

Investigating Oligodendrocyte Biology and Function: Insights from Neurological and Neuromuscular Diseases

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Medicine

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ABSTRACT

Oligodendrocytes (OLs) are the cells responsible for myelin production in the central nervous system (CNS). Myelin serves to increase the efficiency of signal propagation down the axon and is essential for proper communication between the CNS and the periphery. As a result, pathologies affecting the OL, including multiple sclerosis (MS) and multiple system atrophy (MSA), present with a wide range of symptoms including impaired muscle control, loss of coordination, as well as cognitive deficits. While the biology of the OL continues to garner research interest, much remains to be understood about cell function in a healthy context, and also how the biology of these cells goes awry in disease. Our objective was to explore the effects of varying disease models on OL biology and use those findings to further our knowledge on the biology of OL development and regeneration. Here we explore OL function and dysfunction in the context of spinal muscular atrophy (SMA), MSA and MS. We have thoroughly characterized the OL response to SMN-depletion and have determined that SMN is not required for the development of OLs in the neonatal brain. Additionally, we have sought to characterize the endogenous role of MSA-disease relevant protein alpha-synuclein in OL development and have determined that this protein is not required for OL differentiation or CNS myelination. Lastly, we have explored the biology of the OL in the context of the inhibitory milieu it faces during remyelination in MS. We have investigated different pathways that may be involved in mediating signalling of one such inhibitory cue (chondroitin sulphate proteoglycans, CSPGs), and have extended this model to interrogate OL cytoskeletal dynamics in the context of CSPGs. Together, this work uses disease frameworks to investigate basic OL biology, as well as provides insights into how the OL and its interactions with the extracellular milieu should be considered in disease pathogenesis and therapeutic exploration.

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List of Abbreviations

α -syn – Alpha-synuclein
A β – Amyloid beta
AD – Alzheimer's disease
ALS – Amyotrophic lateral sclerosis
ANOVA – Analysis of variance
ARP – Actin-related protein
BSA – Bovine serum albumin
CC3 – Cleaved caspase 3
CDC42 – Cell division control protein 42 homolog
CNP – 2'-3'-cyclicnucleotide 3'-phosphohydrolase
CNS – Central nervous system
CNTF – Ciliary neurotrophic factor
CSPGs- Chondroitin sulfate proteoglycans
DAPI – 4',6-diamidino-2-phenylindole
DCC – Deleted in colorectal cancer
DEG – Differentially expressed gene
DIV – Days *in vitro*
DMEM – Dulbecco's Modified Eagle's Medium
dNTPs – Deoxynucleoside triphosphates
DRG – Dorsal root ganglion neuron
DTI – Diffusion tensor imaging
E(#) – Embryonic day #
EAE – Experimental autoimmune encephalomyelitis
ECM – Extracellular matrix
EM – Electron microscopy
EtBr – Ethidium bromide
F-actin – Filamentous actin
FBS – Fetal bovine serum
FGF-2 – Basic fibroblast growth factor
Fyn – Fyn tyrosine kinase
GCI – Glial cytoplasmic inclusion
GS – Goat serum
H – Hour
HBSS – Hank's Balanced Salt Solution
HD – Huntington disease
HS – Horse Serum
Htt – Huntingtin
IFN γ – Interferon gamma
ILK – Integrin-linked kinase
iPSC – Induced pluripotent stem cell
LiCl – Lithium chloride
LPC – Lysophosphatidylcholine
MAG – Myelin Associated Glycoprotein
MAPK13 – Mitogen-activated protein kinase 13

MBP – Myelin Basic Protein
 MGC – Mixed glial culture
 mHTT – Mutant Huntingtin
 miR – MicroRNA
 min - minute
 MOG – Myelin/oligodendrocyte glycoprotein
 MRI – Magnetic resonance imaging
 MS – Multiple Sclerosis
 MSA – Multiple system atrophy
 Myrf – Myelin regulatory factor
 NAWM – Normal appearing white matter
 NG2 – Neural/glial antigen 2
 N-WASP – Neuronal-WASP
 OL – Oligodendrocyte
 Olig2 – Oligodendrocyte transcription factor 2
 OPC – Oligodendrocyte precursor cell
 OPCAs – Oligodendrocyte precursor cell aggregates
 P(#) – Post-natal day #
 PBS – Phosphate buffered saline
 PBST – PBS/0.05% Tween-20
 PDGF-A – Platelet-derived growth factor A
 PDGF-R α – PDGF-receptor alpha
 Pen/Strep – Penicillin-Streptomycin
 p-GSK3 β – Phosphorylated GSK3 β
 PFA – Paraformaldehyde
 PFF – Pre-formed fibrils
 PKC – Protein kinase C
 PLP – Proteolipid Protein
 PMSF – Phenylmethylsulfonyl fluoride
 PPMS – Primary progressive multiple sclerosis
 PTP σ – Protein tyrosine phosphatase sigma
 Rac1 – Ras-related C3 botulinum toxin substrate 1
 RIPK3 – Receptor interacting protein kinase 3
 Rho – Ras homologous
 RNA – Ribonucleic Acid
 ROCK – Rho-associated kinase
 RPM – Rotations per minute
 RRMS – Relapsing remitting multiple sclerosis
 RT-QPCR – Reverse transcriptase-quantitative polymerase chain reaction
 SCI – Spinal cord injury
 scRNA-seq – Single-cell Ribonucleic Acid Sequencing
 SMA – Spinal muscular atrophy
 SMN – Survival motor neuron
 SN-VTA - Substantia nigra ventral tegmental area
 SNAP25 – Synaptosomal-associated protein 25 kDa
 SNARE – Soluble N-ethylmaleimide sensitive factor attachment protein receptor

SOD1 – Superoxide dismutase 1
SPMS – Secondary progressive multiple sclerosis
T3 – 3,3',5-triiodo-L-thyronine
TDP-43 – Tar DNA binding protein 43
VAMP-2 – Vesicle-associated membrane protein 2
WASP - Wiskott Aldrich Syndrome protein
WAVE – Wiskott Aldrich Syndrome protein family verprolin-homologous protein

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Chapter 1: General Introduction

Chapter 1 Introduction

Oligodendrocytes (OLs) are the cells responsible for myelin production in the central nervous system (CNS). The myelin sheath, which surrounds the neuron, serves to increase the efficiency of signal propagation down the axon and is essential for proper communication between the CNS and the periphery. In addition to this primary role, the myelin sheath is also responsible for protecting the axon from damage, as well as providing metabolic support.

1.1 Oligodendrocyte and Myelin – Early History

The early history of OL and myelin discovery is rife with controversy and disbelief. Despite the distinction of white matter from gray matter dating back to 1543, followed by the first description of myelinated fibers much later in 1717, the term *myelin* was not coined until 1864 by Rudolf Virchow, and still the understanding of myelin at this time remained primitive (Boullerne, 2016). In the early 1900s Santiago Ramón y Cajal, an esteemed neuroanatomist credited with identifying the neuron, was a strong proponent of the hypothesis that myelin was an axonal creation (Boullerne, 2016; Cajal, 1909). In the years to follow, Pio del Rio-Hortega, a student of Cajal's, developed a novel technique that allowed him to identify two new cell types, microglia and OLs, and further proposed that OLs were the makers of myelin in the CNS (Boullerne, 2016; Del Rio Hortega, 1921, 1922). This point was highly contested, as was the existence of the new cell types, most notably by Cajal – an influential voice at the time. Shortly after, Penfield confirmed the presence of OLs and supported the notion that OLs may produce myelin (Penfield, 1924, 1928). Gradually the scientific community grew to accept OLs as a new cell type, and entertained the thought of myelin production, though evidence of the OL-origin of myelin would not come until the late 1950s. The first electron microscope studies of CNS myelination provided the necessary evidence to confirm that OLs were producing the myelin that

would ensheath axons (De Robertis, 1958; Luse, 1956). However, both studies opposed the idea that myelin from OLs would concentrically wrap axons as had been demonstrated for Schwann cells, the myelinating cells of the peripheral nervous system (De Robertis, 1958; Geren, 1954; Luse, 1956). Reports from Bunge et al. in the 1960s strongly supported the concentric wrapping hypothesis of myelin in the CNS, and this became the widely accepted theory of how myelination transpired until recently, when a landmark study from Snaidero and colleagues very elegantly demonstrated mechanisms of myelination, though these will be discussed in depth in Chapter 1.4 of this thesis (M. B. Bunge, Bunge, & Pappas, 1962; Richard P. Bunge & Glass, 1965; Snaidero et al., 2014).

1.2 Oligodendrocyte Precursor Cell Origins and Migration

Oligodendrocyte precursor cells (OPCs) are morphologically simple, proliferative progenitors which give rise to the OL lineage in the CNS. For OPCs to reach the sites where they are required to differentiate and subsequently initiate myelination, they are required to migrate, often long distances, throughout the CNS. The importance of OPC migration is highlighted by the fact that OPCs arise in multiple, but very discrete, niches along the neural tube and it is these OPC populations that are responsible for myelinating the entire CNS. It is now evident that developmental myelination in the spinal cord includes three major migratory events. The first wave of OPCs migrate from the ventral ventricular zone and these OPCs account for the majority of OLs in the spinal cord (Pringle & Richardson, 1993; Warf, Fok-Seang, & Miller, 1991). A second migratory event comprised of OPCs originating in the dorsal ventricular zone and this wave accounts for about 10-15% of OLs in the spinal cord (Fogarty, Richardson, & Kessaris, 2005; Vallstedt, Klos, & Ericson, 2005). A third, smaller wave has been identified and occurs in early post-natal development in the central canal (Ševc, Matiašová, Kútna, & Daxnerová, 2014).

Similarly, generation of OLs in the development of the forebrain can be traced to three distinct OPC populations, responsible for migrating from distinct foci (Kessaris et al., 2006). The first OPCs originate in the ventral forebrain, specifically in the medial ganglionic eminence and the anterior entopeduncular area. These OPCs arise around E12.5 and migrate throughout the telencephalon. This is followed by a second wave around E15 comprised of OPCs originating in the lateral and caudal ganglionic eminences. The third wave of OLs are generated post-natally from within the cortex. Interestingly, ablation of any one of the three OPC populations involved in populating the forebrain has no effect on the final myelination status as neighbouring populations quickly expand to fill areas devoid of OPCs and development proceeds as expected (Kessaris et al., 2006). This indicates that despite the highly organized migratory cascades, functional redundancy exists between OPC populations.

1.2.1 OPC Migration Guidance Cues

It is clear that OPC migration is a remarkable feat given the long distance of travel required before initiating differentiation. Unsurprisingly, a multitude of intracellular and extracellular factors influence the ability of an OPC to migrate. It was once thought that OPCs could only migrate along pre-formed axonal tracts and in some way required either the electrical signals firing down the axon, or signaling molecules expressed on the axon surface (Demerens et al., 1996). However, one study investigated OPC migration following transection of the optic nerve and determined that OPCs are able to populate the optic nerve space and differentiate appropriately into mature OLs even in the absence of viable axons (Ueda, Levine, Miller, & Trapp, 1999). Two well characterized growth factors that serve to promote OPC migration are basic fibroblast growth factor-2 (FGF-2) and platelet-derived growth factor A (PDGF-A). In addition to *in vitro* studies highlighting the pro-migratory effects of PDGF-A, knockout mice

have also been used to investigate this phenomenon *in vivo*, though the results are difficult to interpret due to the fact that PDGF-A also plays a vital role in OPC proliferation (Frost, Zhou, Krasnesky, & Armstrong, 2009; Fruttiger et al., 1999; Milner, Edwards, Streuli, & Ffrench-Constant, 1996). The pro-migratory effects of FGF-2 have been demonstrated *in vitro* using established OPC migration assays as well as *in vivo* transplantation studies (Osterhout et al., 1997). A number of other mitogenic factors are involved in the process of OPC migration in development, the extent of which are beyond the scope of this overview.

It is important to note that heterogeneity exists in many facets of OPC and OL biology, including in the receptors expressed by OPCs and, consequently, the responses to extracellular cues differs among populations of OPCs as well. A remarkable example of this during OPC migration is netrin-1, an extracellular protein initially characterized as an axon guidance cue. Netrin-1 is a complex factor in OPC migration capable of exerting both chemoattractant and chemorepellent functions, depending on the receptors activated in the cell (Jarjour et al., 2003; Spassky et al., 2002; Tsai, Tessier-Lavigne, & Miller, 2003). More specifically, netrin-mediated chemoattraction occurs through engagement of deleted in colorectal cancer (DCC) receptors, while the chemorepellent function is mediated through a combination of DCC and Unc5 receptor engagement (Spassky et al., 2002). What is clear is that the presence of netrin-1 in the developing CNS is required for OPCs to disband from their point of origin and migrate to their target in the spinal cord (Tsai et al., 2003). In addition to netrin-1, a number of extracellular matrix (ECM) components influence the migration of OPCs. Laminin, fibronectin and vitronectin have all been identified as factors that can promote migration, whereas collagen impedes OPC movement (Milner et al., 1996). Tenascin-C and semaphorin 4F both limit OPC motility, while semaphorin 3A and 3F promote migration (Armendáriz et al., 2012; B. W.

Kiernan, Götz, Faissner, & Ffrench-Constant, 1996; Spassky et al., 2002). It must be appreciated that precise expression and situation of chemoattractant and chemorepellent cues is required to cooperatively guide migrating OPCs to their terminal destination.

1.3 Oligodendrocyte Differentiation

Once the OPC has reached the area within the CNS where it is required for myelination, the cell must undergo the tightly regulated process that is OL differentiation. The differentiation gamut is often separated into molecular and morphological differentiation; both processes are essential for appropriate cell function and eventual myelination but the regulation of each is quite different. It should be noted that OL differentiation and CNS myelination will be described in a general manner despite the newly appreciated heterogeneity of OPCs and OLs throughout the CNS (Marques et al., 2016). Though the likelihood that signaling mechanisms and responses to extracellular cues likely varies between OL populations is high, this has not yet been sufficiently explored and thus will not be incorporated into this overview.

1.3.1 Molecular Differentiation of OLs

The maturation of an OL is often tracked through its ability to express certain stage-specific protein markers as the progression from an immature OPC to a differentiated OL includes a coordinated and temporally regulated profile of upregulated and downregulated genes and proteins (Figure 1.1). OPCs are generally characterized through their expression of PDGF-receptor alpha (PDGF-R α) and neural/glial antigen 2 (NG2), among others. The expression of these proteins in the OPC stage are vital as they play important roles in migration and proliferation (Kucharova & Stallcup, 2010; Noble, Murray, Stroobant, Waterfield, & Riddle, 1988; Pringle & Richardson, 1993). One of the first myelin proteins to be expressed in a developing OL is 2'-3'-Cyclic nucleotide 3'-phosphohydrolase (CNP), which has roles in

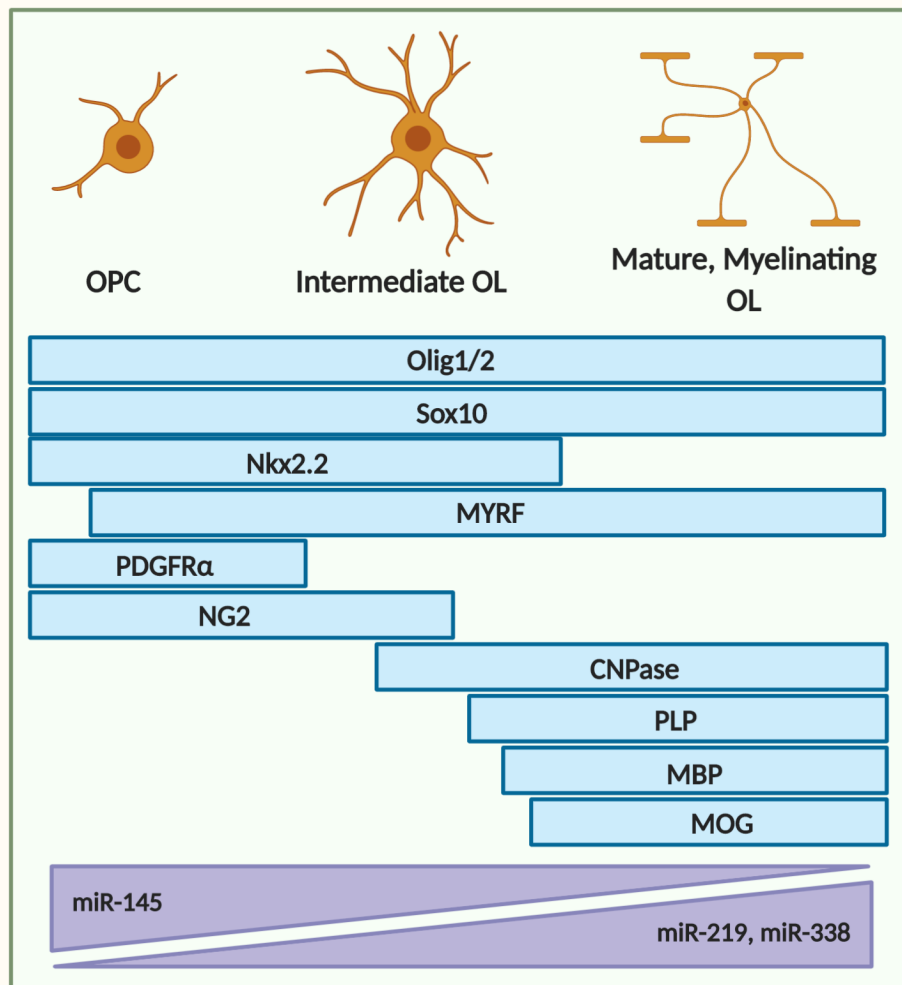


Figure 1.1 Oligodendrocyte maturation – molecular differentiation sequence. Schematic representing the expression sequence of major myelin proteins and microRNAs crucial for the development of the oligodendrocyte as well as transcription factors constitutively expressed throughout. Figure created with BioRender.com.

process extension as well as myelination (J. Lee, Gravel, Zhang, Thibault, & Braun, 2005; Pfeiffer, Warrington, & Bansal, 1993; Snaidero et al., 2014). As the cell continues to mature, it begins expressing myelin-associated glycoprotein (MAG) (Pfeiffer et al., 1993). Finally, the OL expresses the myelin proteins associated with the most mature stage of differentiation, proteolipid protein (PLP), myelin basic protein (MBP) and myelin/oligodendrocyte glycoprotein (MOG) (Pfeiffer et al., 1993). Though this is not an exclusive list of markers for staging OL differentiation, these five proteins are crucial to OL development as well as myelination, and taken together, constitute the major myelin proteins.

Successful orchestration of the molecular differentiation program of OLs requires a shift from transcription factors that facilitate expression of genes that maintain the progenitor state, to those which regulate expression of the major myelin genes. *Nkx2.2* is a transcription factor thought to be required to transition out of the progenitor state and its deletion in the OL lineage leads to an inhibition of OL differentiation and an expansion of the OPC population in the spinal cord (Qi et al., 2001). The most notable transcription factor in mediating OL differentiation is the fairly recently discovered myelin regulatory factor (Myrf). This transcription factor coordinates the activation of OL differentiation through direct interactions with regulatory regions of most major myelin genes (Bujalka et al., 2013). Myrf is required for CNS myelination as its loss results in a block in differentiation and an amyelination phenotype (Emery et al., 2009). Thus, Myrf is now appropriately regarded as the master regulator of CNS myelination.

MicroRNAs (miRs) exert an additional regulatory force on the OL differentiation program. miRs are non-coding RNA molecules that regulate gene expression by binding to the 3' untranslated region of target mRNA molecules. Two miRs have been identified with pro-differentiation effects on the OL lineage: miR-219 and miR-338 (Zhao et al., 2010). Mice

lacking miR-219 exhibited significantly impaired OL differentiation *in vivo*, and a subsequent lack of CNS myelination, highlighting the importance of this miR in the OL differentiation gamut (H. Wang et al., 2017). miR-219 and miR-338 are thought to repress genes that are inhibitory to OL differentiation including those that encourage OPC identity and appear to do so cooperatively (Dugas et al., 2010; H. Wang et al., 2017; Zhao et al., 2010). Oppositely, our lab has discovered that miR-145, which is abundant in OPCs, is required for maintenance of the OPC state with a loss of miR-145 inducing precocious differentiation (S. Kornfeld, Cummings, Fathi, Bonin, & Kothary, 2020). This effect can be attributed, at least in part, to direct repression of *Myrf*, with miR-145 overexpression experiments appearing phenotypically similar to *Myrf* knockouts (S. Kornfeld et al., 2020).

1.3.2 Morphological Differentiation of OLs

The morphological progression from a simple bipolar OPC, to a mature OL with many highly ramified processes and finally to a myelin-producing cell capable of producing membranes with surface areas upwards of $2 \times 10^6 \mu\text{m}^2$ is a remarkable feat and implies that this is a complex yet regulated development (Pfeiffer et al., 1993). In general, the OL cytoskeleton consists of actin filaments and microtubules but lacks intermediate filaments. It was long understood that the growing tips of neurons were structures termed growth cones, which allowed for a high degree of motility and environment sensing to guide axon growth (Gomez & Letourneau, 2014; Omotade, Pollitt, & Zheng, 2017). It is now appreciated that the growing tips of OL processes are highly reminiscent of neuronal growth cones, with very similar cytoskeletal compositions (M. A. Fox, Afshari, Alexander, Colello, & Fuss, 2006; Michalski, Cummings, O'Meara, & Kothary, 2016). More specifically, a growth cone is characterized by the expansion of a filamentous (F)-actin network called the lamellipodium, while numerous fine F-actin

protrusions called filopodia extend out from the lamellipodium into the extracellular space. Actin polymerization in these protrusions is what pushes the cell membrane forward, ultimately driving growth of the process. Bundled microtubules, which essentially form the structure of the branch itself, meet with the lamellipodium and form the central domain of the growth cone, with microtubule assembly and stabilization following the growth initiated by actin protrusions (Lowery & Vactor, 2009; Michalski et al., 2016; Thomason, Escalante, Osterhout, & Fuss, 2020). As the cell matures, branches are stabilized in part by post-translational modifications of tubulin in the microtubule branches, including acetylation (Michalski et al., 2016). Given that these structures represent the motile unit of the OL process, mediators of process outgrowth often regulate F-actin assembly and/or stability, or microtubule stability.

A number of important proteins have been identified with roles in regulating the OL cytoskeleton and process extension. One of the earliest signaling events in the differentiation program is the activation of a Src family kinase highly expressed in OLs, Fyn tyrosine kinase (Fyn). It is evident that OL process extension is reliant on Fyn activity as *in vitro* studies using a dominant negative Fyn greatly impeded OL branching and subsequent myelin membrane formation (Osterhout, Wolven, Wolf, Resh, & Chao, 1999). Fyn can be activated through integrin receptors in the cell membrane, connecting this early mediator of process extension directly to extracellular matrix components, which are well-known to impact OL morphology (Colognato, Ramachandrapa, Olsen, & Ffrench-Constant, 2004; Liang, Draghi, & Resh, 2004). The activation of Fyn can regulate actin through two distinct mechanisms in OLs. Firstly, increased Fyn activity leads to the activation of the Rho (Ras homologous) GTPases CDC42 (Cell division control protein 42 homolog) and Rac1 (Ras-related C3 botulinum toxin substrate 1), both of which have been shown to regulate actin polymerization through Wiskott Aldrich

Syndrome protein (WASP) family-mediated activation of a key actin nucleator complex, actin-related proteins (Arp)2/3 (Higgs & Pollard, 2000; Machesky & Insall, 1998; Miki, Suetsugu, & Takenawa, 1998). In OLs, the WASP family members WAVE1 (Wiskott Aldrich Syndrome protein family verprolin homologous proteins) and Neuronal-WASP (N-WASP) play important roles in process extension, likely through this Arp2/3-mediated actin polymerization mechanism (Bacon, Lakics, Machesky, & Rumsby, 2007; H. J. Kim et al., 2006). Alternatively, Fyn activation has also been shown to induce morphological changes in OLs through the activation of intermediate effector p190RhoGAP, which leads to inhibition of RhoA, as well as downstream inhibition of Rho-associated kinase (ROCK) and finally non-muscle myosin II (NMII) (Kippert, Fitzner, Helenius, & Simons, 2009; Liang et al., 2004; Rajasekharan et al., 2009; H. Wang et al., 2012; Wolf, Wilkes, Chao, & Resh, 2001). This pathway, which mediates actomyosin contractility, is crucial for cell spreading and OL process extension and its overactivation has been consistently linked to stunted morphological development (Kippert et al., 2009; H. Wang et al., 2012; Wolf et al., 2001).

