profiles, it is possible that SCs arising in the CNS of MS patients could be linked with peripheral pathologies that have been not been investigated in detail. Although the link between MS and PNS pathologies is somewhat controversial, histological (Poser 1987) and electrophysiological studies suggest that PNS abnormalities are not that rare in MS patients. According to electrophysiological studies, many MS patients have peripheral sensory lesions, although these are normally subclinical (Couratier, Boukhris et al. 2004, Pogorzelski, Baniukiewicz et al. 2004). If human peripheral pathology stimulated SC proliferation and migration (as is the case in many rodent models), the SCs produced may have a developmental phenotype more capable of traversing the PNS-CNS boundary and responding to axonal remyelination signals than normal mature SCs.

With respect CNS P0-positive re-myelinating cells, we show that similar to what we observe in the PNS, subsets of BLBP-positive SCs produced basement membrane in the spinal cord white matter of MS cases (Fig 3.4). Human spinal cord SCs have not been well characterized, and the
identification of these cells in MS cases has been limited to immunodetection of peripheral myelin and EM observations (Ghatak, Hirano et al. 1973, Itoyama, Webster et al. 1983, Itoyama, Ohnishi et al. 1985, Yamamoto, Kawamura et al. 1991). Here, we extend characterization of spinal cord SCs by using multiple myelin markers, and showing that subsets of these cells express BLBP. Further, although observations of SC in the human and rodent demyelinated spinal cord suggest that SCs predominantly occur in areas lacking astrocytes, (Itoyama, Ohnishi et al. 1985, Ikota, Iwasaki et al. 2010, Zawadzka, Rivers et al. 2010), we show that in the spinal cord of MS patients, SC myelin occurs in GFAP-rich areas (Fig 3.6). Indeed, comparisons of GFAP intensity and abundance of GFAP+ cells in various white matter locations with and without peripheral-type myelin in the MS spinal cord suggested no positive or negative relationship between the occurrence of peripheral-type myelin and GFAP-expressing cells.

Finally our LPC-induced demyelinated lesions in the mouse spinal cord, confirmed that SCs can remyelinate CNS axons in the dorsal column at 3 WPL. No SC were found at 1WPL this confirms previous observations of LPC-induced demyelinated lesions in the dorsal and ventral funiculus of the mouse spinal cord (Zawadzka, Rivers et al. 2010). However, our analysis showed that unlike in the case of human MS, CNS re-myelinating SCs up-regulate P0, but do not retain BLBP expression, all BLBP in the lesion center and periphery being associated with astrocytes. SC were found mostly in the epicenter of the lesion, but could also be found in proximity of PNS sources (next to disrupted DREZ and in association with blood vessels). Our data do not support the hypothesis that SC remyelination preferentially happens in the absence of astrocytes (Zawadzka, Rivers et al. 2010; Itoyama et al., 1985; Blackemore, 1975), at both time-points the lesions were populated by astrocytes, and CNS re-myelinating SC were always found
in astrocyte rich areas that were also BLBP positive. The lesions caused up regulation of BLBP beyond the lesion boundaries - in the central canal, and spinal cord margin, by astrocytes and RG-like cells. Astrocytes became more reactive over time, up-regulating both BLBP and GFAP.

Our data shows that the pattern of expression of BLBP in both SCs and astrocytes in human MS lesions and mouse LPC-induced lesions is very different, as is the pattern of remyelination from SC-like cells. Further studies should aim to characterize human cell types that participate in demyelination repair to investigate human intrinsic differences in pathways related to development and pathology.

**Caveats and Limitations:**

Our observations in the human spinal cord and brain were done in samples from patients with pathology, either MS or spinal cord trauma (non-MS controls). Therefore our observations about BLBP in SCs should be compared with healthy spinal cord roots control, in order to clarify if BLBP is normally expressed by human SCs or is only expressed in response to pathological conditions. Another limitation of this study is the lack of clinical information and medical history of the patients studied, knowing the differences in the progression of the disease as well as type of treatment used for each patient could be helpful for understanding the variation of SCs remyelination in the spinal cord of MS cases.
Chapter 5: Conclusion

5.1.1 Recommendations for future work

5.1.1.1 BLBP in re-myelinating SCs under normal and pathological conditions:

The results presented in this thesis suggest that unlike in the mouse, human re-myelinating SCs express BLBP, and BLBP is more highly enriched in abnormal uncompacted myelin that is produced to different degrees across MS patients, and across different tracts within a single MS patient. In the mouse SC lineage thus far, BLBP is exclusively associated with SC precursors and immature SCs (Mirsky, Woodhoo et al. 2008). Additionally, studies in RG and SCs suggest that BLBP is involved in regulating cell morphology (Feng, Hatten et al. 1994, Hartfuss, Forster et al. 2003, Miller, Li et al. 2003). However, future work should clarify the pattern of expression of BLBP during human SC development as well as during SC response to PNS degeneration and regeneration.