Fyn-mediated effects on F-actin are not the only forces governing process extension in the maturing OL. Alternative pathways, with some crosstalk, have been identified as additional means to regulate F-actin and ultimately contribute to process extension. Our lab has identified a role for integrin-linked kinase (ILK) in OL morphology as its loss leads to impaired process extension (R. W. O'Meara et al., 2013). ILK, in a complex with its obligate binding partners, is known to mediate signaling between integrin activation and the cytoskeleton (Hannigan et al., 1996; Zervas, Gregory, & Brown, 2001). Specifically, in OLs the loss of ILK leads to increased RhoA activity, and its downstream impact on the F-actin rich growth cone is likely similar to the mechanism of Fyn-mediated RhoA inactivation described previously (R. W. O'Meara et al.,

2013). Process extension can also be encouraged through the enhancement of microtubule stability, as the microtubule network forms the backbone of the process and its stability is essential for solidifying the track laid by F-actin protrusions. CNP, one of the major myelin proteins, has the ability to bind tubulin and has been shown to drive microtubule assembly. Interestingly, overexpression of CNP in non-glial cells leads the cell to start extending branched processes, highly reminiscent of the characteristic OL branching network (J. Lee et al., 2005). This supports the importance not only of CNP to OL function, but also of microtubule assembly to OL morphological differentiation.

1.4 Myelination

Increasing speed of electrical signal transduction down an axon is one of myelin's most notable function. To accomplish this, myelin must be formed in discontinuous segments along the axon. These segments are referred to as internodes, and the regions of bare axon between each segment is the node of Ranvier. The nodes of Ranvier contain clusters of voltage-gated sodium channels which allow for action potentials to jump down the axon, from one node to the next, in a process termed saltatory conduction. For this process to be efficient, the formation of myelin from OLs into these concentrically-wrapped, compact myelin segments must be tightly regulated. Though there are still many unanswered questions surrounding this process, much progress has been made in recent years. One of the most intriguing myelination-related questions is the mechanism behind how myelin actually wraps the axon. A number of hypotheses have been proposed, though limitations in imaging tools made it difficult to support any proposed mechanism with confidence. One of the early wrapping hypotheses was the "carpet-crawler" or "jellyroll" method – the same method used by Schwann cells for myelination in the peripheral nervous system. In this method, after initially making contact with the axon, myelin extends in a

lateral fashion until the length of the final myelin segment is reached. This step is followed by radial wrapping of the entire segment to increase the myelin segment's thickness (M. B. Bunge, Bunge, & Ris, 1961; R. P. Bunge, Bunge, & Bates, 1989). This “carpet-crawler” hypothesis was contradicted in the mid-1970s by the finding that myelin is not equally thick across the length of the internode, and myelin was found to be thicker at the point where the OL process meets the sheath than it was at the ends of the internode (Knobler, Stempak, & Laurencin, 1976). A number of other mechanisms were proposed including the notable “liquid croissant” model, which was supported by many at the time. In this model, once the OL process contacted the axon, myelin expanded both laterally and radially at the same time, with new radial layers adding on top of the innermost layer (Sobottka, Ziegler, Kaeck, Becher, & Goebels, 2011).

In their seminal work, Snider and colleagues took advantage of a number of techniques to make tremendous advances in our understanding of myelin wrapping mechanisms. Using high pressure freezing to reduce artifacts and damage to tissue, combined with electron microscopy (EM) and serial block-face imaging, the authors were first able to confirm that the thickest part of the myelin sheath during development is consistently found at the place where the OL process meets the axon. Moreover, the authors determined that the outermost layer of the membranous sheet spans the entirety of the myelin segment, with few indications of winding or wrapping. This is contrasted by the innermost section of the myelin sheath sitting immediately adjacent to the axon it is wrapping, which is the cytoplasm-rich inner tongue. Three-dimensional reconstruction of the EM images demonstrated that the inner tongue wound around the axon many more times than the outermost layer. These findings indicate that myelin wraps radially with movement led by the inner tongue advancing underneath the concentric layers and in the

center of the myelin segment. Lateral extension of the membrane layers towards the nodes of Ranvier then follows (Snaidero et al., 2014) .

1.4.1 Myelin Compaction

Functionally, it is important that myelin segments do not exist as loose wraps around the axon and must instead undergo a process known as compaction to produce the desired final product of tightly wrapped concentric layers of myelin. MBP is the myelin protein thought to drive membrane compaction. This is supported by the findings that MBP-deficient mice seem unable to produce compact myelin sheaths (Readhead & Hood, 1990). Myelin basic protein is aptly named as it is a protein rich in positively charged amino acids, giving it a net positive charge. This positive charge allows the protein to interact and bind with the negatively charged phospholipid membrane (Beniac et al., 1999; Boggs & Moscarello, 1982). It is this feature that is utilized in myelin compaction as MBP binds opposing inner membranes of concentrically-wrapped layers of myelin, bringing them tightly together. Once MBP is bound to two membrane surfaces, it has been shown to laterally cluster with other bound MBP, stabilizing the compact state of myelin (Aggarwal et al., 2013). This formation of a network of cross-linked MBP proteins leads to the extrusion of other proteins out of the compact myelin sheath, as well as the formation of a diffusion barrier which has been demonstrated to limit further entry of proteins (Aggarwal et al., 2013; Aggarwal et al., 2011).

In developing myelin sheaths, myelin compaction appears to operate from the outside inward, such that the innermost wraps of myelin (adjacent to the axon) are the last to compact (Snaidero et al., 2014). Given the fact that MBP has been highlighted as the primary driver of compaction, and the fact that MBP can be found in, and is actually locally translated in the leading edge of myelin, Snaidero and colleagues proposed that there must be a mechanism at

play to limit MBP-mediated myelin compaction in these inner wraps (Ainger et al., 1993; Colman, G., Frey, & Sabatini, 1982; Laursen, Chan, & Ffrench-Constant, 2011; Snaidero et al., 2014). Although this mechanism is not yet fully understood, it seems likely that there is a competitive relationship between MBP and CNP. CNP-deficient mice were found to have significantly fewer myelin sheaths with uncompacted regions than age-matched wild type mice, suggesting that the balance between MBP and CNP levels contributes to regulating myelin compaction (Snaidero et al., 2014). Mechanistically, it is suggested that perhaps CNP is able to limit myelin compaction through its ability to bind to the cytoskeleton, specifically to F-actin, though much remains to be understood regarding this newly-discovered role for CNP (Snaidero et al., 2017).

1.5 Adaptive Myelination

The bulk of CNS myelination occurs in a developmental context, as has been discussed to this point. However, it is now readily accepted that the production of OLs and subsequent myelination continues to take place into adulthood. The first indication that myelin could form in the CNS after the aforementioned developmental waves, was the discovery of OPCs in the adult rodent brain (Ffrench-Constant & Raff, 1986). We now appreciate that 6% of all cells in the adult CNS are OPCs (Dawson, Polito, Levine, & Reynolds, 2003). Further investigation of the adult OPC indicated that these cells do indeed undergo proliferation and differentiation to generate mature, myelinating OLs in the adult rodent CNS (Rivers et al., 2008). At this point, the function of myelination in adulthood remained elusive. OLs and their myelin sheaths were thought to be relatively stable, but it was possible that these adult OPCs were activated to simply contribute to myelin turnover. The alternative hypothesis was that newly formed OLs would myelinate regions previously unmyelinated, referred to as *de novo* myelination. It appears as

though both of these proposed functions are true to some extent. Young and colleagues artfully proposed that if *de novo* myelination were the only purpose, a region such as the optic nerve which is almost entirely myelinated would have very few adult born OLs. By contrast, if myelin turnover were the only function of adult OPCs, all CNS regions should recruit these OPCs to a similar extent. This study concluded that OPCs in the adult were recruited to all CNS regions studied, including the optic nerve, suggesting a role in turnover, but the rate of OPC proliferation in less-myelinated regions was higher than those with an already high density of myelinated fibers, suggestive of a role for *de novo* myelination (Young et al., 2013). These findings were expanded on with the use of beautiful imaging methods to confirm that newly born OLs in the adult rodent brain do contribute by filling in unmyelinated regions of partially myelinated axons (Hill, Li, & Grutzendler, 2018). *De novo* myelination in adulthood does raise a number of questions in terms of why this may be happening and how it may benefit the organism. Borrowing from the concept of neuronal plasticity facilitating learning and formation of memory, it is reasonable to think that myelin plasticity may contribute to these processes as well. In rodent studies, OPC proliferation and oligodendrogenesis have been shown to increase during motor skill learning in rodents (Gibson et al., 2014; McKenzie et al., 2014; L. Xiao et al., 2016). This association between learning tasks and white matter changes has been identified in humans as well through the use of imaging studies (Scholz, Klein, Behrens, & Johansen-Berg, 2009; Taubert et al., 2010). Interestingly, further investigation with rodents indicates that oligodendrogenesis and myelin formation may actually be required for motor skill learning and memory formation (Gibson et al., 2014; Pan, Mayoral, Choi, Chan, & Kheirbek, 2020; Steadman et al., 2020; L. Xiao et al., 2016).

The mechanism by which OPCs in the adult are activated to contribute to myelin plasticity during learning and memory formation has not yet been fully elucidated. It is reasonable to hypothesize that the amount of neuronal activity could mediate the extent of myelination of a given axon. This then raises the consideration of myelination that is dependent on neuronal activity and that which can occur without neuronal activity (activity-dependent and activity-independent, respectively). It has been established that myelination does not require neuronal activity and that an activity-independent, inherent myelination program exists in OLs. Cell culture experiments reveal that OLs are not only capable of wrapping fixed axons, or activity-dead axons, but also electrically-silent nanofibers (Bechler, Byrne, & Ffrench-Constant, 2015; S. Lee et al., 2012). Complimentary to these findings are a number of reports suggesting that myelination can be modified in an activity-dependent manner. Stimulation of neuronal activity *in vivo* leads to increased oligodendrogenesis and myelin formation (Gibson et al., 2014; Mitew et al., 2018). Not only are electrically stimulated axons preferentially myelinated by newly generated OLs, but the myelin sheath is also thicker in these axons relative to unstimulated axons (Mitew et al., 2018). It also bears mentioning that the inverse experiments suggest a similar conclusion such that socially isolated mice, which have been shown to have decreased neuron activity in the prefrontal cortex, also exhibit impaired myelination in the same region (J. Liu et al., 2012). Furthermore, silencing of single axons led to preferential myelination of neighbouring axons over inactive ones, and sheaths that did form on silenced axons were significantly shorter than on active axons (Hines, Ravanelli, Schwindt, Scott, & Appel, 2015). Taken together, these studies suggest that although the myelination program is able to proceed without neuronal activity, myelination can be influenced by neuronal activity and may contribute to learning and memory formation.

1.6 Remyelination

The capacity for repair of many tissues in the human body is in many cases astonishing, and understandably a point of great interest for research. Given the presence of OPCs in the adult CNS and the fact that these OPCs are able to contribute to myelin maintenance and experience-driven myelination, it is unsurprising that OPCs are also able to contribute to repair of myelin damage. Remyelination, or replacement of lost or damaged myelin, has been demonstrated many times over and is now a budding area of research. Early studies of remyelination revealed that the myelin sheaths produced during remyelination were consistently thinner than the sheaths produced during development (Blakemore, 1974). This of course raised questions regarding the functional adequacy of regenerated myelin. Importantly, electrophysiological experiments determined that remyelination was capable of restoring conduction in previously denuded axons (Smith, Blakemore, & McDonald, 1979). Although this metric only assesses one of the now many known functions of OLs, it is generally accepted that regenerated myelin is functional, despite being thinner than developmental myelin (I. D. Duncan, Brower, Kondo, Curlee, & Schultz, 2009; Franklin & Ffrench-Constant, 2017).

One important question regarding remyelination is specifically which cells are capable of contributing to repair – OPCs or pre-existing mature OLs. It was long thought that only adult OPCs could remyelinate a damaged area following a tightly regulated program of activation, migration and finally differentiation in a mature, myelin-producing OL similar to that of the developmental myelination program (Crawford et al., 2016; Franklin & Ffrench-Constant, 2017; Targett et al., 1996; Zawadzka et al., 2010). Much of the research on remyelination to date is focused on adult OPCs and understanding the mechanisms of activation and differentiation following injury. Intriguingly, gene profiling of dormant OPCs in the adult compared to neonatal

OPCs are characterized by a more mature phenotype, but activation of these adult OPCs reverts the gene profiles of these back to a state much more reminiscent of OPCs in development (Moyon et al., 2015). This would indicate that remyelination may occur in a process very similar to that of developmental remyelination, which is also supported by findings that certain transcription factors critical for developmental myelination (*Myrf* and *Zfp488*) are also required for remyelination (G. J. Duncan et al., 2017; Soundarapandian et al., 2011). There is also evidence that the two processes may differ, such as a necessity for some transcription factors in remyelination (*Klf9* and *Stat3*) which appear to be dispensable for developmental myelination (Dugas, Ibrahim, & Barres, 2012; Steelman et al., 2016). While OPCs serving as the initiating cell of remyelination has been the dogma for some time, recent studies have highlighted the potential for adult OLs in repair including an intriguing carbon dating study by Yeung and colleagues (Ian D. Duncan et al., 2018; Yeung et al., 2019). The literature concerning the potential for spared mature OLs to contribute to remyelination is limited and much remains to be investigated.

The most common cause of demyelination in the adult brain, and therefore the most well studied application for remyelination, is multiple sclerosis (MS). Thus, remyelination will be discussed in more detail in the context of MS in chapter 1.7.1 of this thesis.

1.7 Primary Oligodendrocyte Pathologies

1.7.1 Multiple Sclerosis

MS is a chronic neurodegenerative disease of the CNS. While typically considered an autoimmune disease, the etiology of MS remains unknown. It is characterized by demyelination, which results in vastly heterogeneous symptoms which often include impaired muscle control and coordination, difficulties with balance, fatigue as well as cognitive deficits. MS is typically

classified as one of three types, primarily distinguished by disease course and progression of symptoms (R. J. Fox, Bethoux, Goldman, & Cohen, 2006). Relapsing remitting MS (RRMS) is the most common form at diagnosis, and presents as periods of relapse, or onset of symptoms, which are then followed by a period of remission, or dramatic improvement of symptoms. Oppositely, primary progressive MS (PPMS) patients experience gradual symptom onset, but these symptoms are never relieved and instead, accumulate throughout the course of disease. Secondary progressive MS (SPMS) occurs in patients initially diagnosed with RRMS who, as the disease progresses, no longer experience alleviation of symptoms and begin to follow a disease course strikingly similar to PPMS.

Though these subtypes of MS are distinguishable by clinical presentation, it is important to recognize that the cellular and molecular workings of the disease also differ. It is evident that in RRMS, demyelination events (relapses) are followed by remyelination which translates to functional recovery in the patient (remission). As RRMS transitions to a SPMS phenotype over time, as well as in PPMS from the start, the ability to remyelinate denuded axons is limited (Antel, Antel, Caramanos, Arnold, & Kuhlmann, 2012; Franklin & Goldman, 2015). This lack of repair translates to progressive accumulation of disability in the patient, in part due to increased rates of axon degeneration when the protective myelin sheath is not replaced (Franklin & Goldman, 2015). Remyelination presents itself as a thrilling avenue of research with therapeutic potential in MS, particularly in progressive forms of the disease. It is important to elucidate causes of remyelination failure in those MS lesions that are resistant to repair. One study has determined that there are some lesions that do not have sufficient numbers of OPCs within the lesion for remyelination to ensue which would indicate incidents of either defective OPC activation or migration (Boyd, Zhang, & Williams, 2013). However, a number of publications

have demonstrated that OPCs are present in and around most lesions that are resistant to repair, which suggests that OL differentiation and/or the process of myelination are impeded in MS (Chang, Tourtellotte, Rudick, & Trapp, 2002; Wolswijk, 1998). As a result, much of the research on repair in MS thus far has focused on understanding the OL differentiation and myelination block, in the hopes of promoting remyelination.

Inhibition of Remyelination in MS

Pathologically, lesions appearing resistant to remyelination are associated with glial scarring, which refers to a strong presence of reactive astrocytes, and subsequent alterations to the lesion microenvironment (Holley, Gveric, Newcombe, Cuzner, & Gutowski, 2003). The initial recruitment of astrocytes and the factors they produce is with beneficial intent as it serves to stabilize the tissue experiencing damage and, in the case of MS, helps to contain the immune-mediated damage to the site of the lesion (Williams, Piaton, & Lubetzki, 2007). However, if not sufficiently cleared, much of the remnant changes, including aberrant ECM deposition is inhibitory to OL differentiation and/or myelination (Van Horssen, Dijkstra, & De Vries, 2007). The composition of the ECM is crucial for efficient OPC migration and subsequent OL differentiation as ECM components have been found to have tremendous beneficial or inhibitory effects throughout this process.

One pathologically-associated cue garnering research attention in the field of remyelination as well as neuroregeneration, is the presence of chondroitin sulfate proteoglycans (CSPGs). CSPG presence in the CNS is crucial during development (Bandtlow & Zimmermann, 2000; Golding, Tidcombe, Tsoni, & Gassmann, 1999; Masuda et al., 2004). In the context of the MS lesion, CSPGs are highly upregulated as a response to injury (Sobel & Ahmed, 2001). In remyelinating lesions, CSPG levels are much reduced while in those lesions resistant to repair,

high levels of CSPG deposition remain (Lau et al., 2012). CSPGs have been shown *in vitro* to significantly inhibit the morphological differentiation of the OL including stunting process extension and subsequent myelin sheet production (Lau et al., 2012; Siebert & Osterhout, 2011). Thus, it is likely that sustained CSPG presence in an MS lesion impairs the ability of the OPC to differentiate into a mature OL, as well as the OL's ability to remyelinate. Furthermore, animal modeling has demonstrated that inhibiting the astrocytic production of CSPGs results in more efficient OL-mediated repair, supporting the probability of CSPG presence contributing to an OL differentiation block in MS (Keough et al., 2016). CSPG-mediated inhibition of OL differentiation will be discussed in greater detail in Chapter 5 of this thesis.

A number of other alterations to the lesion microenvironment have been identified, also with the thought that these changes may inhibit OL differentiation. Other ECM-related changes include the increased presence of tenascin-C and high molecular weight hyaluronan, both of which have been identified as inhibitory to OL differentiation (Czopka, Von Holst, Schmidt, Ffrench-Constant, & Faissner, 2009; Sloane et al., 2010; Van Horssen et al., 2007). Given that the lesion is the site of demyelination, it should be expected that myelin breakdown products may be found in some capacity. However, inefficient clearance of degenerated myelin and its sustained presence in the demyelinated area has been identified as contributory to impaired differentiation of OPCs into mature, morphologically complex OLs (Baer et al., 2009; Kotter, Li, Zhao, & Franklin, 2006). In the pursuit of therapies that may promote repair it is important to consider how the proposed therapeutic will advantage the OL in navigating the treacherous environment of the MS lesion, or serve to alter the lesion microenvironment to be more conducive to OL differentiation.

1.7.2 Multiple System Atrophy

Multiple system atrophy (MSA) is a rare sporadic neurodegenerative disease that presents with either predominant parkinsonism or cerebellar ataxia depending on the disease subtype, as well as progressive autonomic failure (Gilman et al., 2008). Progression of MSA is very rapid with patients most likely succumbing to the disease within 10 years of diagnosis (Schrage, Wenning, Quinn, & Ben-Shlomo, 2008). There are some symptomatic treatments available for MSA patients however, these few options are limited in efficacy and scope (Wenning & Stefanova, 2009). The lack of therapeutics is most certainly a reflection of how poorly understood MSA disease mechanisms are and the amount of work still to be done.

Pathologically, MSA is characterized by the presence of insoluble protein aggregates in glial cells, most abundantly in OLs, and these are referred to as glial cytoplasmic inclusions (GCIs) (Matyas I. Papp, Kahn, & Lantos, 1989). These GCIs contain a multitude of proteins including heat shock proteins and ubiquitin, but alpha-synuclein (α -syn) is the most abundant component, classifying MSA as an α -synucleinopathy (Chiba et al., 2012; Jellinger, 2018; Murayama et al., 1992; Tu et al., 1998). Further investigation of MSA pathology has revealed widespread demyelination at autopsy, though most consistently impacting select brain regions including the striatonigral region and cerebellar white matter (Matsuo et al., 1998; Wenning, Stefanova, Jellinger, Poewe, & Schlossmacher, 2008). A prevalent OL cell death phenomenon has also been captured in post-mortem tissue samples as highlighted by a number of markers of apoptosis (Probst-Cousin, Rickert, Schmid, & Gullotta, 1998).

Unsurprisingly, the OL is not the only cell type affected in MSA. Protein aggregates have also been found in neurons, and degeneration of neurons is an established feature of MSA certainly contributing to disease progression (Arima, Murayama, Mukoyama, & Inose, 1992; Tu

et al., 1998). Given the clear interconnectedness of OL and neuron health and viability, it can be difficult to discern which degenerative event is the primary pathology and which is secondary. MSA is suggestive of a primary oligodendroglialopathy due to the pattern and severity of neurodegeneration closely following the distribution and density of GCIs in OLs (M. I. Papp & Lantos, 1994; Wenning et al., 2008). It is thus hypothesized that neuron loss is a consequence of losing OLs and myelin, which have well established roles in neuroprotection and providing energetic support to axons. Adding to the complex cellular pathology of MSA are the contributions of astrogliosis and microglial activation and the accumulation of GCIs in these cell types as well (Ishizawa et al., 2004; Radford et al., 2015). These elements constitute a state of neuroinflammation in the MSA brain. MSA pathology has also been proposed to include mitochondrial dysfunction, impaired autophagic processes, iron deposition and oxidative stress (Dexter et al., 1991; M. J. Lee et al., 2019; Monzio Compagnoni & Di Fonzo, 2019; Monzio Compagnoni et al., 2018). The plethora of pathological characteristics identified makes it difficult to hypothesize which phenomena are implicated in the development and progression of the disease, compared to those which are secondary or tertiary pathologies. As such, a greater understanding of disease mechanisms and this interplay is required in order to benefit those affected by MSA.

1.8 Oligodendrocytes in Neurodegenerative Diseases

As the understanding of the OL's biology and its diverse and vital roles in the CNS grew, so too did the appreciation that OLs may play important roles in diseases of the CNS that were classically thought of as diseases of the neuron. Along with the other resident cells of the CNS, such as astrocytes and microglia, OLs were not historically a necessary consideration in diseases for which the most notable pathology was degeneration of neurons. However, myelin defects and

OL-intrinsic pathologies have been of great interest recently for many neurodegenerative diseases including amyotrophic lateral sclerosis (ALS), Alzheimer's disease (AD) and Huntington disease (HD).

1.8.1 OLs in ALS

ALS has historically been considered a disease of the motor neuron as its primary pathology is degeneration of both upper and lower motor neurons (Rowland & Shneider, 2001). This leads to a clinical manifestation of muscle weakness and eventual paralysis. Mutations have been discovered in a number of different genes that lead to the development of ALS including *SOD1*, *TDP-43*, *FUS* and *C9orf72*; only 10% or fewer cases are considered familial ALS, meaning that there is known history of the disease in the family, while the remainder of cases are considered sporadic (M. C. Kiernan et al., 2011; Rowland & Shneider, 2001). Early indications of white matter damage in ALS patients were discovered through the exploitation of imaging techniques, namely diffusion tensor imaging (DTI). White matter abnormalities were identified not only in the corticospinal tract, but also in the motor and extramotor tracts in the brain in many cases of familial and sporadic ALS (Agosta et al., 2010; Sach et al., 2004; Sarro et al., 2011). Analysis of post-mortem spinal cord tissue revealed a consistent reduction in the myelin protein MBP when compared to controls (Kang et al., 2013). Moreover, multiple groups have identified that TDP-43 (Tar DNA binding protein) inclusions in the cytoplasm, a defining pathological hallmark in motor neurons in ALS, occur in OLs as well (Philips et al., 2013; Tan et al., 2007). However, this connection is complicated by the findings that TDP-43, is required for myelination and survival of OLs but not motor neurons (J. Wang et al., 2018).

Most of the studies linking OLs to ALS do so using animal models of the disease and not only support OL-intrinsic defects in response to ALS-associated mutations, but also that OL-

specific phenotypes influence the course of neurodegeneration. In a mouse model often used to study ALS (SOD1^{G93A} mice) increases in OL death and degeneration have been reported with a concomitant increase in OPC proliferation rates such that the absolute numbers of OL-lineage cells remain relatively unchanged in these animals, though many of the newly formed OLs failed to differentiate fully and demonstrate morphological abnormalities. Importantly, increased incidence of OL cell death were detected prior to the appearance of overt motor neuron loss (Kang et al., 2013; Philips et al., 2013). Similar findings were demonstrated in a zebrafish model, where the same SOD1 mutation was conditionally expressed under the MBP promotor, restricting its expression to mature OLs. In this model, there was increased OL apoptosis as well as loosening of myelin wraps around the axon (S. Kim et al., 2019). A particularly interesting angle of this research is the influence of OLs on motor neuron degeneration. In the zebrafish model described above, expression of mutant SOD1 (superoxide dismutase 1) in mature OLs resulted in motor neuron degeneration and neuromuscular junction abnormalities following onset of OL-specific phenotypes (S. Kim et al., 2019). The impact of OLs on motor neuron health in ALS was also explored utilizing human induced pluripotent stem cells (iPSCs) from ALS patients. All co-cultures of iPS-derived OLs from ALS patients with wild type mouse motor neurons induced significantly more motor neuron death than iPS-derived OLs from non-ALS individuals (Ferraiuolo et al., 2016). Taken together, these findings support the notion that not only are OLs affected in the course of ALS, but also that OL defects may precede obvious neurodegeneration and could influence the severity of disease.

1.8.2 OLs in AD

Alzheimer's disease (AD) is a neurodegenerative disease of the elderly and is the leading cause of dementia (Ballard et al., 2011). Clinically, AD presents with memory loss, as well as

other displays of cognitive dysfunction and personality changes (Ballard et al., 2011; L. Fan et al., 2020). Hallmarks of AD pathology include the presence of amyloid plaques, which are depositions of aggregated amyloid- β (A β), and the presence of neurofibrillary tangles, which are primarily composed of aggregated tau, a microtubule-associated protein. It is hypothesized that the presence of these aggregated proteins contributes to neuronal cell death and grey matter loss, resulting in the manifestation of AD (Ballard et al., 2011; L. Fan et al., 2020). Additional hypotheses continue to be put forward and explored, as does the notion that non-neuronal cell types, including OLs, may play important roles in AD pathology and progression of disease. Imaging studies, including the use of DTI or magnetic resonance imaging (MRI), have demonstrated white matter abnormalities not only in patients diagnosed with AD, but also those with pre-clinical AD, individuals who are cognitively normal but present evidence of A β deposition (Bendlin et al., 2012; Benitez et al., 2014; Brickman et al., 2012; Dean et al., 2017; Fischer, Wolf, Scheurich, & Fellgiebel, 2015). Post-mortem AD tissue has been used to demonstrate that A β deposits can be found in white matter in addition to those known to be found in grey matter (Roher et al., 2002). Post-mortem tissue has also supported the involvement of OL and myelin defects in AD by exhibiting reductions in mature myelin markers, such as MBP and PLP, as well as decreased NG2 immunoreactivity and number of Olig2 (Oligodendrocyte transcription factor 2)-positive cells (Behrendt et al., 2013; Nielsen et al., 2013; Roher et al., 2002). Interestingly, deeper investigation of the A β plaque environment of these tissues revealed a much higher incidence of OPCs exhibiting a senescence phenotype than in healthy, age-matched controls (P. Zhang et al., 2019).

A number of mouse models are routinely used to study various elements of AD including the effects of A β deposition and neurofibrillary tangle formation at the cellular and molecular

levels. Numerous studies have used mouse models to identify myelin and OL aberrations including myelin swellings, thinner myelin sheaths and decreases in essential myelin markers (MBP, CNP) in select brain regions (Behrendt et al., 2013; Chu et al., 2017; Desai et al., 2009; Gu et al., 2018; X.-H. Sun et al., 2018; Wu et al., 2017). Further exploration of the effects of A β on OL vitality *in vitro* in cell lines as well as neurosphere-derived OL cultures revealed reductions in differentiation capacity and increases in cell death (Desai et al., 2010; J.-T. Lee et al., 2004). Despite a high number of studies reporting detrimental impacts of A β peptides on OL and myelin health, there are conflicting findings in this field. Firstly, some studies using the same mouse models as alluded to previously have reported increases in the myelin markers MBP and CNP in particular brain regions of these animals (Behrendt et al., 2013; Quintela-López et al., 2019; Wu et al., 2017). Moreover, one study in particular reported that A β treatment increased differentiation and survival of primary OL cultures derived from rat. Specifically, the authors determine not only that A β appears to increase the morphological complexity of these cells *in vitro*, but also that A β treatment increased translation of MBP in the distal processes of OLs (Quintela-López et al., 2019). The contradictory nature of these findings certainly warrants greater investigation into the impact, positive or negative, of A β -deposition and any other AD-related pathology on OLs and myelin.

1.8.3 OLs in HD

HD is a neurodegenerative disease caused by mutations in the huntingtin (*Htt*) gene which result in the production of a toxic, mutant huntingtin (mHTT) protein (Macdonald, 1993). HD is clinically characterized by chorea, balance and gait disturbances, as well as cognitive deficits including impaired processing speed and executive function (Ross et al., 2014). Pathologically, HD is most notably characterized by neurodegeneration in the caudate and

putamen following accumulation of mHTT (Vonsattel et al., 1985). Recently, and with growing appreciation, white matter abnormalities and myelin degeneration have been detected in human HD patients (Rosas 2018, Phillips 2016, Fennema-Notestine 2004). Importantly, imaging studies on pre-symptomatic individuals identified as gene-positive with a certainty of developing HD revealed white matter abnormalities that could not be detected in age-matched healthy controls (Rosas 2018).

Animal modelling has furthered the understanding of myelin abnormalities and OL involvement in HD. Teo and colleagues investigated two different murine models of HD (YAC128 and BACHD) and found similar myelin-related phenotypes in both (Teo et al., 2016). Most notably, myelin thickness was reduced in both models relative to controls prior to onset of behavioural phenotypes, as well as a reduction of myelin-related genes across all time points studied. Interestingly, a follow up study created a model in which mice carry the full-length human mutant *Htt* gene but blocked its expression specifically in OL lineage cells by crossing it with an NG2-Cre mouse model. This model was sufficient to rescue the myelin phenotype observed in those animals where OLs do express the mutant *Htt* gene, as well as some of the behavioural measures (Ferrari Bardile et al., 2019). These results indicate that the observed myelin defects in HD models are not simply caused by neuronal pathologies but are actually intrinsic to the OL. In support of these findings, the specific expression of mutant *Htt* in OLs, through the use of the PLP promoter, results in a reduced expression of crucial myelin proteins (Huang et al., 2015). Further biochemical analysis in this work revealed that mutant *Htt* can bind *Myrf* (master regulator of most myelin-genes) and affects its transcriptional activity, again supporting the notion of OL-intrinsic pathologies in HD (Huang et al., 2015).

1.9 Neurodegenerative Diseases with Unknown OL Involvement

1.9.1 Spinal Muscular Atrophy

Spinal muscular atrophy (SMA) is an inherited neuromuscular disorder with a range in disease presentation but notably resulting in skeletal muscle atrophy and in many cases, paralysis. SMA is caused by mutations in the *Survival Motor Neuron 1* (*SMN1*) gene (Lefebvre et al., 1995). Humans also have *SMN2*, an inverted duplication of *SMN1* with a C to T transition at position 6 within exon 7. This single nucleotide difference favours the exclusion of this exon from the transcript, ultimately yielding a highly unstable, truncated protein (Lorson, Hahnen, Androphy, & Wirth, 1999). However, *SMN2* also produces low amounts of full-length SMN. As such, the copy number of *SMN2* inversely correlates with disease severity ranging from Type I-IV, representing individuals with most severe to least severe disease, respectively (Oskoui, Darras, & De Vivo, 2017). A number of models exist to study SMA in the laboratory, most of which are mouse models. Unlike humans, mice only carry one SMN gene and its homozygous deletion is embryonic-lethal (Schrack et al., 1997). Thus, establishing models with various amounts of SMN production, resulting in a range of severities reflected, required creativity. Models that are frequently used include the *Smn*^{-/-};SMN2 severe model and the *Smn*^{2B/-} model which is more intermediate in severity. The *Smn*^{-/-};SMN2 model is a homozygous deletion of *Smn* with supplementation of human *SMN2* while the *Smn*^{2B/-} model uses a knock-in allele to alter splicing in a manner similar to that which occurs in human *SMN2* (Mélissa Bowerman, Murray, Beauvais, Pinheiro, & Kothary, 2012; Monani et al., 2000).

Research using model systems coupled with clinical research of human patients has led to much advancement in our understanding of SMA disease progression and its underlying pathology. Pathological reduction of SMN notably results in the loss of lower motor neurons in

the spinal cord. Being the hallmark pathology of this disease, SMA has classically been considered a “motor-neuron disease” with muscle weakness as well as other organ system involvement considered secondary to denervation. However, an abundance of recent findings identifying intrinsic defects in a variety of cell types and organ systems upon loss or depletion of SMN are pushing the field to accept SMA as a multi-system disorder (Deguise, Patitucci, Ebert, Lorson, & Kothary, 2017). Cell-intrinsic defects have been identified in the liver, pancreas, heart and skeletal muscle in SMA (M. Bowerman et al., 2014; Melissa Bowerman, Kathryn J. Swoboda, et al., 2012; Boyer et al., 2014; Heier, Satta, Lutz, & Didonato, 2010; Shababi et al., 2010; Shafey, Côté, & Kothary, 2005; Szunyogova et al., 2016; Vitte et al., 2004). Additionally, SMA models have revealed defects in cortical neurons, astrocytes and Schwann cells (G. Hunter, Aghamaleky Sarvestany, Roche, Symes, & Gillingwater, 2014; Gillian Hunter et al., 2016; McGivern et al., 2013; Rindt et al., 2015; Wishart et al., 2010). Investigations into the effect of SMN depletion on OLs is limited, though one study did identify a change in the expression of some myelin markers in the spinal cord using one SMA mouse model (Ohuchi et al., 2019). This finding alone is insufficient to claim an OL-intrinsic pathology in SMA, thus identifying a valuable avenue to explore.

1.10 Rationale

The well-orchestrated process of OL differentiation and myelination is a point of great interest for many research groups, particularly with the focus of providing regenerative therapies for the most well-known demyelinating disease – MS, but also with the intention of better understanding disease mechanisms for the other debilitating oligodendroglial pathologies. While the biology of the OL is starting to achieve much needed attention, much remains to be understood about how these cells function in a healthy context, including developmental and adaptive

myelination, but also how the biology of these cells goes awry in disease. This is particularly true for diseases that are classically considered primarily diseases of the neuron, despite increasing evidence of multi-cell involvement. Shifting away from these neuron-centric ideas, we can begin to appreciate how OLs are similar to, or different from other cell types in the CNS, especially in disease states. Our objective is to explore the effects of varying disease models on OL biology and use those findings to further our knowledge on the biology of both OL development and regeneration.

1.11 Aims

1. Explore the effects of Smn depletion on OL development
2. Understand the role of alpha-synuclein in OL development
3. Investigate the inhibition of OL differentiation by chondroitin sulfate proteoglycans

Chapter 2: Materials and Methods

Chapter 2: Materials and Methods

2.1 Mouse Models

All animals used in this work were cared for according to the Canadian Council on Animal Care guidelines. Ethical approval for experiments conducted was obtained from the University of Ottawa Animal Care Committee. Breeding maintenance for *Smn*^{-/-};*SMN2* and *Smn*^{2B/2B} mouse lines was approved under protocol OHRI1948 while breeding maintenance for all other lines was conducted under protocol OHRI1949. All experimentation using *Smn*^{-/-};*SMN2* and *Smn*^{2B/2B} mouse lines was approved under protocol OHRI1927 and all others were approved under protocol OHRI1947. All animals were given water and chow *ad libitum*.

FVB *Smn*^{+/-};*SMN2* mice were obtained from Jackson Laboratories and were bred to generate the severe SMA mouse model (*Smn*^{-/-};*SMN2*). A less severe model of SMA (*Smn*^{2B/-}) was used in Chapter 3.3.6 only for *in vivo* optic nerve analysis. This model was generated through the crossing of *Smn*^{+/-} mice with *Smn*^{2B/2B} mice to obtain *Smn*^{2B/+} and *Smn*^{2B/-} mice. *Ilk*^{fl/fl} mice (generously provided by Dr. Rene St-Arnaud, McGill University) were bred to homozygosity with the tdTomato-EGFP (*mT/mG*) reporter strain to yield the *Ilk*^{fl/fl};*mT/mG* line (R. W. O'Meara et al., 2013). Primary OL cultures were derived from these mice for the added benefit of a cell-intrinsic reporter (signified as TdTomato). Alpha-synuclein knockout mice (*Sncα*^{-/-} (Cabin et al., 2002)) on a C57BL/6 background were generously provided to us by Dr. Michael Schlossmacher (Ottawa Hospital Research Institute, Ottawa, ON). Wildtype C57BL/6 mice were used as controls for *Sncα*^{-/-} experiments, as well as for additional experiments on their own. Sprague-Dawley rats were purchased from Charles River. P1-P3 pups were used for the establishment of primary rat OL cultures. MiR-145^{-/-} mice generated on the C57BL/6

background were generously provided by Dr. Eric Olson. P0-P2 pups were used to establish primary mouse OL cultures.

2.1.1 Chronic Cuprizone Model and Fasudil Treatment

We established the chronic cuprizone demyelination model in wildtype mice as has been done previously (Hibbits, Yoshino, Le, & Armstrong, 2012). Cuprizone was delivered to the animals through their diet. Normal chow supplemented with 0.2% w/w cuprizone (oxalic bis(cyclohexylidene-hydrazide) was purchased from the same company as their normal chow (Envigo). The diet is exactly the same as the normal chow (control-fed mice) with the only difference being the addition of cuprizone. Mice were switched onto the cuprizone diet at 8 weeks of age and fed *ad libitum* for 12 weeks, with food changes occurring every other day to maintain stability of the diet. At 12 weeks, the mice on the cuprizone diet were changed back to normal chow for a 5-week recovery period. Fasudil treatment to assess the impact of ROCK inhibition in remyelination began when mice were changed back to normal chow. For those animals on normal chow for the duration of the experiment, Fasudil treatment began when they reached 20 weeks of age. Fasudil (Fasudil, Mono HCl Salt, $C_{14}H_{17}N_3O_2S \cdot HCl$; LC Laboratories) was administered at a dose of 30 mg/kg via the drinking water in a light-protected bottle as has been done previously (Takata et al., 2013). To ensure correct dosing, drinking volumes for the animals were tracked daily for two weeks prior to treatment. Animals were weighed daily and the average weight of the animals in the cage was used to calculate appropriate dosing based also on the average amount of water consumed in 24 h. Drinking water was changed daily to ensure stability of the treatment and accurate assessment of water consumption. Those animals in the control group receiving only normal drinking water were also weighed daily to control for the stress of human handling as a factor.

2.1.2 Behavioural Analysis – Rotarod

Behavioural analysis was conducted on mice 8 weeks of age prior to diet changes (baseline), as well as after 12 weeks of cuprizone or normal chow and again after 5 weeks of return to normal diet. All animals were minimally handled and disturbed aside from rotarod testing and were allowed to acclimate to the room in which testing took place for at least 30 mins prior. The rotarod test was chosen as a measure of motor skill and balance. The apparatus (IITC Life Science) consisted of a rotating rod on which the mice were placed. A fall off of the rod was automatically detected by the apparatus and metrics were recorded including the time spent on the rod as well as the calculated distance traveled based on the number of rotations the mouse remained on the rod. The apparatus was programmed such that the starting speed was 1 rpm, speed ramped up to a maximum of 45 rpm steadily over the course of 1 min and max speed was maintained for 1 min. Testing was performed on two consecutive days, with three trials performed per day and inter-trial intervals maintained at 10 min. Interpretation of data was done using the first trial on the second day to assess coordination and balance without a confounding learning variable.

2.2 Cell Culture

2.2.1 Primary Culture - Mouse

Primary OL cultures were established from mice as described previously (R. O'Meara, Ryan, Colognato, & Kothary, 2011). Briefly, a mixed glial culture (MGC) was established from cortices removed from neonatal mice (P0-P2). Cortices were digested for 20 min at 37°C with papain solution, comprised of 1.54 mg/mL papain (Worthington), 360 µg/mL L-cysteine, 60 µg/mL DNase I (Roche) in Minimum Essential Media (Gibco). Mouse MGC media, composed of Dulbecco's Modified Eagle's Medium (DMEM; Wisent), 10% fetal bovine serum (FBS), 1%

GlutaMAX (Life Technologies), and 0.33% Penicillin-Streptomycin (Pen/Strep; Gibco), was added to the tissue to quench the digest. Trituration of the tissue followed, allowing for gentle mechanical digestion. Tissue suspensions were centrifuged at 1200 rotations per minute (RPM) for 5 min, followed by removal of supernatant and replacement with fresh mouse MGC media. Careful resuspension at this stage allows for the isolation and subsequent removal of a DNA pellet in the sample. Cell suspensions were then seeded in Poly-L-lysine (PLL; Sigma-Aldrich)-coated T25 vent cap flasks at 37°C and 8.5% CO₂ for 6-7 days and moved to 5% CO₂ for a remaining 3-4 days, totalling a 9-11 day growth period. 75% MGC media changes were completed every 2-3 days, with the second, and all subsequent media changes, being supplemented with 5 µg/mL insulin. Enriched OPC cultures were generated by shaking flasks for 16-17h at 37°C, 5% CO₂. OPCs used for migration experiments follow a different procedure from this point forward (See 2.2.6 Migration Assay). At this point for all other experiments, media containing OPCs was collected and transferred to coated cell culture plates (Corning) and allowed to settle for 30 min at 37°C, 5% CO₂. Media was then collected and OPCs pelleted by centrifugation (1200 RPM for 5 min). Resuspension of the OPC pellet was done using mouse OL differentiation media, and cells were then seeded in the same media. Mouse OL Differentiation media was composed of DMEM, 0.5% FBS, 1% GlutaMAX, 2% B27 (Gibco), 0.33% Pen/Strep, 100 µg/mL bovine serum albumin (BSA), 0.5 µg/mL holo-transferrin, 60 ng/mL progesterone, 400 ng/mL 3,3',5-triiodo-L-thyronine (T3), 400 ng/mL L-thyroxine, 16 µg/mL putrescine, 5 ng/mL sodium selenite and 50 ng/µL ciliary neurotrophic factor (CNTF; Peprotech). OPCs differentiated at 37°C, 8.5% CO₂ for up to six days, depending on the experiment.

2.2.2 Primary Culture – Rat

Primary OL cultures were established from rats as described previously (Chen et al., 2007). Briefly, MGCs were generated from cortices dissected from P1-P3 Sprague-Dawley rat pups. Cortices were digested for 15 min at 37°C in a trypsin solution composed of 1% Trypsin (Sigma-Aldrich), 10.7 µg/mL DNase I in Hank's Balanced Salt Solution (HBSS; Invitrogen). Rat MGC media, composed of 20% FBS, 2% GlutaMAX, 0.5% Pen/Strep in DMEM was added to digested tissue for trituration. Tissue suspensions were left on ice for 10 min, filtered through a 70 µm cell strainer, then incubated in rat MGC media in PLL-coated T75 vent cap flasks at 37°C, 5% CO₂. MGCs were left to grow for 7-10 days, with 75% media changes with rat MGC media every 2-3 days. Enriched OPC cultures were obtained using the shake off method. MGCs were shaken for 18-20 h at 37°C, 5% CO₂ after which the media, containing suspended OPCs, was collected and allowed to settle on coated culture plates for 30 min. Media was collected from culture plates and OPCs were pelleted through centrifugation at 100 x g for 10 min, then resuspended in SATO media. SATO media was made up in DMEM, and comprised of 5 µg/mL insulin, 1 µg/mL BSA, 0.05 µg/mL apo-transferrin, 0.03 µg/mL sodium selenite, 0.25 µg/mL D-biotin, 0.01 µg/mL hydrocortisone, 2% GlutaMAX and 0.5% Pen/Strep. OPCs were plated for further experimentation. To maintain OPC identity, cells were plated in SATO medium supplemented with 0.01 µg/mL fibroblast growth factor (b-FGF; Millipore) and 0.01 µg/mL platelet-derived growth factor (PDGFAA, Millipore). To encourage differentiation, OPCs were plated in SATO medium supplemented with 15 nM T3, 0.01 µg/mL CNTF and 5 ng/mL N-acetyl L-cysteine (Sigma-Aldrich).

2.2.3 Oli-Neu Cultures

Oli-neu cells were incubated in SATO medium with 1% horse serum in DMEM at 37°C and 10% CO₂ (Jung et al., 1995). Media was changed every 2-3 days. Cells were differentiated with the addition of 1 mM dbcAMP (Sigma) to the media. To passage cells, plates were incubated for 1 min with 0.05% trypsin at 37°C, followed by the addition of 10% horse serum in DMEM to inactivate trypsin. Detached cells were spun at 1200 RPM for 3 min, then replated in SATO medium as above.

2.2.4 Plating Substrates

OPCs were collected from the above methods and seeded onto coated coverslips for differentiation. For all experiments where OPCs were derived from *Smn*^{+/-}; *SMN2* mice and their controls, coverslips or 6-well plates were coated with human placental merosin (laminin; 10 µg/mL; Millipore). To analyze the effects of CSPGs on OLs, coverslips were pre-coated with PLL coated with a mixture of chicken extracellular chondroitin sulfate proteoglycans (10 µg/mL, Millipore CC117) combined with MaxGel™ ECM (1:100, Sigma) made up in DMEM overnight at 37°C, 8.5% CO₂. Control substrates in these experiments are always coated with MaxGel™ ECM (1:100) in DMEM only, overnight at 37°C, 8.5% CO₂. To create a CSPG ring, as in the CSPG spot assay, coverslips were pre-coated in PLL and washed. CSPGs were mixed in equal ratio with laminin, and 3 µl of that mixture was dropped into the centre of the coverslip and allowed to dry. Coverslips were then coated fully with laminin, as above, to provide a permissive substrate around the CSPG spot. All experiments using OPCs derived from *Sncα*^{-/-} mice and their wildtype counterparts used coverslips or 6-well plates that were pre-coated with PLL, washed and then coated with MaxGel™ ECM (1:100) in DMEM overnight at 37°C, 8.5% CO₂. The experiment using Oli-neu cells involved coating 6-well plates with PLL.