The main limitation with studying human SCs is the source: ethical considerations as well as limited availability of prenatal and early postnatal human tissue make the study of SC development difficult (Kaplan, Odaci et al. 2009). Additionally, the harvesting of adult SCs from donors results in functional peripheral nerve losses (Hood, Levene et al. 2009). An attractive alternative source of human SCs is skin-derived precursors (SKPs), which can be made from adult neural crest-derived and mesodermal-derived dermal precursors (Krause, Dworski et al. 2014). SKP-derived SC can be isolated and differentiated into functional SCs, and can re-myelinate rodent PNS and CNS axons (Biernaskie, McKenzie et al. 2006, McKenzie, Biernaskie et al. 2006). SKP-derived SCs closely resemble peripheral nerve-derived SCs, and they can re-myelinate embryonic DRG neurons in culture (Krause, Dworski et al. 2014), two features which
make them a good candidate for an in vitro model in which to analyze the expression of BLBP. In vitro studies could facilitate a variety of experiments to investigate the pattern of BLBP expression at different stages such as; SC differentiation, SC myelination and SC de-myelination under normal and pathological conditions. Additionally BLBP blocking antibodies could potentially be used to test its importance for the axon-SC association.

5.1.1.2 Analysis of cell and pathologic heterogeneity in lesions with endogenous SCs in the spinal cord of MS patients:

The work of this thesis shows that endogenous SCs can re-myelinate spinal cord axons in MS patients. SC remyelination has also been reported in some spinal cord lesions in human cases as well as in animal models of demyelination and spinal cord injury (Ghatak, Hirano et al. 1973, Blakemore 1978, Itoyama, Webster et al. 1983, Itoyama, Ohnishi et al. 1985, Jasmin and Ohara 2002, Guest, Hiester et al. 2005, Ikota, Iwasaki et al. 2010, Zawadzka, Rivers et al. 2010). However, further investigation should identify the cues and lesion environment that promote endogenous SCs to remyelinate, as this is of high therapeutic interest. Although previous studies suggested that the absence of astrocytes is important for SCs to remyelinate (Itoyama, Ohnishi et al. 1985, Ikota, Iwasaki et al. 2010, Zawadzka, Rivers et al. 2010), our data shows that that endogenous SCs can remyelinate in GFAP-rich areas. Additionally, we observed that not all demyelinated axons are remyelinated by SCs, and the amount of SC remyelination across patients is variable. Given the heterogeneity of MS lesions (Lucchinetti, Bruck et al. 2000), a systematic comparison of the cell composition (glia, precursor cells and immune cells), and immunological activity (active versus inactive lesions) in MS lesions with and without SCs could shed some light on the factors that are important for SC remyelination.
5.1.1.3 Analysis of immune cells associated with endogenous SCs in the spinal cord of MS patients:

One aspect that escaped the scope of this study was a close examination of the immune component in these MS cases. It has been shown that the level of immune activity and immune components varies across MS lesions, and it is well understood that these factors could affect remyelination (Lucchinetti, Bruck et al. 2000). In our studies, mouse microglia appeared very active in the demyelinating lesions – diminishing as remyelination was established. Closer analysis of the immune infiltrates present in MS lesions that contain P0+ myelin may be extremely informative for understanding the pathological state of the lesions that had CNS remyelinating SCs.

5.1.2 Concluding statement

We observed marked differences in the pattern of expression of a key developmentally-regulated protein (BLBP/FABP7) between mouse and human SCs and spinal cord RG. While BLBP is highly expressed by mouse spinal cord RG, we did not detect BLBP in the aged human spinal cord RG. Despite the fact that BLBP is highly conserved among vertebrates, and is widely used as a marker for RG during the development, our study suggest that the BLBP is not retained in the adult MS or non MS spinal cord. Additionally BLBP was detected in re-myelinating SC in the MS and non-MS rootlets, as well as in the MS spinal cord, but not in the mouse SC. Our study therefore suggests that BLBP expression may be retained through development from SC precursor to some myelinating phenotypes in human SCs. Furthermore BLBP is enriched in pathologic uncompacted myelin rings in MS and non MS PNS, suggesting a role for BLBP in SC pathology. The role of BLBP in PNS myelin and abnormal myelin should be further clarified.
Finally endogenous SC can extensively re-myelinate the MS spinal cord but there is high variation in the amount of PNS-type myelin in the MS spinal cord. In addition, reactive astrocytes do not appear to limit SC myelination in MS, but further research should clarify the cues and environment that allow SC to re-myelinate CNS axons in the MS spinal cord.
References