2.2.5 Cell Treatments

Various cell treatments were used while the OLs were undergoing differentiation. Cell culture plates were left in the incubator during all treatments as mouse-derived primary OLs are highly sensitive during differentiation and do not tolerate removal from this environment well. First, an InSolution Rho Kinase Inhibitor (Calbiochem) was constituted in water. Ten μl of differentiation media was removed from the well and replaced with 10 μl of treatment solution. Stock solution was made such that the 10 μl treatment added into the 1 mL of differentiation media would result in a final concentration of 10 μM . Control wells were treated with 10 μl of vehicle (water). The same paradigm was followed for Blebbistatin (InSolution Blebbistatin, Calbiochem) except that the compound was reconstituted in DMSO and the appropriate vehicle delivery of 10 μl of DMSO served to control the experiment. Stock solution was made such that the 10 μl treatment added into the 1 mL of differentiation media would result in a final concentration of 30 μM . Glycogen synthase kinase-3 beta (GSK3 β) inhibition was achieved through treatment with either 1 mM or 2 mM LiCl in PBS. For those experiments where CSPGs were added later in differentiation, as a soluble treatment, the same paradigm was followed such that 10 μl of media was removed from the wells of differentiating OLs, and 10 μl of 1 $\mu\text{g}/\mu\text{l}$ CSPG mixture or 10 μl of MaxGelTM ECM were added.

2.2.6 Small RNA Transfection

miR-219 mimic and scrambled miR mimic (Applied Biological Materials) were used in rat-derived primary OL cultures at a concentration of 30 nM. Cells were transfected using INTERFERin siRNA transfection reagent (Polyplus). Briefly, 30 pmol of miR mimic was prepared in 50 μl Opti-Mem serum-free media (Thermo-Fisher) and vortexed, after which 1.5 μl INTERFERin was added and vortexed again. The mixture was left for 10 min at room

temperature to allow lipid:RNA complexes to form. For every 1 mL of cell media, 50 µl of transfection mixture was added and cells were returned to incubate at 37°C and 5% CO₂ until DIV5, at which point they were removed for fixation and analysis.

2.2.7 Migration Assay

OL migration was assessed *in vitro* using the mouse OPC aggregate migration protocol described previously (O'Meara, Cummings, Michalski, & Kothary, 2016). MGCs were established as above (See 2.2.1 Primary Culture – Mouse). During the high-speed shake overnight, suspended clumps of OPCs, or OPC aggregates (OPCAs), are generated. The media suspension was passed through a 40 µm cell strainer to collect OPCAs, which were then backwashed onto sterile Petri dishes. OPCAs were seeded on coverslips coated with human placental merosin (laminin-2 (Ln-2); Millipore) and allowed to migrate for 10 h in migration media. Migration media was comprised of 48 h conditioned MGC media passed through 0.22 µm filter, 2% B27, 100 µg/mL BSA, 5 µg/mL insulin, 0.5 µg/mL holo-transferrin, 60 ng/mL progesterone, 400 ng/mL T3, 400 ng/mL L-thyroxine, 16 µg/mL putrescine, 5 ng/mL sodium selenite, 10 ng/µL PDGFAA (PeproTech), 50 ng/µL CNTF and 1 µg/mL aphidicolin (Sigma-Aldrich). Phase contrast images of OPCAs were taken at the time of seeding to record diameter.

2.2.8 *In vitro* Myelination Co-culture Assay

OL/dorsal root ganglion neuron (DRG) co-cultures were derived as described previously (R. O'Meara et al., 2011). Briefly, MGC cultures were established as usual. The following day, DRGs were dissected from the spinal cords of P5-P10 wild type mice and transferred to HBSS. DRGs were pelleted through centrifugation at 1200 RPM for 5 min at 4°C. DRGs were digested using pre-warmed DRG papain solution, comprised of 1.54 mg/mL papain and 360 µg/mL L-cysteine in HBSS, for 10 min at 37°C. Following centrifugation (1200 RPM, 5 min, 4°C), papain

solution was removed and Collagenase A solution, comprised of 4 mg/mL Collagenase A (Gibco) in HBSS, was added and incubated for 10 min at 37°C. DRGs were centrifuged again (1200 RPM, 5 min, 4°C), and supernatant was replaced with DRG media (10% FBS and 1% Pen/Strep, made in DMEM). DRGs were triturated using a BSA-coated pipette, after which they were passed through a 50 µm cell filter into DRG media. DRGs were incubated at 8.5% CO₂ for approximately 1.25 hours, after which they were collected, spun down (1200 RPM, 5 min) and resuspended in fresh DRG media. Isolated DRGs were seeded on Ln-2 coated coverslips and cultured for nine days. Purified OPCs were then seeded on a dense neurite bed and allowed to differentiate for six days.

2.3 Immunocytochemistry (Immunofluorescence)

Coverslips were fixed with 3% paraformaldehyde (PFA) at room temperature for 15 minutes. Coverslips were then washed with phosphate buffered saline (PBS), permeabilized with 0.1% Triton X-100, blocked with 10% goat serum (GS) and incubated with primary antibodies in blocking solution at 4°C overnight (Table 2.1). Coverslips were then washed with PBS and incubated with Alexa Fluor-conjugated secondary antibodies (Alexa-488, Alexa-555, Alexa-647; Invitrogen) and/or incubated with rhodamine phalloidin (Invitrogen) for F-actin staining. Samples were counterstained with Hoechst or 4'6-diamidino-2-phenylindole (DAPI) nuclear stain and mounted in Dako mounting medium.

Table 2.1: Primary antibody specifications used for immunocytochemistry, immunohistochemistry and western blotting.

Antibody	Species	Company, Catalogue Number	Concentration (ICC)	Concentration (IHC)	Concentration (WB)
Alpha-Synuclein	Rabbit	Cell Signalling (NEB), 2628	1:50		
Cleaved Caspase 3 (Asp175)	Rabbit	Cell Signaling, 9661	1:400		
CNPase	Mouse	Abcam, ab6319			1:1000
GSK3 β	Rabbit	Cell Signalling, 9315			1:1000
pGSK3 β (serine 9)	Rabbit	Cell Signalling, 9336			1: 1000
Ki67	Rabbit	Leica Biosystems, KI67P-CE	1:500		
Myelin Associated Glycoprotein	Mouse	Millipore, MAB1567	1:100		
Myelin Basic Protein	Rat	Bio-Rad, MCA4095	1:100	1:100	1:1000
NG2	Rabbit	Millipore, AB5320	1:250		
Olig2	Rabbit	Millipore, AB9610	1:500		
Olig2, clone 211F1.1	Mouse	Millipore, MABN50	1:200		
Alpha-Tubulin	Rabbit	Cell Signalling, 2144			1:50000

2.4 Immunohistochemistry (Immunofluorescence)

Immunohistochemistry was either performed on sections following cryopreservation or on those that had been paraffin-fixed and then deparaffinized.

Cryosections: Whole brain samples were dissected and fixed overnight in 4% PFA at 4°C followed by overnight cryopreservation in 30% sucrose/PBS at 4°C and then embedded in a 1:1 mixture of 30% sucrose/OCT (Sakura). Thirty µm coronal sections were cut and prepared for staining. Briefly, coronal sections were washed with PBS, permeabilized with 0.5% Triton X-100, and blocked in 10% GS, 1% BSA and 0.2% Triton X-100. Primary antibodies were diluted in 1% GS, 1% BSA and 0.2% Triton X-100 and incubated overnight at 4°C. Sections were then washed, incubated with Alexa Fluor conjugated secondary antibodies (Invitrogen), counterstained with DAPI nuclear stain, and mounted in Dako mounting medium.

Paraffin-Embedded Sections: Whole brain samples were dissected and fixed for 48 h in 4% PFA and transferred to 70% ethanol. Samples were then processed by the Histology Core Facility, Department of Pathology and Lab Medicine (University of Ottawa). Samples were paraffin embedded using a LOGOS microwave hybrid tissue processor. Paraffin blocks were sectioned at a 20 µm thickness with a microtome and mounted on slides. Slides were then deparaffinized using a series of dehydration and rehydration steps. Briefly, slides underwent 3 changes of xylene substitute (Histo-Clear, National Diagnostics), followed by 2 rounds in a 50/50 mixture of ethanol and Histo-Clear. Slides were then rehydrated following a stepwise procedure of 100%, 95%, 70% and 50% ethanol, and lastly in water. Slides were then prepared for staining. They were permeabilized in 0.5% Triton-X-100 for 20 min, rinsed in PBS and blocked in the following solution: 1% BSA, 10% GS, 0.2% Triton-X-100 made up in PBS, for 1 h at room temperature. Primary antibodies were incubated overnight at 4°C in antibody solution:

2% BSA, 10% GS, 0.2% Triton-X-100 made up in PBS. The only antibody used for immunohistochemistry was MBP (1:100). The following day, sections were washed with PBS, incubated for 1 h at room temperature with AlexaFluor secondary antibodies (1:200) in antibody solution. Sections were washed, counterstained with DAPI nuclear stain, and mounted in Dako mounting medium.

2.5 Tissue Ultrastructural Analysis Sample Preparation

Vertebral columns from P5 mice (*Smn*^{-/-}; *SMN2* and *Smn*^{+/-}; *SMN2*) and optic nerve samples from P21 mice (*Smn*^{2B/-} and *Smn*^{2B/+}) were dissected and fixed overnight at 4°C in Karnovsky's fixative (4% PFA, 2% glutaraldehyde and 0.1 M sodium cacodylate in PBS, pH 7.4). After fixation, the lateral funiculus of the white matter from each L1 spinal cord was cut under a stereomicroscope into a segment of less than 1 mm of length with a surgical blade. All segments were subsequently washed twice in 0.1 M sodium cacodylate buffer for 1 h and once overnight at room temperature. Segments were post-fixed with 1% osmium tetroxide in 0.1 M sodium cacodylate buffer for 1 h and subsequently dehydrated in an ascending concentration of alcohol. Samples were infiltrated gradually in 100% Spurr resin. Finally, segments were embedded in fresh liquid Spurr resin and polymerized at 70°C overnight. Ultrathin sections (80 nm) from the white matter of the L1 spinal cord were cut with an ultramicrotome and collected onto 200-mesh copper grids. These sections were stained with 2% aqueous uranyl acetate and with Reynold's lead citrate.

2.6 Imaging

Epifluorescent images were acquired using an Axio Imager M1 microscope with an AxioCamHR HRm Rev.2 camera and Axiovision 4.8.2 software. Confocal images were taken using a Zeiss LSM 510 Meta DuoScan microscope and Zen 8.0 software. Phase contrast imaging

was conducted using an Axiovert 200M microscope equipped with an AxioCamHR HRm Rev.2 camera and Axiovision 4.6 software. Electron micrographs were taken with a transmission electron microscope (Hitachi 7100) at 4,000x magnification.

2.7 Quantification

All images were analyzed in ImageJ and analysis was blinded for all experiments.

2.7.1 Sholl Analysis

Sholl analysis was used to quantify the size and complexity of the maturing OL. Using the ImageJ *Concentric Rings* plugin, 10 concentric circles were overlaid at equal increments from the cell body. Branch intersections with each ring were counted manually. Data is depicted either as total intersections, which summates the intersections of all 10 rings, or as a breakdown of intersections per ring for a more detailed view of morphology.

2.7.2 Membrane Area

Membrane area was quantified by manual tracing of MBP immunofluorescent staining in cells late in development (DIV4-DIV6). Membrane areas presented are the average of 40-60 cells per group.

2.7.3 Migration Assay

To quantify migration, an exclusion zone 1.5x the seeding diameter of the OPCA was digitally overlaid using Photoshop. Only OPCs outside of this exclusion zone were counted. Concentric rings were then digitally overlaid with diameters increasing by increments of 100 μm (100 μm , 200 μm , 300 μm and 400 μm). Migration distance was quantified as the number of OPCs falling within each concentric ring and depicted as the percentage of OPCs in each ring. The total number of OPCs migrated was summated regardless of distance travelled and used as a measure of migration initiation.

2.7.4 In vitro Myelination Co-culture

Co-localized regions of MAG and neurofilament were measured using the integrated density plugin of ImageJ software. Neurite density was quantified as the mean gray value of the entire neurofilament signal in a given image.

2.7.5 G-ratio Measurement

Axon and total fibre diameter from wild type and *Smn*^{-/-}; *SMN2* white matter from L1 spinal cords were measured from electron micrographs. All diameter measurements were traced manually using ImageJ software. A total of 135-155 myelinated axons were measured from 12 electron micrographs by genotype. G-ratios were calculated as the ratio of axon diameter relative to the total fibre diameter, representing the inner and outer limits of the myelin sheath, respectively.

2.7.6 MBP Area – Tissue

Using ImageJ, threshold levels were set to be consistent among all images and the *Analyze Particles* plugin was used to assess the extent of MBP staining. Given that the region of interest for this experiment was the cortex and corpus callosum, images were standardized such that the same area of tissue was quantified in each section. Generated from this method was the area of MBP staining in the select region of interest.

2.8 RNA Expression Analysis

2.8.1 RNA Isolation

For all RNA experiments, cells were differentiated in 6-well plates. Total RNA was extracted using the RNeasy Mini Kit (Qiagen), and manufacturer's protocols were followed. RNA was eluted in RNase-free water (Qiagen). Concentrations were determined either using a nanophotometer spectrophotometer (MBI Lab Equipment) or with Qubit HS RNA Assay

(ThermoFisher). For mRNA analysis, total cDNA was constructed using the RT² First Strand Kit (Qiagen) as per the manufacturer's protocol.

2.8.2 Realtime-Quantitative Polymerase Chain Reaction (RT-QPCR): mRNA

PrimePCR pre-optimized primers (Bio-Rad) were used according to the manufacturer's protocol. Two negative controls (no cDNA, water only) were included in each plate. As per protocol, amplification was performed at 95°C for 5 min, followed by 40 cycles of 95°C for 5 s and 60°C for 30 s using a Bio-Rad CFX Connect. QPCR results were quantified by the $\Delta\Delta C_t$ method using CFX Maestro software (Livak & Schmittgen, 2001).

2.8.3 PCR Microarray

RNA was collected from primary OLs treated with either ECM or CSPGs and extracted as above. Gene expression was assessed using the RT² ProfilerTM PCR Array Mouse Cytoskeleton Regulators (Qiagen, PAMM-088Z). cDNA was made as above using 250 ng of RNA. Execution of qPCR reaction was performed as per the manufacturer's protocol. A total of 5 biological replicates were run for each group (ECM- and CSPG-treated). Relative expression was calculated using the RT² ProfilerTM PCR Data Analysis software using the $\Delta\Delta C_t$ method.

2.8.4 RNA Sequencing

RNA sequencing (RNA-seq) was conducted on DIV5 primary mouse OLs derived from wildtype and *Sncα*^{-/-} animals. Three biological replicates were collected and run for each genotype (N=3). Quality control and sequencing were performed by Stem Core Laboratories (Ottawa Hospital Research Institute). RNA concentrations were determined with the Qubit HS RNA Assay and RNA integrity was assessed using the Fragment Analyzer HS NGS assay (Agilent). The TruSeq Stranded mRNA Library Prep (Illumina) was used for library construction and was sequenced using the NextSeq 500 High Output 75 cycle kit (Illumina).

Bioinformatics analysis was performed by the Ottawa Bioinformatic Core Facility (Ottawa Hospital Research Institute). Generated reads were assigned to transcripts using Salmon v0.12.0 (Patro, Duggal, Love, Irizarry, & Kingsford, 2017). Transcript abundances were imported to R for analysis with DESeq2. First, data was filtered to retain only genes with 5 or more counts in two or more samples which retained 21,756 genes for further analysis. DESeq2 was used to calculate principle component analysis, hierarchical clustering and fold change (Love, Huber, & Anders, 2014). Importantly, expression differences were calculated using the lfcShrink function (Zhu, Ibrahim, & Love, 2019). Benjamini-Hochberg adjustment was applied in DESeq2 and adjusted p-values were used to identify significantly changed genes as those with adjusted p-value of < 0.05 (Benjamini & Hochberg, 1995).

2.8.5 RT-QPCR: miR

Reverse transcription for miR-219-5p and snU6 were completed following Biggar et al., with some modifications (Biggar, Kornfeld, & Storey, 2011). Briefly, 300 ng total RNA was incubated with 250 nM stem-loop primer. The annealing reaction proceeded at 95°C for 5 min, then at 60°C for 5 min, after which samples were centrifuged and kept on ice for 1 min. Reverse transcription was done using M-MLV Reverse Transcriptase (Invitrogen), 5x First Strand Buffer (Invitrogen), 100 mM dithiothreitol (Invitrogen) and premixed deoxynucleoside triphosphates (dNTPs) at final concentrations of 25 μ M each in RNase-free water. The following protocol was used for reverse transcription using an Eppendorf Mastercycler: 16°C for 30 min, 60 cycles of 20°C for 30 s, 42°C for 30 s and 50°C for 1 s, followed by 85°C for 5 min. Primer sequences used were: miR-219-5p stem-loop primer 5'-

CTCACAGTACGTTGGTATCCTTGTGATGTTTCGATGCCATATTGTACTGTGAG

AGAATTGC-3', miR-219-5p forward 5'-ACACTCCAGCTGGGTGATTGTCCAAACGC-3', snU6 stem-loop primer 5'-CTCACAGTACGTTGGTATCCTTGTGATGTTTCGATGCCATATTGTACTGTGAGAAAA ATATGGAACGCTT-3', snU6 forward 5'-ACACTCCAGCTGGGGTGCTCGCTTCGGCAGCACATA-3', universal reverse 5'-CTCACAGTACGTTGGTATCCTTG TG-3'. Primers were obtained from AlphaDNA.

Amplification of miR-219-5p and snU6 cDNA was done using specific forward primers and a universal reverse primer complementary to the stemloop portion of the cDNA. The qRT-PCR reaction consisted of 12.5 μ l 2x SsoFast EvaGreen Supermix (Bio-Rad), 0.8 μ l each of 25 μ M forward and universal primer, and 4 μ l cDNA in RNase-free water. Samples were amplified using the following protocol: 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min, using a Bio-Rad CFX Connect. All samples were run in technical triplicate. Relative expression was calculated using the CFX Manager software or CFX Maestro software using the $\Delta\Delta$ Ct method.

2.9 Protein Expression Analysis

2.9.1 Sample Lysis

Protein lysates were collected either from adherent cells in culture or flash frozen tissue.

Adherent cells: Cells were cooled on ice and washed with ice cold PBS. Ice cold RIPA lysis buffer (Cell Signaling) supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF) was added to the cells at which point cells were lifted from the plate with a cell scraper and lysate was collected. Lysates were then centrifuged at high speed to remove insoluble material.

Flash frozen tissue: Tissues were immersed in ice cold RIPA lysis buffer (Cell Signaling) supplemented with 1 mM PMSF. All tissues were then homogenized and transferred to Eppendorf tubes for centrifugation at high speed, after which the protein lysate was collected.

2.9.2 Western Blotting

Equal amounts of protein lysate (15-40 µg) were electrophoresed on polyacrylamide gels (8-15%) and transferred to a PDVF membrane at 0.25 amps for 60-90 mins. Membranes to be visualized using fluorescence were blocked for 1 h at room temperature using Odyssey Blocking Buffer (Li-Cor Biosciences) and those to be visualized using chemiluminescence were blocked with 5% milk powder in TBST (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.1% Tween-20) for 1 h at room temperature and incubated with primary antibody overnight at 4°C in blocking buffer (See Table 2.1 for antibody specifications). Membranes were washed with TBST. For fluorescent western blotting, membranes were incubated at room temperature for 1 h in secondary antibody (IRDye 680RD and 800CW; Li-Cor Biosciences) at 1:20,000 in Odyssey blocking buffer. After washing with TBST, membranes were visualized using the Li-Cor Odyssey CLx Infrared Imaging System. Band intensities were quantified within the program. For chemiluminescent western blotting, membranes were incubated with HRP-conjugated secondary antibodies for 1 h at room temperature, washed with TBST and treated with ECL (Thermo Scientific). Following development, films were scanned and densitometric analysis was performed using ImageJ software to quantify band intensity. Results for all experiments were normalized to alpha-tubulin levels.

2.9.3 Sandwich ELISA: Quantification of Alpha-Synuclein

384-well MaxiSorp plates (Nunc, Inc) were coated with capturing antibody, Syn1 (BD Biosciences) diluted 1:500 in coating buffer (NaHCO₃ with 0.2% NaN₃, pH 9.6) overnight at 4°C. Plates were washed with PBS/0.05% Tween-20 (PBST) and then blocked for 1 h at 37°C in blocking buffer (1.125% fish skin gelatin; PBST) and washed again with PBST. Samples were loaded, in duplicate and at two dilutions, and incubated at room temperature for 2 h.

Biotinylation of hSA4 antibody (MJFR1; Abcam) was completed using 200 µg Sulfo-NHS-LC Biotin (Pierce), then added to the plate for 1 h at 37°C. Following 5 washes with PBST, ExtrAvidin phosphatase (Sigma) diluted in blocking buffer was applied for 30 min at 37°C. Colour development was performed using fast-p-nitrophenyl phosphate (Sigma) and monitored at 405 nm every 2.5 min for up to 60 min. Saturation kinetics were examined for identification of time point(s) where standards and sample dilutions were in the log phase. Protein levels determined by ELISA were normalized to total protein levels as determined using bicinchoninic acid (BCA) protein assay.

2.10 Statistical Analysis

All statistical analyses were performed using Prism 7 GraphPad software with the exception of q-RT-PCR experiments, which were analyzed using CFX Maestro software (Bio-Rad). All statistical tests are noted in figure legends, where appropriate. Briefly, pair-wise comparisons were done using two-tailed Student's t-test. Comparisons that involved two conditions or more were done using either one-way or two-way analysis of variance (ANOVA) followed by either Tukey's or Bonferroni's post-hoc testing. Significance was attributed with p-values less than 0.05 and are indicated on all figures with asterisks (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$).

Chapter 3: Oligodendrocyte Development and CNS Myelination are Unaffected in a Mouse

Model of Severe Spinal Muscular Atrophy

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Model of Severe Spinal Muscular Atrophy

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Author Contributions

Conceived and designed experiments: RWO, RK. Performed experiments: RWO (All except Figure 3.7), SEC (All except Figure 3.7), YDR (Figure 3.7), J-PM and M-OD (Assisted with Figure 3.6). EM and SG assisted with animal care, line maintenance and genotyping of animals. RWO, SEC and RK interpreted and analyzed the results. SEC wrote the manuscript and RWO, EM, JPM, MOD and RK contributed with editing. RK was the primary supervisor of this work.

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3.1 Introduction and Rationale

Spinal muscular atrophy (SMA) is an often-fatal childhood-onset neurodegenerative disease of genetic origin (Pearn, 1978). It is relatively common among paediatric genetic diseases with an incidence around 1 in 10,000 live births (Verhaart et al., 2017). The major pathological feature of SMA is the loss of alpha motor neurons in the spinal cord, translating to progressive proximal muscle weakening and atrophy. Loss-of-function mutations or deletions in the *Survival Motor Neuron 1* gene (*SMN1*) are the underlying cause of SMA (Lefebvre et al., 1995). Humans also possess *SMN2*, an inverted duplication of *SMN1* that harbours a C to T transition at position 6 within exon 7 that favours the exclusion of this exon from the transcript. This transcript yields a highly unstable, truncated protein (Lorson et al., 1999). However, *SMN2* also produces low amounts of full-length SMN, such that the copy number of *SMN2* inversely correlates with disease severity (Lorson et al., 1999).

Mice possess only one *Smn* gene and its loss leads to early embryonic lethality (Schrank et al., 1997). A mouse model that closely recapitulates the most severe form of SMA was developed by Monani and colleagues, through supplementation of *Smn* knockout mice with a human *SMN2* transgene (*Smn*^{-/-};SMN2) (Monani et al., 2000). Study of this model of severe SMA has facilitated the understanding of the role of *Smn* in different cell types, and its importance in the maintenance of lower motor neuron function. Insufficient *Smn* levels result in disorganization of the neuronal growth cone structure and subsequent stunting of axon extension (McWhorter, Monani, Burghes, & Beattie, 2003; Rossoll et al., 2003). Due to the similarities in growth cone morphology between neurons and OLs, it is important to address the effect of low *Smn* levels in OLs. Additionally, *Smn*-depletion contributes to an increase in astrocyte reactivity, as well as functional defects in astrocyte signalling and growth factor secretion (McGivern et al.,

2013; Rindt et al., 2015). Astrocyte involvement in SMA not only presents the question of how other glial cells within the CNS respond to *Smn* deletion, but astrocyte defects may also directly influence establishment and maintenance of CNS myelin. Furthermore, myelin defects in the peripheral nervous system have been reported in multiple models of SMA, characterized by intrinsic defects in the Schwann cell, which is responsible for peripheral myelination (G. Hunter et al., 2014; Gillian Hunter et al., 2016). The key role played by the OL in supporting motor neuron function, along with the similarities to other affected cell types mentioned above, led us to investigate the effect of *Smn* depletion on OLs.

3.2 Results

3.2.1 Low Smn levels do not affect OPC growth or molecular differentiation *in vitro*

The ubiquitously expressed Smn protein is known to play a role in various cellular functions including the proper formation of elaborate axonal growth cones in motor neurons (Rossoll et al., 2003). The presence of dynamic, ‘growth cone-like’ process tips in OLs is increasingly recognized as the mechanism for orchestrating the highly branched network in the differentiating cell (Michalski et al., 2016). Due to the commonalities between neuronal and OL growing tips, it is reasonable to speculate that Smn plays a role in the OL differentiation gamut. To investigate the role of Smn in this process, primary OLs harvested from *Smn*^{-/-}; *SMN2* mice were compared to that of control littermates. Western blots confirmed that the amount of Smn protein was reduced in our mixed glial culture system (Supplementary Figure 3.1).

To assess OPC maturation, primary OLs were allowed to differentiate for three and six days *in vitro* (DIV3 and DIV6; Figure 3.1A). Progression through the OL differentiation paradigm can be tracked via the expression of many common maturation markers: NG2 proteoglycan for early, MAG for mid and MBP for late stage differentiation. The proportion of OLs expressing each marker at the chosen time points *in vitro* was consistent between *Smn*^{-/-}; *SMN2* and wild type cultures (Figure 3.1B,C). It should be noted that OL differentiation followed a typical expression pattern such that NG2 is expressed early and was lost as MAG was induced but with overlapping expression during this time. MBP followed MAG in expression; however, MAG continued to be expressed throughout the remainder of OL differentiation. Therefore, the differentiation pattern of the OLs assessed from both wild type and *Smn*^{-/-}; *SMN2* cultures was as expected, with almost complete co-expression of MAG and MBP by DIV6. In addition to the proportion of cells expressing each marker, it was also of interest to assess the

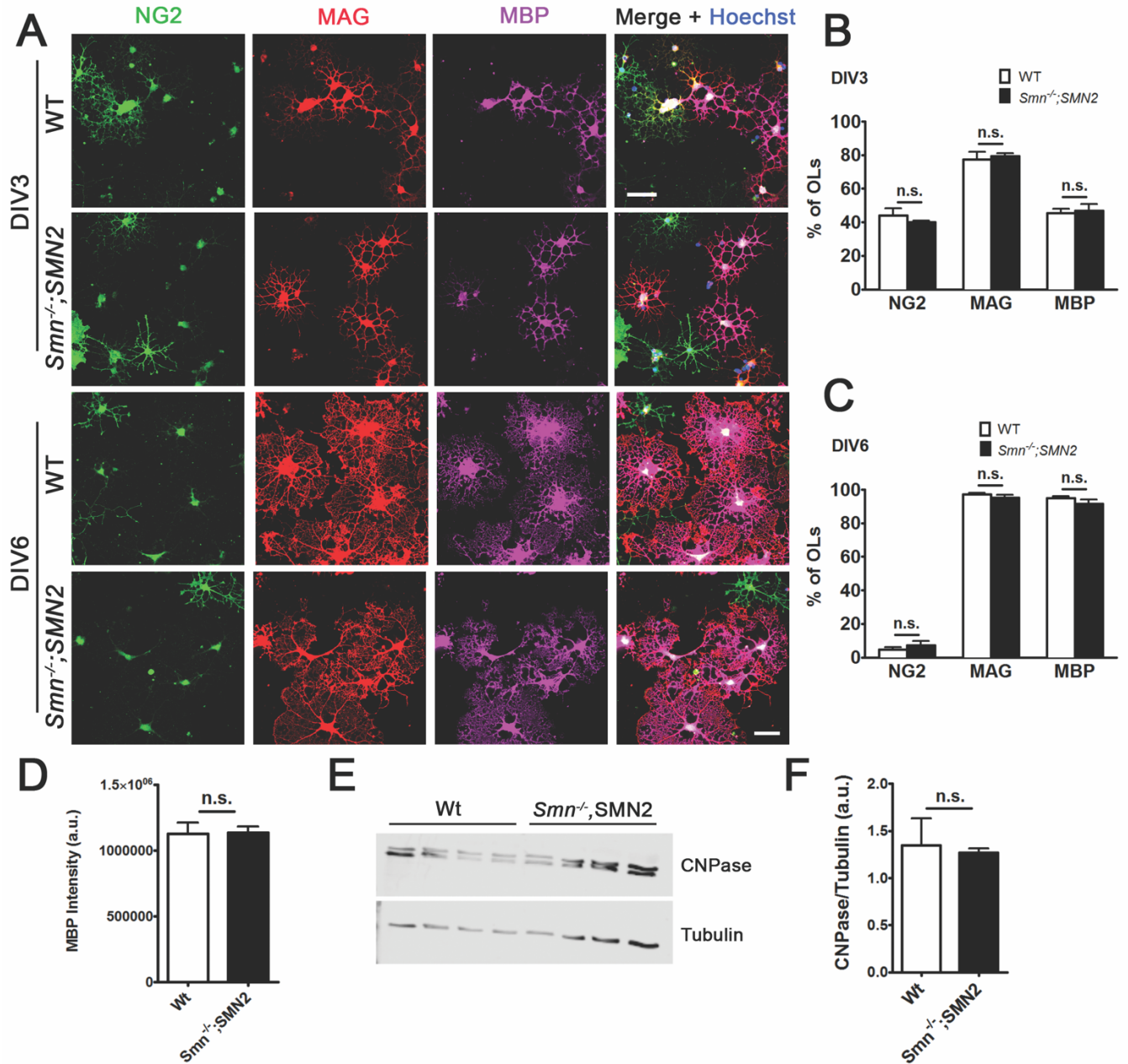


Figure 3.1 Oligodendrocyte differentiation and marker expression is unaltered upon *Smn* depletion. (A) Immunofluorescence micrographs of OL primary cultures from neonatal *Smn*^{-/-};SMN2 or WT control mice after DIV3 and DIV6 of differentiation demonstrating the ability of both genotypes to progress through the molecular differentiation program. (B,C) Quantification of the percent of OLs expressing NG2, MAG and MBP at DIV3 and DIV6 for mutant and WT

cultures. **(D)** Quantification of the intensity of MBP expression from DIV3 immunofluorescence images. **(E)** Fluorescence western blot analysis of CNPase and α -tubulin from primary OLs at DIV5. **(F)** Quantification of total CNPase normalized to α -tubulin (from blot pictured in E). Data represent the mean $\pm$ standard error of the mean (SEM). Unpaired two-tailed Student's *t*-test; n.s. indicates no significant difference; *N* = 3 (except for E and F where *N* = 4). Scale bar, 50 μ m; applicable to all images.

relative amount of these vital myelin proteins within the positive-expressing OLs. The intensity of the fluorescence signal for MBP-positive OLs at DIV3 was quantified, using the integrated density plugin of ImageJ software and no difference between genotypes was observed (Figure 3.1D). Moreover, the expression level of CNPase, an alternative OL lineage marker, was similar between genotypes as determined by western blot (Figure 3.1E). Thus, reduced *Smn* levels do not appear to affect the typical OL molecular maturation progression *in vitro*.

To further interrogate the *Smn*^{-/-}; *SMN2* OPC cultures, cell death and proliferation assays were conducted. The percentage of OPCs expressing Ki-67 was analysed as a marker of proliferation. We did not observe any significant difference in the expression of Ki-67 in differentiating OPCs after 4h (Figure 3.2A,B). Furthermore, we assessed the incidence of apoptotic cell death in these cultures using cleaved caspase 3 (CC3) as a marker. There was no significant difference in the percentage of CC3-positive OLs between the genotypes at DIV3, suggesting that *Smn* reduction does not influence survival of OL-lineage cells *in vitro* (Figure 3.2 C,D). This correlates with the data above suggesting that *Smn* depletion does not impact OPC development.

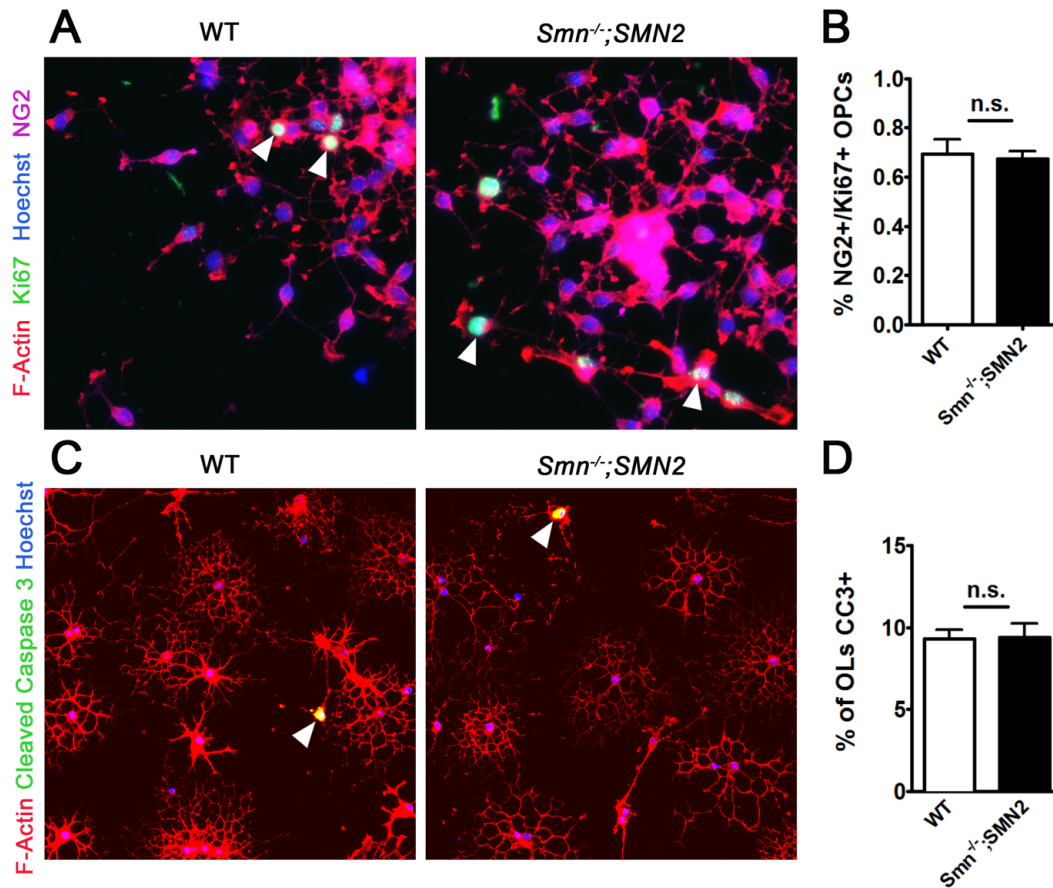


Figure 3.2 The proportion of OPCs undergoing proliferation or of OLs undergoing cleaved caspase 3-mediated cell death is similar in wild type and *Smn*^{-/-};*SMN2* cultures. (A)

Immunofluorescence micrographs of *Smn*^{-/-};*SMN2* or WT after 4 h of differentiation demonstrating similar expression of Ki67 in NG2-positive OPCs (arrowheads). (B)

Quantification of the percent of OPCs that are double-positive for NG2 and Ki67, indicating proliferative precursor cells at 4 h of differentiation. (C) Immunofluorescence micrographs of *Smn*^{-/-};*SMN2* or WT OL cultures at DIV3 highlighting similar incidence of cleaved caspase 3

in MAG-positive OLs. (D) Quantification of the percent of OLs that are cleaved caspase 3-

positive (arrowheads) at DIV3 in each genotype. Data represent the mean $\pm$ SEM. Unpaired two-tailed Student's *t*-test; n.s. indicates no significant difference; *N* = 3.

3.2.2 Smn depletion does not impair the migratory capacity of primary OPCs

Successful myelination by the mature OL is highly dependent on the migration of OPCs to the unmyelinated axon. As such, we sought to determine the impact of Smn depletion on the migration of OPCs. We took advantage of a previously described *in vitro* OPCA migration assay developed for the primary murine system (O'Meara et al., 2016). Following seeding of individual OPCAs, OPCs were allowed to undergo radial migration for 24h, at which point they were fixed for quantification. Our analysis revealed that the number of *Smn*^{-/-}; *SMN2* OPCs capable of reaching each concentric ring, overlaid 100 µm apart, was comparable to wild type (Figure 3.3A,B). The total number of OPCs migrated, irrespective of distance, can be used as a measure of the ability to initiate migration. There was no significant difference in the total number of OPCs migrated from the seeded aggregate between genotypes (Figure 3.3C). Of note, the diameter of the initial OPCA at seeding time was also consistent (Figure 3.3D). Taken together, these results indicate that reduced levels of Smn do not contribute to a migratory phenotype in OPCs, including initiation and extent of migration.

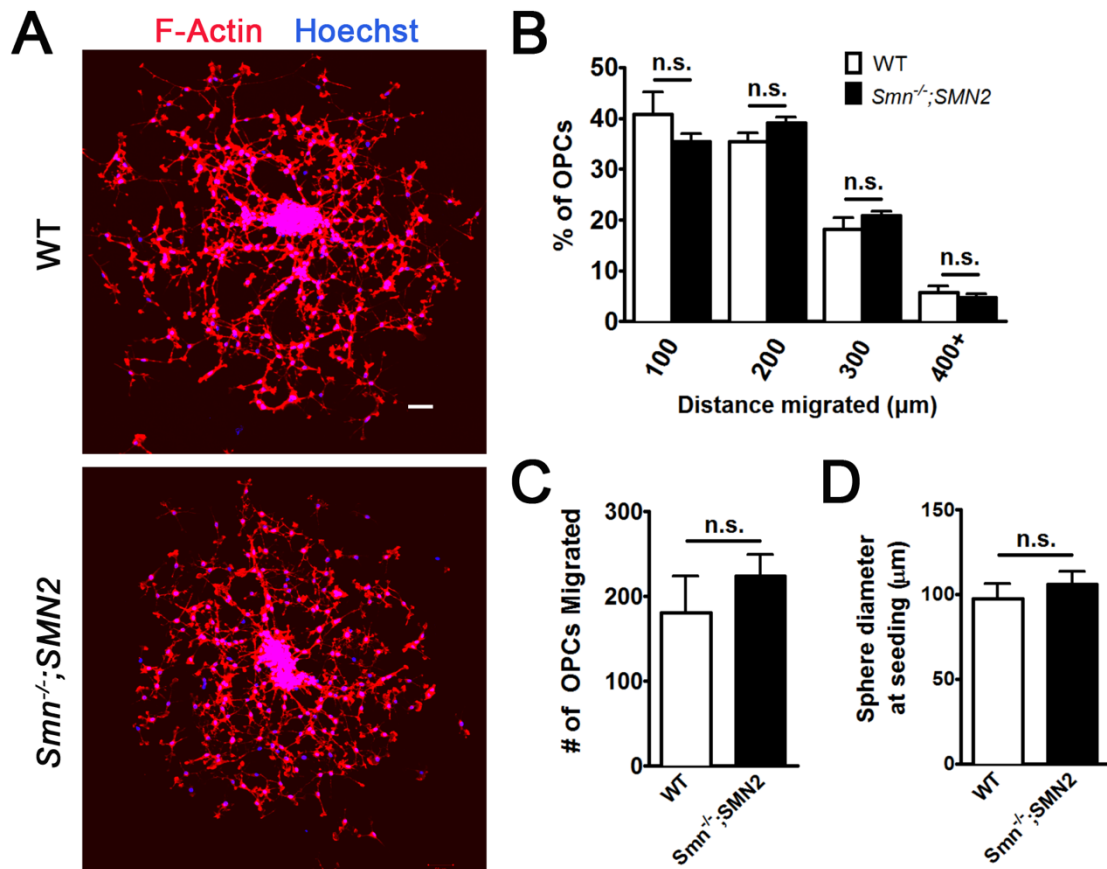


Figure 3.3 OPC migration *in vitro* is normal in *Smn*^{-/-};SMN2-derived cultures. (A)

Immunofluorescence micrographs of OPCAs from *Smn*^{-/-};SMN2 or WT mice following 10 h of migration. **(B)** Quantification of the percentage of OPCs within rings placed 100, 200, 300 or 400 μm away from the original OPCA. There was no difference in the distances migrated by *Smn*^{-/-};SMN2 OPCs at 10 h compared to WT. **(C)** Quantification of the total number of OPCs migrated from the OPCA, indicating migration initiation. *Smn*^{-/-};SMN2 OPCs are equally capable of initiating migration as WT. **(D)** Sphere diameter of the initial OPCA at seeding time is similar in both genotypes. Data represent the mean +/- SEM. Repeated measures two-way ANOVA with Bonferroni post-tests (B) or unpaired two-tailed Student's *t*-test (C); n.s. indicates no significant difference; *N* = 3. Scale bar, 50 μm; applicable to all images.

3.2.3 Reduction in Smn level does not inhibit morphological maturation of OLs in primary culture

A crucial component of OL development is the extension of a complex network of processes and the subsequent formation of membranous sheets. This transformation is collectively referred to as morphological maturation and is required for efficient myelination. To quantify morphological maturation, primary OLs were binned into one of four stages as has been previously reported based on their increasing complexity *in vitro* (Figure 3.4B,C) (R. W. O'Meara et al., 2013). At DIV3, the culture is expected to be predominantly stage 3 OLs, which have formed a highly complex network of processes and represent an intermediate OL. As differentiation continues to DIV6, OLs are predominantly stage 4, which are those producing a membranous sheet reminiscent of myelin. Stage 4 cells are considered to be morphologically mature OLs. Quantification of primary OLs using this staging system revealed no significant difference in morphological differentiation between *Smn*^{-/-}; *SMN2* and wild type at DIV3 and DIV6. Importantly, the OLs derived from control animals exhibited the expected morphological complexities at each time point, such that the majority of OLs at DIV3 were highly branched (stage 3) with progression to a predominantly membranous culture (stage 4) at DIV6. Additionally, Sholl analysis was applied to OLs at DIV3. This measure quantifies the number of times a process of a given cell intersects with concentric rings digitally overlaid 10 µm apart. There was no significant difference between genotypes in the number of intersections across each of the ten rings, nor was a difference detected in the total number of process intersections (Figure 3.4D).

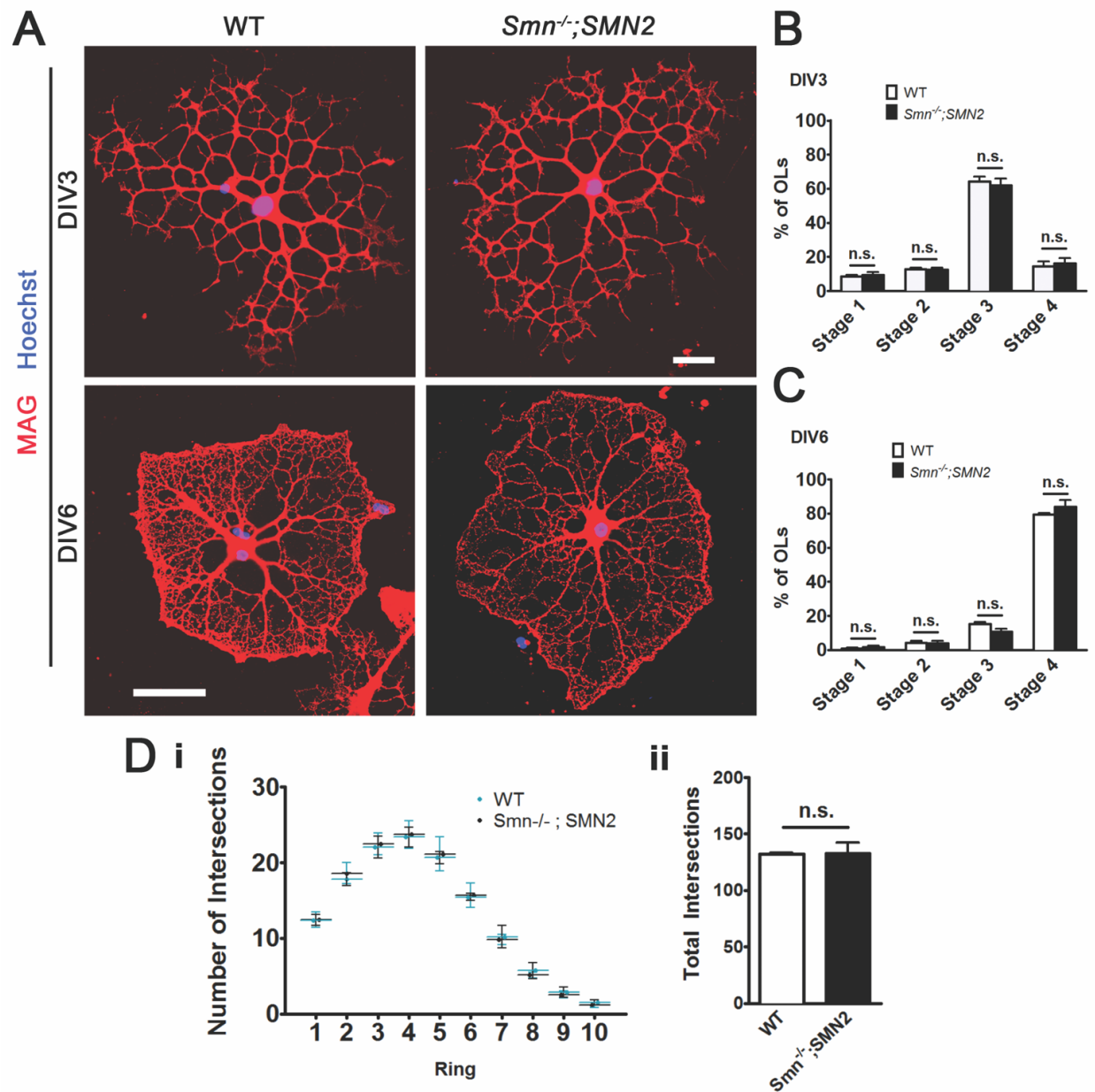


Figure 3.4 Loss of Smn does not affect morphological differentiation of OLs. (A)

Immunofluorescence micrographs depicting representative morphologies of OLs at DIV3 and DIV6 derived from *Smn*^{-/-};SMN2 or WT mice. **(B, C)** A four-tiered staging scheme was employed to quantify the morphological differentiation of OLs *in vitro*. In this method, OLs with predominantly primary, secondary or tertiary branching were binned as stage 1, 2 or 3, respectively. OLs producing any amount of myelin membrane were binned as stage 4. A typical

stage 3 OL is depicted in (A) at DIV3, while the DIV6 OLs shown in (A) are considered stage 4. Quantification revealed no difference in the morphology of *Smn*^{-/-}; *SMN2* OLs relative to WT at either DIV3 (B) or DIV6 (C). Quantification of Sholl analysis completed on DIV3 OLs depicting the number of cell process intersections with concentric rings overlaid 10 μ m apart (**D**). Quantification revealed no difference in the size or complexity of the cells when represented either by the number of intersections across each ring (i) or the sum of all intersections (ii). Data represent the mean $\pm$ SEM. Unpaired two-tailed Student's *t*-test or repeated measures two-way ANOVA with Bonferroni post-tests; n.s. indicates no significant difference; *N* = 3. DIV3 scale bar, 20 μ m; DIV6 scale bar, 50 μ m; applicable to all images within the given time point.

3.2.4 Smn depletion does not impact OL myelinating capacity *in vitro*

Smn depletion appears to have no impact on OPC development or migration, however the effect of Smn depletion on functional OL myelination remained to be addressed. To this end, we used an OL/DRG co-culture system. The extent of myelination can be quantified in this assay by the co-localization of the myelin marker MBP with neurofilament, a neuron-specific marker. At DIV6 of co-culture, *Smn*^{-/-}; *SMN2* OLs were capable of contacting and subsequently enwrapping neighbouring neurites with MBP^{+ve} membrane to the same extent as OLs derived from control littermates (Figure 3.5A,B). Importantly, the neurite density in these cultures was consistent between genotypes, eliminating this as a confounding variable (Figure 3.5C). Cumulatively, these results suggest that Smn depletion in the OL does not affect *in vitro* myelination.

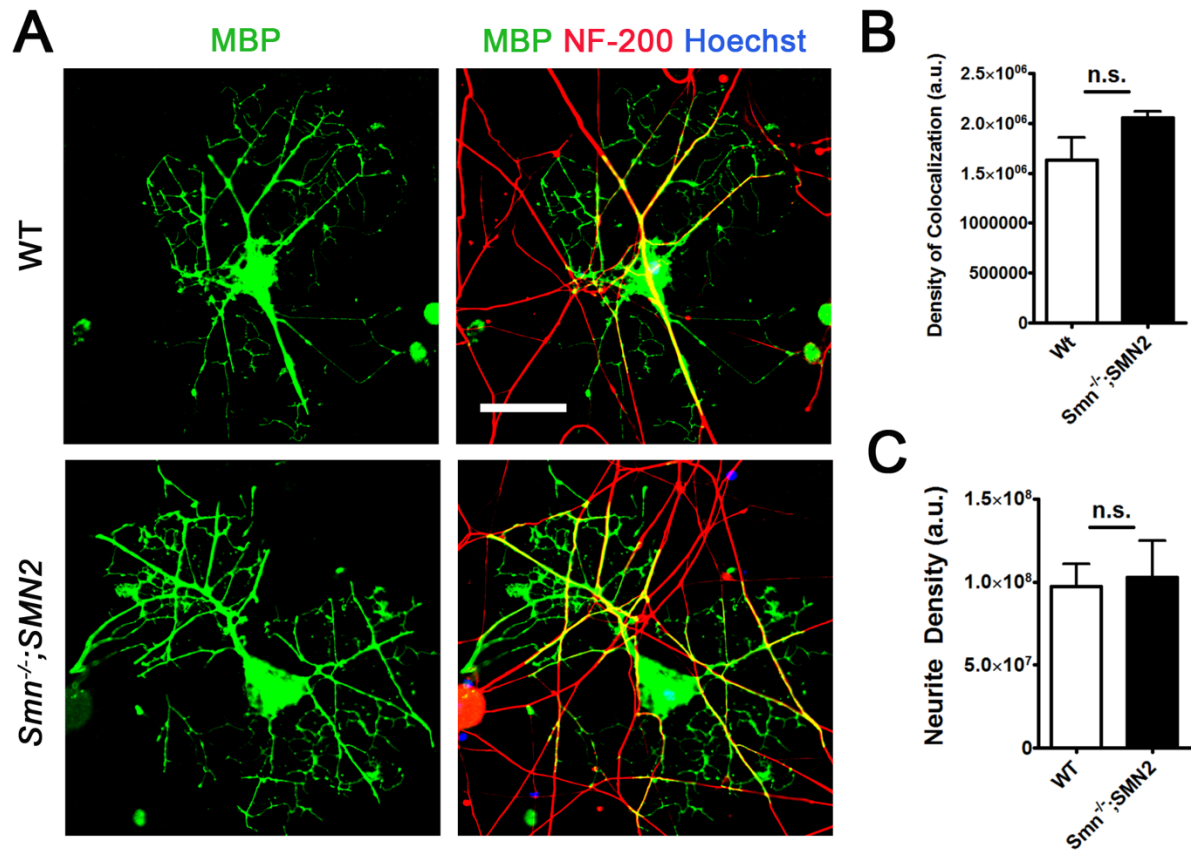


Figure 3.5 Smn depletion does not affect the ability of OLs to myelinate in an OL/DRGN co-culture system. (A) Immunofluorescence micrographs of primary OLs (MAG-positive, green) derived from *Smn*^{-/-};SMN2 or WT in culture with dorsal root ganglion neurons (DRGNs; neurofilament-positive, red) at DIV6. (B) Quantification of the ability of OLs to co-localize with DRGNs, used as a measure of the *in vitro* ability to ensheath axons with myelin membrane. Represented as an arbitrary measure of MAG and neurofilament co-localization, as determined using ImageJ software. (C) Neurite density does not differ between *Smn*^{-/-};SMN2 and WT co-cultures. This indicates that the data was not confounded by differences in assay establishment. Data represent the mean $\pm$ SEM. Unpaired two-tailed Student's *t*-test; n.s. indicates no significant difference; *N* = 3. Scale bar, 50 μ m; applicable to all images.

3.2.5 Smn depletion does not influence OL maturation *in vivo*

Analysis of OLs *in vitro* is essential to discern possible intrinsic developmental defects in an Smn-deficient context. However, the relevance of the OL to the overall SMA phenotype requires *in vivo* analysis of OL development and function. We assessed cortical white matter, specifically the corpus callosum, for the presence of mature (MBP-expressing) OLs.

Quantification of the number of MBP-positive cells relative to the area of the corpus callosum indicated that the number of mature OLs in the white matter of *Smn*^{-/-}; *SMN2* mice did not differ from wild type (Figure 3.6). Therefore, we concluded that the ability to progress through the typical OL maturation gamut *in vivo* was not compromised by Smn depletion.

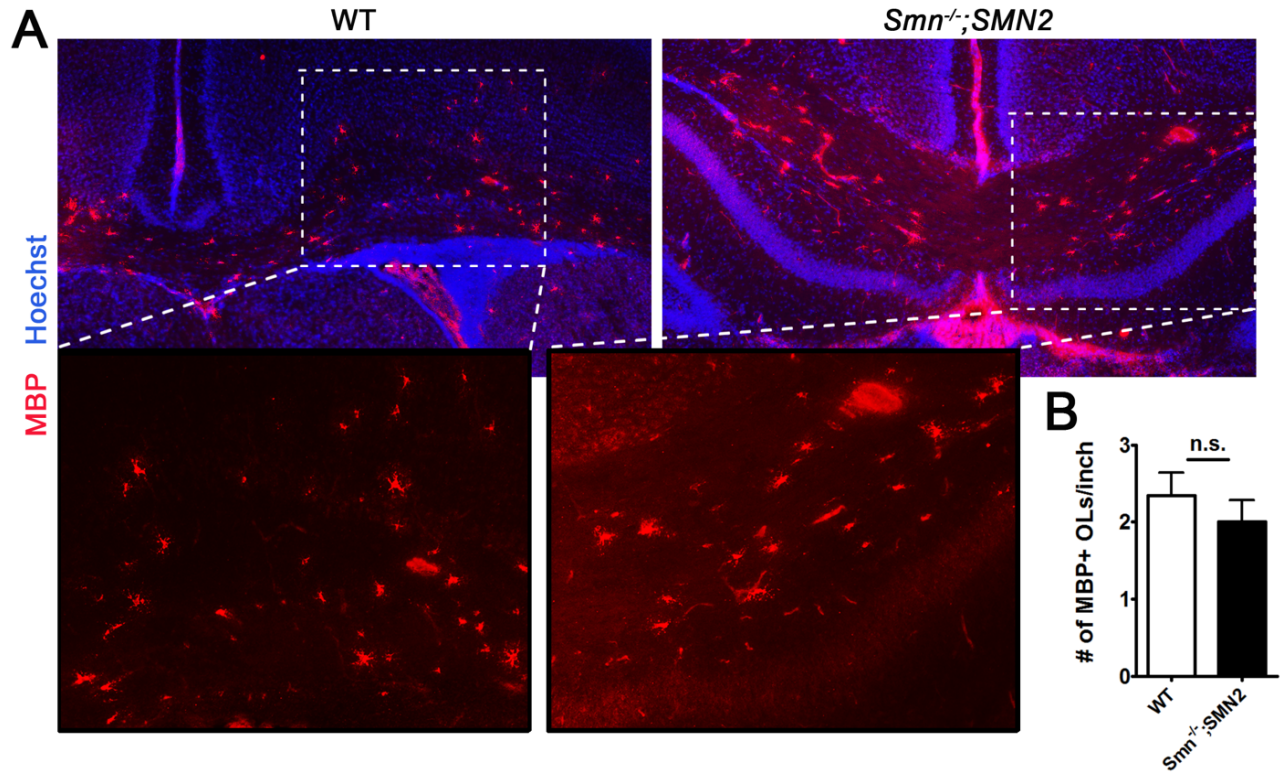


Figure 3.6 *Smn*^{-/-};SMN2 mice have equivalent numbers of mature OLs in the corpus callosum as WT littermates. (A) Immunofluorescence micrographs depicting similar incidence of MBP-positive OLs in the corpus callosum of WT and *Smn*^{-/-};SMN2 mice at P5. Boxed regions have been magnified. (B) Quantification of the number of MBP-positive OLs within the corpus callosum, and normalized to the area of the quantified region. Data represent the mean $\pm$ SEM. Unpaired two-tailed Student's *t*-test; n.s. indicates no significant difference; *N* = 3.

3.2.6 CNS myelination is unaffected in *Smn*^{-/-}; *SMN2* mice

In addition to confirming the ability of OLs to mature and express the appropriate lineage markers *in vivo*, it is imperative to investigate the effects, if any, of *Smn* depletion on overall myelination in the CNS. We, therefore, assessed myelin ultrastructure in the spinal cords of P5 mice. Qualitatively, white matter ultrastructure, as seen through electron microscopy, appeared similar between the *Smn*^{-/-}; *SMN2* mice and wild type controls (Figure 3.7A). G-ratios were calculated for each genotype as a quantitative measure of *in vivo* myelination. This measures the ratio between the inner and outer diameters of the myelin sheath, such that a g-ratio of 1 would represent a completely amyelinated axon and smaller values represent thicker myelin. The average g-ratios for spinal cord are remarkably similar between the two genotypes; *Smn*^{-/-}; *SMN2* g-ratios averaged 0.787, while g-ratios for wild type controls averaged 0.786 (Figure 3.7C). It is important to note that typical g-ratio values for murine spinal cord are reportedly 0.79, which is consistent with our findings. Finally, g-ratios plotted by axon calibre from spinal cord tissue of P5 wild type and *Smn*^{-/-}; *SMN2* mice also showed no difference upon linear regression analysis (Figure 3.7D).

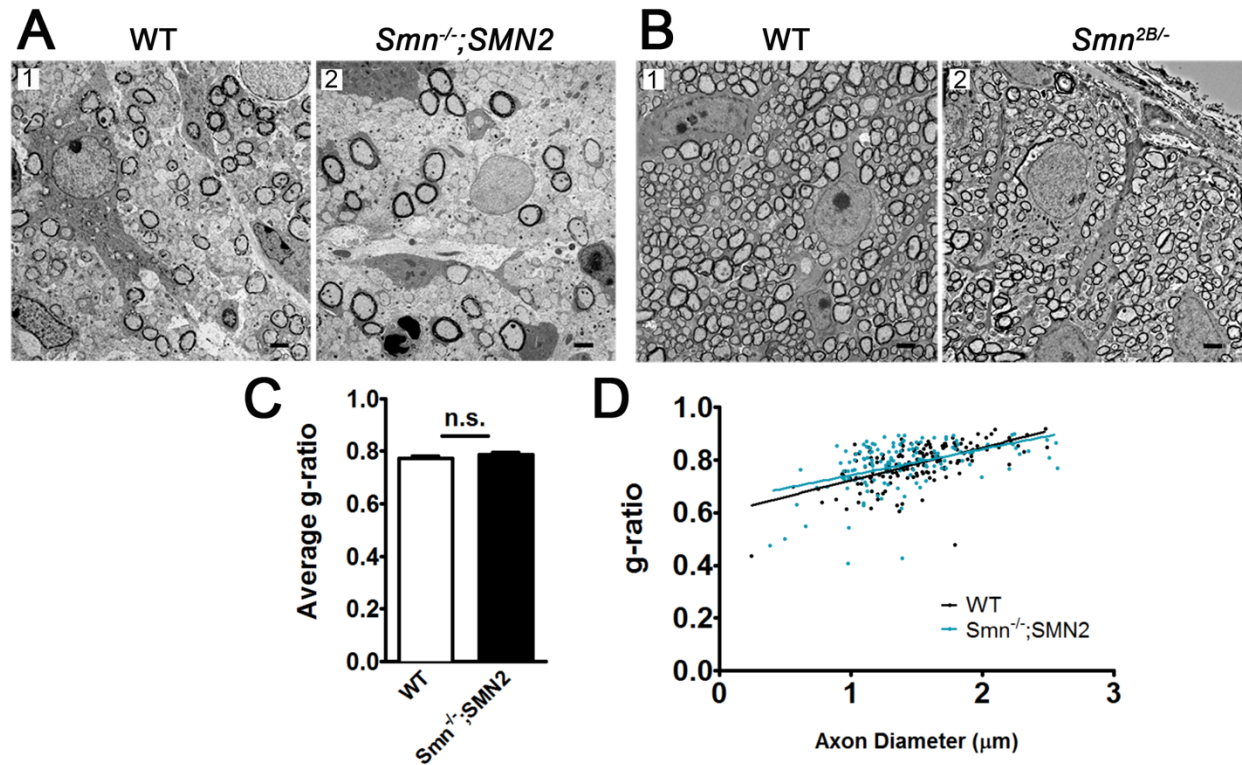


Figure 3.7 Normal myelination in the spinal cord and optic nerve of symptomatic *Smn*-deficient mice. Electron micrographs showing glial cells and many myelinated axons of different calibers in transverse sections of the white matter from the L1 spinal cords of wild type and severe *Smn*^{-/-};SMN2 mice at P5 (**A**) as well as optic nerve from wild type and *Smn*^{2B/-} mice at P21 (**B**). Magnification 4,000x for all images and scale bar is 2 μm for all panels. (**C**) Quantification of the average g-ratio in spinal cord of P5 wild type and severe *Smn*^{-/-};SMN2 mice. Data represent the mean +/- SEM. Unpaired, two-tailed Student's *t*-test; n.s. indicates no significant difference; *N* = 3. (**D**) G-ratios plotted by axon caliber from spinal cord tissue of P5 wild type and *Smn*^{-/-};SMN2 mice. Data represent individual measurements, *N* = 153 and 137 for wild type and *Smn*^{-/-};SMN2, respectively. Comparison non-significant by linear regression analysis.

To confirm that myelination in the spinal cord is representative of central myelination as a whole, we performed similar studies in the optic nerve. The optic nerve is myelinated much later in development than the spinal cord. The animals in the severe model of SMA do not survive past P6, at which point the optic nerve remains almost completely amyelinated. As a result, we investigated the ultrastructure of the optic nerve at P21 in a less severe model of SMA (*Smn*^{2B/-}). Again, qualitative analysis revealed little difference between the affected animals and their wild type counterparts (Figure 3.7B). Collectively, these results indicate that depletion of *Smn* does not have any impact on the developmental myelination program in the CNS, as represented by spinal cord and optic nerve in two different animal models of SMA.

3.3 Discussion of Results

It is well established that the Smn protein is ubiquitously expressed with vital housekeeping roles in many cell types. In the CNS, Smn function extends beyond splicing and snRNP assembly to include β -actin mRNA transport and maintenance of neuronal growth cones (Glinka et al., 2010; Rossoll et al., 2003; Todd et al., 2010). The necessity of Smn function to development is apparent as complete Smn knockout models exhibit pre-implantation defects and early embryonic lethality (Schrack et al., 1997). It is also clear that reductions in Smn levels can lead to dramatic effects on life expectancy and motor function integrity, enforcing the fundamental role of this protein (Lefebvre et al., 1995).

Although SMA is largely considered a disease of the motor neuron, the deleterious effect of Smn-depletion on other cell types is evident and gaining acceptance in the field. Defects in the development of muscle, a crucial component of the motor unit, have been reported in a variety of SMA models. Importantly, these defects are not simply a consequence of motor neuron atrophy but are also due to intrinsic changes following Smn depletion (Boyer et al., 2014; Shafey et al., 2005). Moreover, in the peripheral nervous system of mouse models of SMA, myelination defects have been detected and can be attributed to defects in the developing Schwann cell (G. Hunter et al., 2014; Gillian Hunter et al., 2016). Exploration of the CNS in models of SMA has uncovered inherent abnormalities in cortical neuron development, as well as astrocyte reactivity and signalling (McGivern et al., 2013; Monani et al., 2000; Rindt et al., 2015; Wishart et al., 2010). Furthermore, the characterization of the impact of Smn depletion has expanded to include non-neuronal systems. Notably, intrinsic pancreatic defects have been identified in the context of SMA, as have defects in kidney, liver, spleen and thymus development (M. Bowerman et al., 2014; Melissa Bowerman, Kathryn J. Swoboda, et al., 2012; M.-O. Deguise et al., 2017; Nery et

al., 2019; Szunyogova et al., 2016). This clear trend towards the discovery of unique defects in multiple cell types contributing to SMA pathogenesis led to the hypothesis that Smn-depletion would affect OL development and subsequent myelination processes.

Through extensive investigation of OLs in a severe model of SMA, it was surprising to see that Smn reduction did not affect OL growth, maturation or function. This is contrary to our expectations and, to our knowledge, this apparent lack of a phenotype has not been seen in any other cell type studied to date. Previous studies have particularly underscored the importance of Smn function in developmental processes, which correlates well with high Smn expression in pre- and neonatal stages (Gabanella, Carissimi, Usiello, & Pellizzoni, 2005; Kariya et al., 2014). Importantly, this is a crucial time for OL development and myelination. Thus, it was expected that Smn depletion would at least delay OL maturation, if not completely inhibit this process.

It is possible that the reduction of Smn in OLs has impacted an aspect of cell growth, maturation or function, but cannot be detected through the sensitivity of the selected assays. For example, only cleaved caspase 3-mediated apoptosis was explored as a means of cell death and therefore it remains a possibility that reduced levels of Smn in the OL would increase the incidence of death occurring by an alternative mechanism. However, similar measures have been successfully employed to parse out defects in OL development following other genetic and signalling manipulations, such as deletion of integrin-linked kinase and inhibition of N-WASP and Fyn kinase (Bacon et al., 2007; Colognato et al., 2004; R. W. O'Meara et al., 2013; O'Meara et al., 2016). Therefore, it is likely that the chosen assessments and time points would be sufficient to detect a biologically relevant effect of Smn depletion on OLs.

An alternative explanation for the lack of change in OLs following Smn loss is the concept of Smn threshold (Mélissa Bowerman et al., 2012). It is not only established that slight

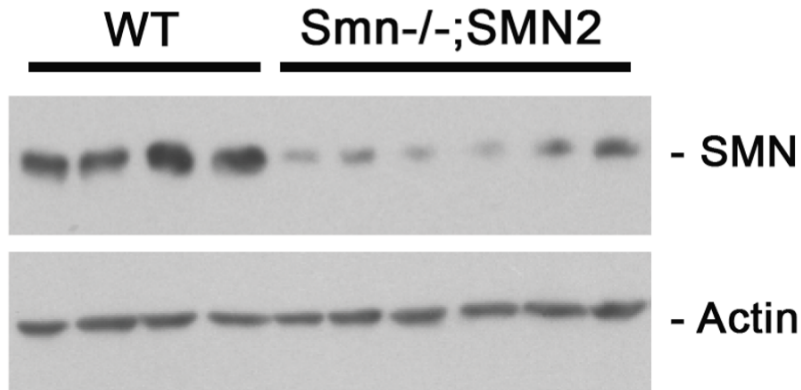
alterations to Smn levels can dramatically improve or worsen phenotype, but also that cell types are differentially susceptible to Smn depletion. It is widely accepted that motor neurons are highly sensitive to Smn depletion and exhibit phenotypic abnormalities at much higher levels of Smn than other tissue types. Moreover, differential susceptibility within the neuromuscular pathology has been well characterized. For example, it is clear that the motor units of abdominal muscles are more vulnerable to deficient Smn levels than those associated with cranial muscles (Murray et al., 2008). On the notion of differential susceptibility, this study does not have the capacity to discern subtle defects present in a subset of OLs, such as those responsible for myelinating lower motor neurons. Due to the limitations of the primary cell culture technique employed, conclusions regarding the impact of Smn depletion could only be made in OLs derived from the cortex. In a different mouse model of SMA (SMN Δ 7), a general reduction in OL presence was detected in the lumbar spinal cord as assessed by NG2 and MBP expression by western blot (Ohuchi et al., 2019). This would suggest that development of OLs in this particular region of the spinal cord may be affected by Smn reduction. However, our assessment of myelin ultrastructure with electron microscopy was completed on sections from the lumbar region of the spinal cord. The lack of overt phenotype in this measure does not support the potential for vulnerable subpopulations of OLs in the lower regions of the spinal cord.

The seemingly insignificant impact of Smn depletion on OLs may also be explained by the induction of an alternative compensatory pathway, mitigating any developmental delays or defects. While this is improbable, as compensatory pathway activation has not been detected in similar essential cell types upon loss of Smn to the extent that no defects are detectable, it remains a possibility that should be further investigated. A number of disease modifiers have been identified whose expression have been shown to alter the severity of the disease, including

plastin 3 (Oprea et al., 2008). Perhaps deeper transcriptomic analysis would identify unique expression of a known or novel disease modifier in OLs, serving as protection in this cell type. Conversely, the molecular mechanisms mediating the effects of Smn depletion in other cell types may not be vital to OL development, thus explaining the seemingly normal growth of this cell type. However, this is unlikely as Smn reduction in other cell types impacts crucial pathways for OL growth, including the Rho family GTPases (Melissa Bowerman, Murray, Boyer, Anderson, & Kothary, 2012; Liang et al., 2004). Smn depletion correlates with increased RhoA activation in skeletal muscle, and inhibition of this activity has been sufficient to restore the expression of lineage markers and reverse growth defects (Melissa Bowerman, Lyndsay M. Murray, et al., 2012).

In summary, we have taken advantage of a severe mouse model of SMA to perform a comprehensive characterization of OL functionality *in vitro* and *in vivo* upon Smn depletion. Our results indicate that unlike many neural cell types, Smn depletion does not impair proper development of OLs. Upon reduction of Smn, OLs remain capable of executing the complete differentiation program, retaining migration abilities and maintaining both *in vitro* and *in vivo* myelination capacity. These results have unveiled a novel system to exploit in the discovery of disease modifiers or compensatory mechanisms to further our understanding of differential susceptibility in SMA.

3.4 Supplementary Material



Supplementary Figure 3.1 Smn levels are lower in mixed glial cultures derived from *Smn*^{-/-};SMN2 mice compared to wildtype. Protein lysates collected from mixed glial cultures established from *Smn*^{-/-};SMN2 and wildtype mice clearly confirm that Smn protein is reduced in the *Smn*^{-/-};SMN2 model.

Chapter 4: Alpha-Synuclein is Not Required for OL Differentiation or CNS Myelination

Chapter 4: Alpha-Synuclein is Not Required for OL Differentiation or CNS Myelination

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Author Contributions

Conceived and designed experiments: SEC, JT, MS, RK. Performed experiments: SEC performed all experiments in this chapter with the following assistance - SK (Figure 4.3.1A, as well as cell culture contributions throughout), NL (ELISA, Figure 4.3.1C). YDR (assisted with tissue preparation for Figure 4.3.4E). SEC and RK analyzed and interpreted the results. JT and MS provided administrative, technical and material support. RK was the primary supervisor of this work.

4.1 Introduction and Rationale

MSA is hallmarked by accumulation of α -syn in OLs, in glial cytoplasmic inclusions (GCIs), resulting in demyelination and subsequent degeneration of axons and neurons. Although MSA is considered a synucleinopathy and its primary pathology is thought to be the presence of insoluble α -syn aggregates in OLs, little is known about the source of α -syn or the mechanism driving its dysregulation. Additionally, the function of endogenous α -syn in a healthy OL is not yet clear. This is perhaps unsurprising given the controversial findings over the years surrounding endogenous expression of α -syn in OLs. The conclusion that OLs do not express α -syn was reached in two separate reports based on the inability to detect *SNCA* mRNA transcripts in OLs from human tissue, including MSA patient tissue (Miller et al., 2005; Solano, Miller, Augood, Young, & Penney, 2000). This remains a commonly held belief in the field, despite a significant body of evidence to support the expression of α -syn in OLs, at both the gene and protein level (Asi et al., 2014; Djelloul et al., 2015; Kaji et al., 2018; Mori, Tanji, Yoshimoto, Takahashi, & Wakabayashi, 2002; Richter-Landsberg, Gorath, Trojanowski, & Lee, 2000). The discrepancy regarding endogenous α -syn in OLs is important to reconcile as the dogma that OLs do not express the protein has fueled the prion-like hypothesis of α -syn transmission between cell types. While this may be correct, the logic that OLs must receive α -syn from external sources due to a lack of endogenous expression is inherently flawed if expression of α -syn can be detected in OLs themselves.

Progress is being made regarding the elucidation of α -syn function in other cell types and systems through the use of α -syn knockout models. α -syn knockout mice have been reported numerous times as appearing phenotypically similar to controls with no overt effects on reproduction, survival or basic movement (Abeliovich et al., 2000; Cabin et al., 2002). The role

of α -syn in neurons has been of particular interest due to its relatively high expression in synaptic terminals coupled with the pathological association of α -syn with the degeneration of neurons in Parkinson's disease (PD) (Clayton & George, 1999; Totterdell, Hanger, & Meredith, 2004). A number of roles have been proposed for α -syn in the neuron including regulation of synaptic vesicle endocytosis and exocytosis, maintenance of synaptic vesicle pool size, regulation of synapse size and potentially synaptic plasticity (Abeliovich et al., 2000; Ben Gedalya et al., 2009; Cabin et al., 2002; Kokhan, Afanasyeva, & Van'Kin, 2012; Murphy, Rueter, Trojanowski, & Lee, 2000; Nemani et al., 2010; Vargas et al., 2014; Vargas et al., 2017). α -syn also has hypothesized roles in the formation of SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor)-complexes, detection and maintenance of membrane curvature, dopamine synthesis and/or transport, and phospholipid membrane organization, among others (Burre, Sharma, & Sudhof, 2012; Burre et al., 2010; Madine, Doig, & Middleton, 2006; Perez et al., 2002; Westphal & Chandra, 2013). α -syn has also been implicated in immune system development by a number of groups with α -syn knockout animals exhibiting decreased numbers of mature T and B cells, and a concomitant decrease in spleen, thymus and lymph node size (Shameli et al., 2016; Tashkandi, Shameli, Harding, & Maitta, 2018; W. Xiao, Shameli, Harding, Meyerson, & Maitta, 2014). The immune cells of the CNS, microglia, are impacted by α -syn loss as well such that microglia derived from α -syn knockout animals appear to be more reactive but display decreased phagocytic ability (Austin, Floden, Murphy, & Combs, 2006).

Overexpression studies have commonly been used in an attempt to infer α -syn function. These studies have proposed a number of different functions and properties of α -syn including its ability to bind DNA and/or histones, bind certain metals and interact with the cell's autophagic machinery among others (Goers et al., 2003; Ma et al., 2014; McDowall & Brown, 2016; Tanik,

Schultheiss, Volpicelli-Daley, Brunden, & Lee, 2013; Xilouri, Vogiatzi, Vekrellis, Park, & Stefanis, 2009). However, overexpression of α -syn is more representative of a pathological synucleinopathy state, rendering it difficult to use these findings to infer physiological function. In the context of MSA, a number of groups have explored the effects of α -syn overexpression in OLs. Overexpression of α -syn in OLs *in vivo* is associated in most studies with demyelination, secondary axonal degeneration, astrogliosis and microglial activation following the deposition of α -syn aggregates, highly reminiscent of human MSA pathology (Hoffmann et al., 2019; Kahle et al., 2002; Shults et al., 2005; Stefanova et al., 2007; Yazawa et al., 2005). *In vitro*, some studies report morphological deficits and reductions in major myelin gene and protein expression levels in OLs overexpressing α -syn (Ettle et al., 2014). Perhaps more physiologically relevant to disease are those studies exploring the response of OLs treated with human α -syn pre-formed fibrils (PFFs), known to trigger α -syn aggregation. Not only was OL health and function detrimentally affected by PFF treatment and α -syn aggregate formation, but also an interesting upregulation of endogenous α -syn expression was identified in OLs following administration of PFFs (Kaji et al., 2018; Mavroei et al., 2019). Furthermore, experimentation using wildtype and α -syn knockout models indicates that endogenous expression of α -syn within OLs is required for α -syn aggregation, as well as its associated detrimental effects on OL health, following PFF treatment (Mavroei et al., 2019).

While the α -syn overexpression studies in OLs do not offer any insight into the role of endogenously expressed α -syn, they do provide an impetus for its exploration. The finding that endogenous α -syn expression is required in the OL for α -syn aggregation to occur suggests that specific deletion of endogenous α -syn in OLs may serve as a potential therapeutic avenue for MSA. Before exploring this idea further, we must first identify any physiological role for α -syn

in OLs to avoid any unintended consequences of its removal. The lack of overt phenotype in mice devoid of α -syn expression initially led to the hypothesis that the physiological roles of this protein were minimal, yet its pathological implications were significant. It is becoming increasingly evident that α -syn does in fact have functional relevance in a variety of cell types, with its loss affecting a number of vital systems and pathways. The function of α -syn in other cell types in the CNS, such as microglia and neurons, have been explored by a number of groups as discussed above, yet despite the pathological context of MSA linking α -syn to OLs, it remains to be understood what physiological role α -syn plays in OLs. Based on the deficits observed in neighbouring CNS cell types upon loss of α -syn, we hypothesized that endogenous α -syn is required for OL development and CNS myelination and that α -syn knockout mice would exhibit myelination deficits and/or impairments in the OL differentiation program.

4.2 Results

4.2.1 Mouse- and rat-derived OLs express α -syn

Due to the divisive history surrounding the detection of endogenous α -syn, we sought to determine α -syn expression levels in our rat- and mouse-derived OL primary cultures over time. Gene expression profiles for *Snca* expression throughout a time course of OL differentiation were established for both rat and mouse-derived primary cultures by qPCR (Figure 4.1A,B). We are able to detect α -syn expression in differentiating OLs in both model systems, supporting a growing body of evidence that α -syn is in fact expressed in this cell type at levels high enough for detection.

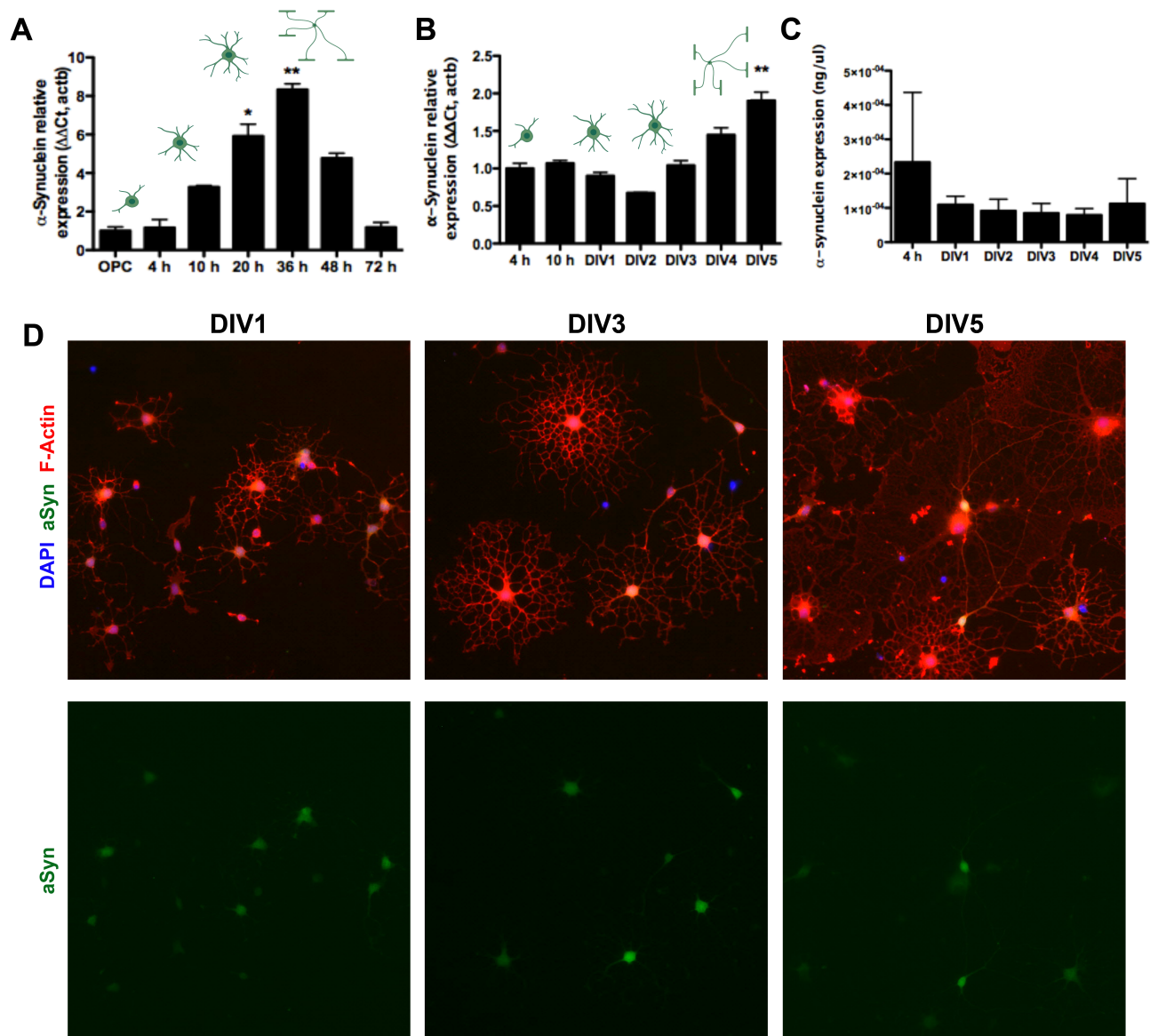


Figure 4.1. Temporal expression patterns of alpha-synuclein in differentiating OLs. (A) α -synuclein mRNA expression levels assessed by qRT-PCR in primary OLs derived from rat with morphological reference points superimposed. Expression of α -synuclein is relative to expression in OPCs. Analyzed by $\Delta\Delta Ct$, normalized to *actb*, N=3, *p < 0.05, **p < 0.01. Significance relative to OPCs. One-way ANOVA with Tukey's post-hoc (B) α -synuclein mRNA expression levels assessed by qRT-PCR in primary OLs derived from mouse with morphological reference points superimposed. Expression of α -synuclein is relative to expression at 4 h. Analyzed by

$\Delta\Delta\text{Ct}$, normalized to *actb*, N=4, $^{**}p < 0.01$. One-way ANOVA with Tukey's post-hoc.

Significance relative to 4 h. (C) α -synuclein protein expression levels assessed by ELISA in primary OLs derived from mouse. α -synuclein levels from ELISA are normalized to total protein levels from BCA assay. No significant changes were detected across the differentiation time course. Data represent the mean $\pm$ SEM, N = 4. One-way ANOVA with Tukey's post-hoc. (D) Immunofluorescence micrographs of mouse-derived OLs throughout differentiation demonstrating α -synuclein expression. Graphics superimposed (A, B) were created with BioRender.com.

Exploring the rat model system first, RNA was collected from cells maintained as OPCs prior to the induction of differentiation, as well as at six time points following the onset of differentiation (Figure 4.1A). α -syn gene expression is low in OPCs and begins to steadily increase in time following the induction of differentiation. Expression levels appear to peak somewhere between 20 and 48 h of differentiation, with the highest levels in our assay detected at 36 h post-differentiation onset. Expression then returns to levels comparable to that of the OPC by 72 h of differentiation, which is a timepoint reflecting mature, myelin producing OLs *in vitro*. This temporal pattern of expression in primary rat OLs very closely mirrors the results published in previous reports using a similar model system (Richter-Landsberg et al., 2000).

We also sought to confirm the expression profile of α -syn throughout the course of mouse-derived OLs differentiating *in vitro* (Figure 4.1B). Not only is inter-species variability in expression an interesting avenue to explore, but also it was essential that we establish a baseline for the mouse system given that the model we are using to interrogate α -syn function is an α -syn knockout mouse model. The expression of α -syn mRNA in mouse-derived OLs remains at a low level for the first three days of differentiation, only beginning to increase after four days of differentiation. The expression of α -syn continues to increase and is significantly higher at DIV5 than the basal level maintained up to and including DIV3. The time points in rat- and mouse-derived OLs cannot be compared directly with one another as the differentiation timeline *in vitro* is not identical. To this end, schematic representations of the cell's maturity at each timepoint have been superimposed on the expression profiles. Analyzing the data in this way is a more functional view as *Snca* expression can be more accurately associated with hallmarks of OL differentiation as opposed to time. Although the expression profile between species is still not identical, we see more congruence between models. Interestingly, it appears as though increasing

Snca expression is associated with time points seeing increases in mature, myelin producing OLs *in vitro*.

In addition to gene profiling, a sandwich-ELISA assay was used to detect protein levels in mouse-derived primary OLs. Mouse-derived primary OLs were again sampled daily from day zero (four hours) up to, and including, DIV5 for protein collection, as was done for the gene expression profile. Surprisingly, α -syn protein levels remained consistent across all timepoints (Figure 4.1C). Furthermore, consistent α -syn expression was detected in differentiating mouse OLs by immunofluorescence at DIV1, DIV3 and DIV5 (Figure 4.1D).

4.2.2 Investigating the role of α -syn in OL differentiation *in vitro*

Not only is it clear that OLs endogenously express α -syn as determined in the previous section (Figure 4.1), the levels of *Snca* are also variable throughout the course of differentiation with expression levels reaching their peak at timepoints that correspond to important changes in the maturation of this cell type (Figure 4.1A,B). This suggests that α -syn may be functionally relevant to the OL differentiation gamut. To investigate the role of α -syn in the differentiation process of this cell type, primary OL cultures were established from neonatal *Snca* knockout mice (*Snca*^{-/-}) and compared to cultures established from neonatal wildtype mice. As described previously, there are a number of facets that can be explored to characterize the overall ability of the OL to differentiate *in vitro* including morphological complexity, expression of major myelin proteins and production of myelin membrane.

Firstly, a valuable indicator of OL maturation is the ability of the cell to express major myelin genes and proteins. While the expression of all major myelin genes and proteins is important to gauge the health and functionality of the cell, the expression of MBP at the protein level is of particular interest in this context. MBP is known to be translated locally, requiring

efficient transport of *Mbp* transcripts to the periphery of the cell. Assessing MBP protein levels in *Snca*^{-/-} OLs would not only indicate maturity status of the cells *in vitro* but would also be an indirect indicator of the cell's ability to transport *Mbp* transcripts to the appropriate location for translation (Ainger et al., 1993). Given the known involvement of α -syn in SNARE complex formation and intracellular trafficking in other cell types, MBP expression is an important developmental milestone to assess (Burre et al., 2010; Gitler et al., 2008; Thayanidhi et al., 2010). Primary OLs from each genotype were allowed to differentiate for four days *in vitro* (DIV4), at which point they were fixed and labeled for MBP immunoreactivity. Quantification of MBP-positive OLs at this time point was completed and is represented as the percentage of OL-lineage cells (Olig2-positive) that are also MBP-positive (Figure 4.2B). In this comparison, there was no difference in the percentage of OLs expressing MBP between genotypes, indicating that *Snca*^{-/-} OLs are equally capable of expressing this major myelin protein in development.

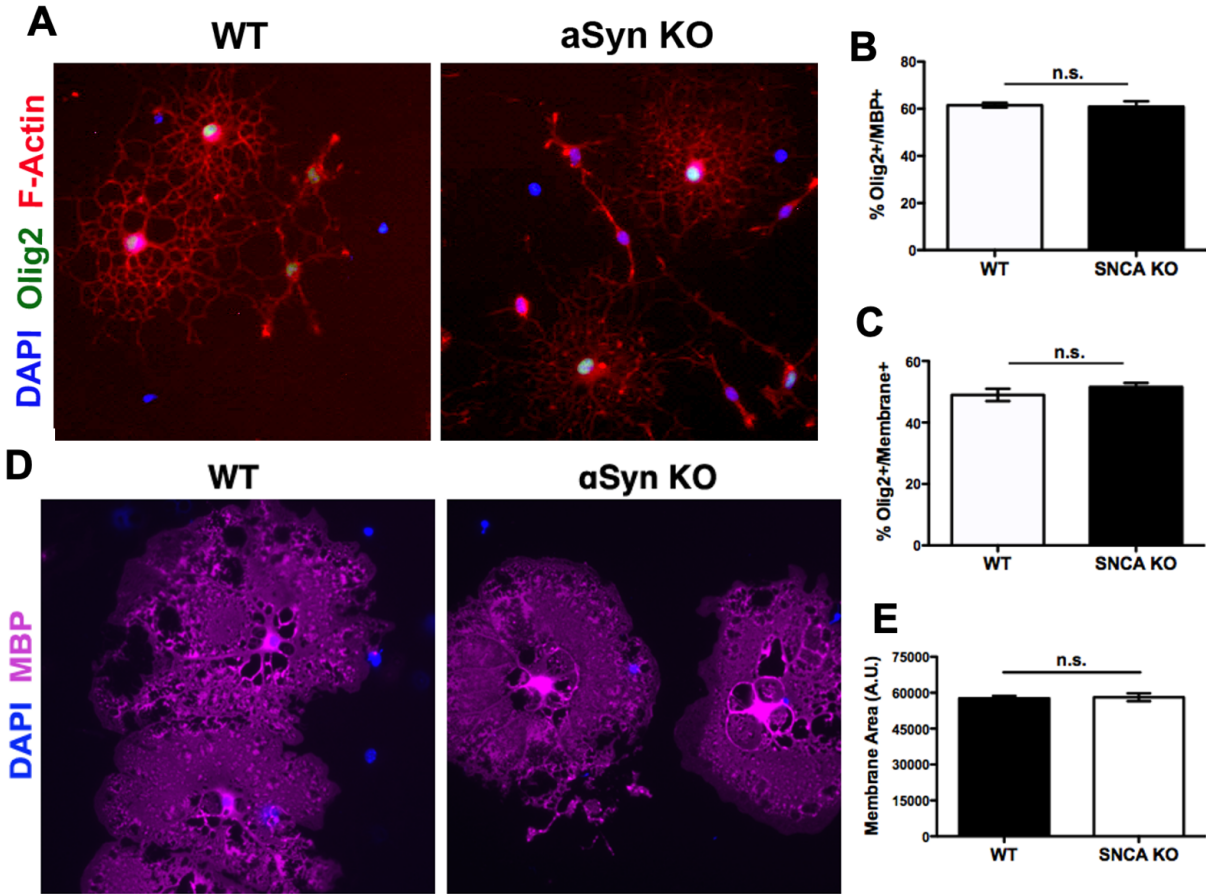


Figure 4.2 OLs lacking endogenous alpha-synuclein are capable of differentiation *in vitro*.

(A) Immunofluorescence micrographs of wildtype (WT) and *Snca*^{-/-} (aSyn KO) primary OLs fixed at DIV2. *Snca*^{-/-} OLs are evidently capable of extending processes and elaborate branching networks similar to wildtype. (B) Molecular differentiation was assessed through quantification of the number of DIV4 OLs expressing MBP (detected through immunofluorescent staining) and is represented as the percentage of Olig2-positive cells expressing MBP. (C) Morphological differentiation at DIV4 is assessed by the number of OLs producing a myelin membrane-like sheet *in vitro* and is represented as a percentage of Olig2-positive cells that are producing membrane. (D) Representative immunofluorescence micrographs of wildtype (WT) and *Snca*^{-/-} (aSyn KO) primary OLs fixed at DIV5. It is visually evident that *Snca*^{-/-} OLs are capable of

extending elaborate membranous sheets *in vitro* as mature cells, similar to that of WT. This is quantified as membrane area by genotype (E). Data represent the mean $\pm$ SEM, N=3. Unpaired, two-tailed Student's t-test, n.s. is used to indicate *not significant*.

To assess the impact of α -syn loss on OL health and function, it is also essential to characterize the morphology of the cell. Morphologically, intermediate OLs are recognized by their elaborately branched process network while more mature OLs are known for the production of myelin membrane. *In vitro*, this morphological differentiation process is similar. Intermediate OLs were qualitatively assessed from both wildtype and *Snca*^{-/-} mice. Primary OLs established from both mouse models were capable of extending processes and no overt defects in branching were detected (Figure 4.2A). We quantitatively assessed morphology in mature OLs through their ability to produce myelin sheets, as well as the extent of their elaboration. OLs cultured from both *Snca*^{-/-} and wildtype mice are equally capable of producing myelin sheets, represented as the percent of Olig2⁺ cells producing membrane at DIV4 (Figure 4.2C). Moreover, the amount of myelin produced per cell, represented as the area of the myelin sheet, at DIV5 is also unchanged between genotypes (Figure 4.2 D,E). Taken together, these findings suggest that α -syn expression in OLs is dispensable for morphological development.

4.2.3 Transcriptomic analysis reveals little impact of α -syn loss in OLs

Thus far, the results indicate that α -syn is not required for the execution of the OL differentiation program *in vitro*. To identify pathways in the cell that may be impacted by the loss of α -syn, or compensatory changes following its loss, we applied whole transcriptome analysis using RNA-seq in primary OLs derived from either wildtype or *Snca*^{-/-} mice. Based on the α -syn gene expression data presented previously (Figure 4.1B), RNA-seq was conducted on OLs cultured in differentiation media for five days. All samples and runs in this experiment passed necessary quality control measures such that all samples were included in the analysis.

Unexpectedly, very few differentially expressed genes (DEGs) were identified between the two genotypes in this analysis. Collectively, 16 DEGs were identified that met the cut off

criteria of a log₂ fold change greater than or equal to 1.5 and $p > 0.05$ (Figure 4.3A). Of these, seven DEGs were annotated genes that have not yet been given canonical names and their functions remain unknown as of yet. The remaining nine DEGs consisted of three upregulated genes in *Snca*^{-/-} OLs relative to wildtype and six downregulated genes (Figure 4.3B, C). Those upregulated in *Snca*^{-/-} OLs were *Mmrn1*, *Sh2d6* and *Trim43a* and the downregulated were *Snca*, *Ppp1r17*, *Cybb*, *Itprid1*, *Ripk* and *Cd84*. The strong decrease in *Snca* detected in this assay was expected and provides validation of the *Snca*^{-/-} mouse model as this model was created through excision of exons four and five as opposed to full gene deletion (Cabin et al., 2002).

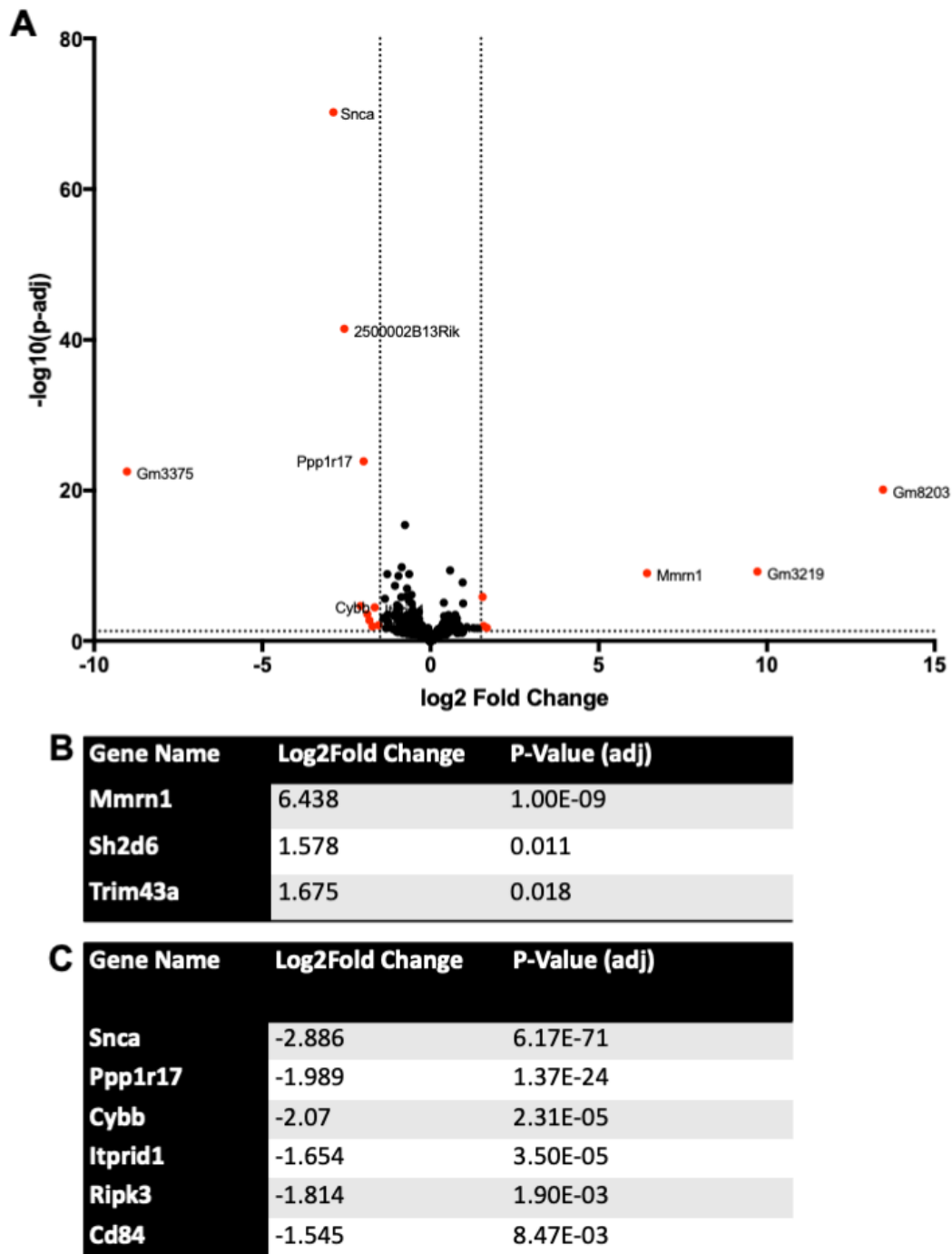


Figure 4.3 RNA-seq reveals a surprisingly small number of gene expression differences between wildtype and *Snca*^{-/-} DIV5 OLs. (A) Volcano plot presentation of identified transcripts by RNA-sequencing conducted on primary mouse OLs collected from *Snca*^{-/-} mice and

differentiated to day 5 (DIV5) compared to those at the same timepoint collected from wildtype mice. Plot represents log2 fold change vs -log10 p-value and cutoff lines represent $p < 0.05$ and log2 Fold change $> 1.5x$. Fold change calculated with DESeq2, p-values adjusted using Benjamini-Hochberg method. Genes exceeding these cutoff criteria in *Snca*^{-/-} OLs relative to wildtype are highlighted in red. N = 3 for each group. **(B, C)** All differentially expressed genes with known canonical names in the literature listed with those genes upregulated in *Snca*^{-/-} OLs **(B)** separated from downregulated genes **(C)**.

4.2.4 Global knockout of α -syn does not impair CNS myelination *in vivo*

The data presented thus far clearly demonstrate that the loss of α -syn in a primary OL culture model has limited impact on the cells and their function. However, the mouse model that we have obtained is a global α -syn knockout and effects of α -syn loss have been reported in other CNS cell types such as microglia, astrocytes and neurons (Abeliovich et al., 2000; Austin et al., 2006; Cabin et al., 2002; Castagnet, Golovko, Barceló-Coblijn, Nussbaum, & Murphy, 2005). It is well accepted that neighbouring cell types can have tremendous impacts on OL differentiation and signalling, as well as the process of myelination. Therefore, it was of interest to explore the effect of global α -syn loss on CNS myelination *in vivo*. The expression of α -syn in the CNS is not uniform and enrichment of this protein in a number of regions has been identified, including the striatum, hippocampus, thalamus, substantia nigra and, to some extent, the cerebral cortex (Li, Henning Jensen, & Dahlström, 2002). Moreover, there is variability in *Snca* gene expression among OL populations within the mouse CNS as shown in single-cell RNA sequencing (scRNA-seq) studies. Interestingly, many of the subpopulations of OLs in the CNS as determined by such studies do appear to segregate spatially to some extent, though certainly with overlap. *Snca* was detected in all subpopulations identified. However, expression was most enriched in a particular mature OL population which was found to populate the substantia nigra ventral tegmental area (SN-VTA), followed closely by the dorsal horn of the spinal cord and the striatum (Marques et al., 2016). This regional heterogeneity prompted the consideration of brain region when exploring the effects of α -syn loss on CNS myelination *in vivo*.

Myelin-specific protein expression is often used as a metric to infer *in vivo* myelination status as differences in these markers, such as MBP, are able to reflect not only the presence or absence of the OL but are also proportional to the amount of myelin membrane produced. To this

end, whole brain tissue was collected from wildtype and *Snca*^{-/-} mice at various ages (P15, P21, 6 weeks) and western blots for MBP protein were completed. Combining forebrain and midbrain tissue together to achieve a broad view of MBP levels in the brain revealed no difference at any age sampled (Figure 4.4A). Further separation of brain regions, including assessment of the metencephalon, the telencephalon and then the combined diencephalon and mesencephalon revealed no difference in MBP expression at all ages sampled (Figure 4.4 B,C,D). Together, analyses on all described brain regions and age divisions revealed no significant alterations to MBP levels in *Snca*^{-/-} mice.

While proteomic analysis by western blot is an efficient metric to capture the general state of myelin-protein expression, the ability to discern regional impacts of α -syn loss on myelination requires a much more discriminatory dissection than the crude separation done here. To this end, immunofluorescent labeling of MBP in sagittal whole brain sections from wildtype mice were compared to *Snca*^{-/-} mice (Figure 4.4E). Qualitative analysis of these sections indicates that MBP expression is unchanged throughout the brain, even in regions where α -syn expression is known to be high.

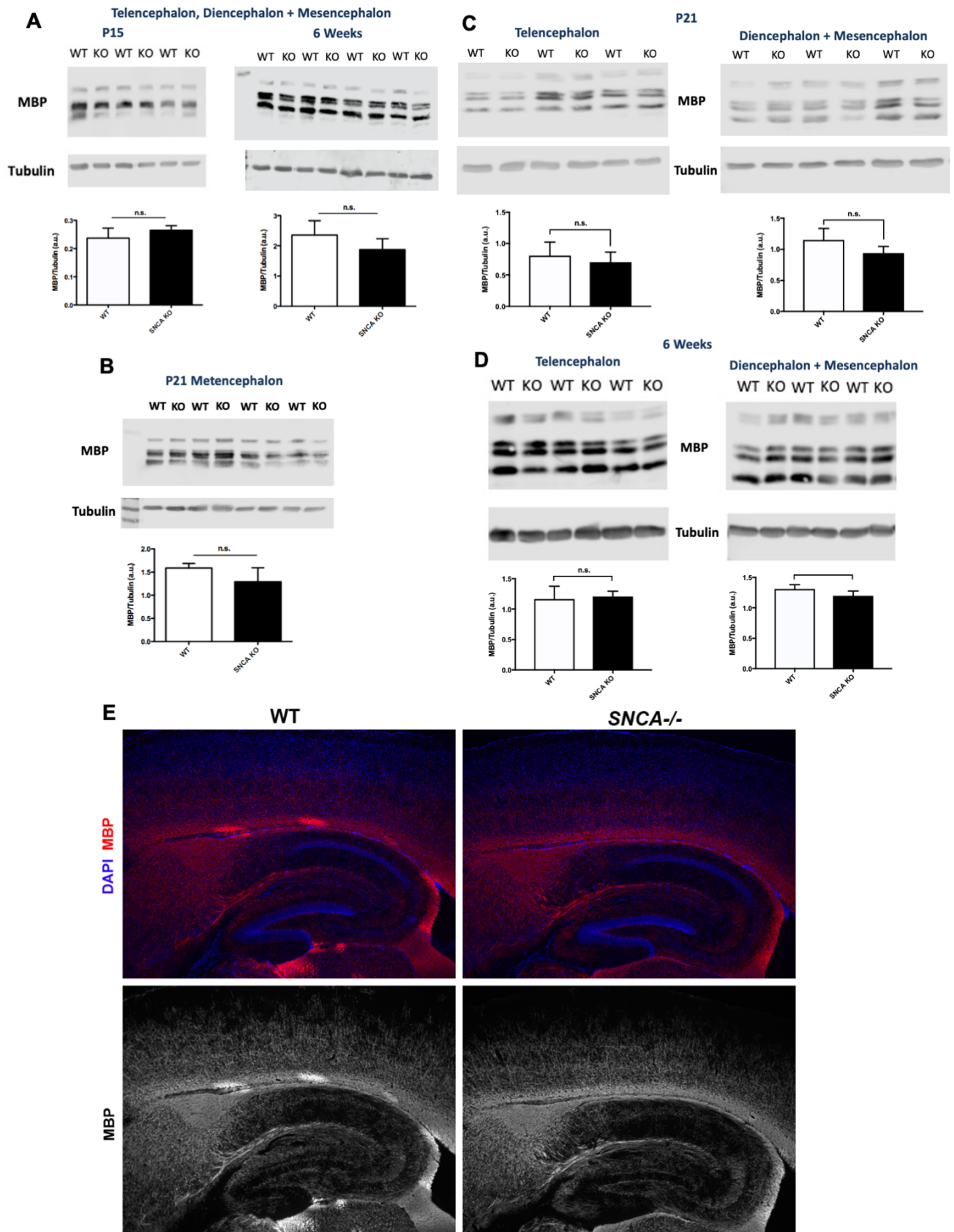


Figure 4.4 CNS myelination *in vivo* is unaltered by global alpha-synuclein knockout.

Representative western blots and associated quantification of MBP expression (normalized to alpha-tubulin) in CNS tissue collected from wildtype and *Snca*^{-/-} mice at various ages (**A-D**). Combined samples of telencephalon, mesencephalon and diencephalon were assessed for mice aged P15 and 6 weeks (**A**), while further separation of telencephalon from the mesencephalon and diencephalon were completed for mice aged P21 (**C**) and 6 weeks (**D**). Metencephalon tissue was analyzed from P21 animals only (**B**). MBP was not significantly altered in *Snca*^{-/-} tissue for any of these subdivisions or time points. Data represent the mean +/- SEM. Unpaired two-tailed Student's *t*-test; n.s. indicates no significant difference; *N* = 3 - 6. (**E**) Representative immunofluorescence micrographs of sagittal brain sections collected from wildtype (WT) and *Snca*^{-/-} mice depict similar MBP expression and do not demonstrate any overt differences in myelination status.

4.3 Discussion of Results

α -syn is a protein of interest for OL biology due to its pathological localization to OLs in MSA. Despite a growing body of literature supporting physiological roles of α -syn in other cell types, appreciation for endogenous α -syn function in OLs outside of an MSA context is lagging. Evidence of endogenous α -syn expression in OLs now outweighs those reporting its absence, though the field remains divided on this subject (Djelloul et al., 2015; Kaji et al., 2018; Richter-Landsberg et al., 2000). In this study, we have confirmed that primary OL cultures derived from both rat and mouse express α -syn at the transcript level, and in mouse-derived OLs at the protein level. Following the confirmation that murine OLs express α -syn, we sought to investigate the functionality of this protein by assessing OL differentiation and viability *in vitro* when α -syn is absent. These experiments focused on investigating maturation of *Snca*^{-/-} OLs, both molecular and morphological, in culture. Remarkably, OLs do not require α -syn to achieve all major developmental milestones assayed including the expression of major myelin proteins as well as the formation of highly branched networks followed by production of myelin sheets. Furthermore, transcriptomic analysis uncovered surprisingly few DEGs between wildtype and *Snca*^{-/-} OLs *in vitro*. Finally, *in vivo* analysis of MBP expression, indicative of myelination status, also revealed little difference between genotypes. Taken together, these findings suggest that although α -syn is expressed in OLs, it is not required for the cell to differentiate and complete myelination in a developmental context.

Given the general opinion in the field that OLs do not express α -syn endogenously, despite a body of evidence supporting its expression, our study began with confirmation of expression in our murine models. Previous studies have reported mRNA expression in primary OL cultures derived from the rat. In comparison to the data generated here, the pattern of

expression throughout differentiation in that report was nearly identical (Richter-Landsberg et al., 2000). It was important that we determine the α -syn expression profile in mouse-derived OLs, as this has not been reported previously and our study takes advantage of an α -syn knockout mouse model. In mouse OLs, α -syn expression remained low before increasing at late stages of differentiation. One could speculate that this pattern is simply delayed relative to the rat, and an extension of the time course would reveal an eventual drop in expression. It is equally plausible that the mouse system is sufficiently unique from the rat that a different expression pattern exists, though sampling of mouse OLs at later time points is required to compare the two. Parallels can be drawn between these expression levels in the cell to the known expression profile of α -syn at the tissue level throughout CNS development in the rodent. Although the absolute timeline of α -syn expression is dependent on the region sampled, generally the mRNA levels are low at early post-natal timepoints (P0-P2), followed by a sharp rise, a plateau, and finally a significant drop around P15, which results in low levels of the transcript maintained throughout adulthood (Kholodilov et al., 2002; Petersen, Olesen, & Mikkelsen, 1999). The mRNA expression time course produced in our study, particularly that of the rat-derived OLs, reflects this same pattern, albeit on a different time scale.

The most striking outcome of the current study is the resounding lack of effect of α -syn deletion in OLs. This study presents a number of experiments which serve to quantitate the ability of OLs devoid of α -syn to differentiate and develop into mature, myelin producing cells. Although these were robust metrics of maturity and cell function, it is certainly plausible that the loss of α -syn was impacting the OL in ways that could not be detected in these assays. For this reason, we conducted RNA-sequencing on *Snc α ^{-/-}* and wildtype OLs. Due to the wide array of cellular processes for which α -syn has been implicated in other cell types, it was shocking that

whole transcriptome analysis of these cells uncovered only 9 known DEGs. Though the majority of the 9 identified DEGs are remarkably disconnected from one another, three of the genes downregulated in *Snca*^{-/-} OLs (*Cybb*, *Ripk3* and *Cd84*) all have established roles in cell death and inflammation (Dragovich & Mor, 2018; Holtman et al., 2015; Kumar et al., 2016; Moriwaki & Chan, 2013; Yan et al., 2007). The expression of all three genes has been confirmed in OLs by other databases, but it is difficult to speculate on how the gene expression changes identified here may impact the OL as the literature regarding their endogenous functions in the OL itself is sparse (Marques et al., 2016). An appreciation for potential immune-related functions in the OL lineage is growing as OPCs have recently been identified as having antigen-presenting capacity. This antigen-presentation phenomenon was found in OPCs after treatment with the cytokine interferon-gamma (IFN γ) (Kirby et al., 2019). Interestingly, microarray analysis revealed that *Snca* was one of the top 250 DEGs in OPCs 24 h following IFN γ relative to control treated OPCs at the same time point. As such, the possibility that α -syn may play a role in mediating the OL's responses to immune challenges or stress should be explored further.

Perhaps even more notable are the lack of changes to certain genes in this experiment. For example, it is interesting to note that neither *Snca* nor *Sncg* are altered in the *Snca* knockout OLs. There are a number of studies suggesting a lack of phenotype following the loss of one synuclein family member can be attributed to compensation by the others (Chandra et al., 2004; Connor-Robson, Peters, Millership, Ninkina, & Buchman, 2016; Robertson et al., 2004). While still possible for either *Snca* or *Sncg* to compensate for the loss of *Snca* at their basal expression levels, compensatory functions often correlate with increased demand on a given protein and thus, increased gene expression. Therefore, the lack of significant change to either of the other synuclein genes is interesting to consider. Additionally, the fact that none of the major myelin

genes were differentially expressed in knockout OLs compared to wildtype corroborates the phenotypic characterization of these OLs. These data collectively support the notion that α -syn is dispensable in isolated OLs developing in steady-state conditions, without challenge or stress.

It is particularly interesting, based on the literature, that OL morphology appears normal in *Snca*^{-/-} OLs *in vitro*. Connections between α -syn's known binding partners in other cell types and their function in OLs supported the hypothesis that α -syn loss may impair process extension. Experiments on the neuron have identified that α -syn is involved in SNARE complex formation (Burre et al., 2010). In the neuronal context, SNARE complexes are essential for synaptic vesicle docking and membrane-fusion, permitting neurotransmitter release into the synaptic cleft (J. Sun et al., 2019; C. Wang et al., 2016). The SNARE complex linked to this function in neurons is comprised of the proteins synaptosomal-associated protein 25kDa (SNAP25), syntaxin and vesicle-associated membrane protein 2 (VAMP-2), and α -syn has been shown to bind directly to the N-terminus of VAMP-2 (Burre et al., 2010; J. Sun et al., 2019; Weber et al., 1998). VAMP-2 in the context of OL biology appears to play an important role, though not one that mirrors its function in the neuron. VAMP-2 is a known cargo of myosin Va, and both proteins are required for the OL to morphologically differentiate as expected (Sloane & Vartanian, 2007). When VAMP-2 is unable to reach the distal tips of the OL growing processes, either as a result of myosin Va knockout or the deletion of VAMP-2 itself, OLs present with stunted morphologies and their growth cones exhibit smaller lamellae. The sparing of OL morphological differentiation in our α -syn knockout experiments (Figure 4.2) informs us that despite α -syn interacting with VAMP-2 in other systems, it is not required and likely not involved in essential VAMP-2-dependent OL branching and growth.

While the results of this study suggest that α -syn is not required for OL differentiation *in vitro* or developmental myelination *in vivo*, it is not clear whether α -syn would play a functional role in the OL during demyelination and remyelination. A recent study investigated the impact of α -syn loss on two models in which demyelination occurs: cuprizone-induced demyelination and experimental autoimmune encephalomyelitis (EAE) (Kuhbandner et al., 2020). These models both result in demyelination, though cuprizone does so by targeting the OL itself while EAE produces an immune response towards OLs resulting in their destruction. Kuhbandner and colleagues determined that *Snca*^{-/-} mice were less severely impacted by induction of EAE than wildtype littermates, but both genotypes responded equally to the cuprizone model. As these were both conducted in global α -syn knockout models, as opposed to cell-specific, these findings are likely less indicative of an inherent resistance to EAE in OLs caused by α -syn deletion and rather represent differences in the peripheral immune response which is ultimately responsible for inducing demyelination. It remains to be determined whether α -syn-null OLs have inherently different responses to immune mediators than wildtype OLs. This is an interesting prospect to consider though, especially given the few DEGs identified in our transcriptomic analysis that seem to be related to the immune response. Additionally, it should be noted that the cuprizone model also allows for measurement of remyelination, or repair, following the toxin-induced demyelination. α -syn knockout animals appeared equally capable of remyelination following cuprizone removal, which not only supports our findings that CNS myelination is spared, but also adds an additional dimension as remyelination and developmental myelination have unique considerations.

Thus far, the theme of this work is a conflict between a considerable amount of evidence from the literature suggesting the plausibility for α -syn to play an important role in at least

certain OL populations, and an apparent lack of phenotype upon its loss. The broader α -syn community is also plagued by similar contradictions. The consensus is that α -syn knockout mice generally appear phenotypically normal, initially suggesting a relatively niche role for α -syn in general physiology. Neurons from *Snc α ^{-/-}* mice, whether *in vitro* or *in vivo*, appear largely functional and viable with subtle defects detectable during certain assays (Abeliovich et al., 2000; Cabin et al., 2002; Robertson et al., 2004). However, knockdown of α -syn in mature neurons results in more dramatic effects (Benskey et al., 2018; Gorbatyuk et al., 2010; Khodr et al., 2011; Murphy et al., 2000). This is intriguing as one would expect a knockout model, where α -syn is completely absent throughout development as well as throughout maturity, to have an exacerbated phenotype relative to a model in which α -syn expression is knocked down in mature cells only. This is suggestive either of a role for α -syn in maintenance of mature cells, or that α -syn's functions can be compensated for when lost early in development but that these compensatory changes are somehow unable to support the cell when α -syn is lost in mature stages. Regardless, this is an important consideration for the present study, given the lack of phenotype in OLs developing in the absence of α -syn. It would be of great interest to investigate the impact of a conditional knockout model that would selectively remove α -syn from mature OLs in adult mice to better understand the parallels between α -syn function, or lack thereof, in neurons and OLs. It is also an important experiment to conduct due to the potential therapeutic relevance of the current study. Pre-formed fibrils of α -syn can trigger α -syn aggregation in OLs, reminiscent of what can be seen in an MSA context, but endogenous α -syn in the OL is required for this process (Kaji et al., 2018; Mavroei et al., 2019). This is indicated by the finding that *Snc α ^{-/-}* OLs treated with pre-formed fibrils do not contain aggregates, while wild type or α -syn-overexpressing OLs do (Mavroei et al., 2019). This poses a very intriguing therapeutic avenue

to mitigate the formation of GCIs in MSA OLs – remove endogenous α -syn from OLs. However, this is only a viable option if OLs would be unaffected by the loss of α -syn itself. Though the results of our study indicate that this may indeed be the case, it is essential to explore whether mature OLs are impacted by the removal of α -syn in adulthood.

Taken together, the findings presented in this chapter support an alternative hypothesis than initially proposed; α -syn does not seem to be required for the execution of the standard OL differentiation and developmental CNS myelination programs. Thus far, these findings provide interesting insight into differences in functionality for this protein between the neuron and OL, including the implications addressed regarding SNARE-complex formation and trafficking systems. Additionally, this study provokes thought on removing endogenous α -syn from OLs as a potential therapeutic avenue in MSA to slow the accumulation of α -syn aggregates. However, future studies are first required to explore the prospective function of α -syn under conditions of stress and immune challenge. Importantly, the effects of α -syn removal in mature, adult OLs should also be explored to gain a better understanding of any deleterious impacts of manipulating endogenous α -syn as a therapeutic strategy.

**Chapter 5: Chondroitin Sulfate Proteoglycans *In Vitro* Provide a Model to Explore OL
Process Retraction and Cytoskeletal Responses but are Not a Suitable Model for Drug
Target Screening**

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Author Contributions

Conceived and designed experiments: SEC, RK. Performed experiments: SEC, with the following assistance and collaborative contributions – SK (Figure 5.1C) as well as assistance in cell culture, YDR (assisted with tissue preparation for Figure 5.5A). SEC and RK analyzed and interpreted the results. RK was the primary supervisor of this work.

5.1 Introduction and Rationale

Multiple sclerosis (MS) is characterized by immune-mediated loss of the myelin sheath normally surrounding axons. OPCs are highly capable of repairing any damage incurred to myelin in a healthy individual, as well as those with RRMS (Goldschmidt, Antel, Konig, Bruck, & Kuhlmann, 2009; Patrikios et al., 2006). This process includes the migration of OPCs to the site of demyelination or lesion site in MS, followed by differentiation into mature OLs and, finally, wrapping the denuded axon. This process is referred to as remyelination and although the myelin sheath produced in this repair is thinner than developmental myelin, it is sufficient to result in functional recovery (Bramow et al., 2010; I. D. Duncan et al., 2009; Mei et al., 2016). In stark contrast, progressive forms of MS (PPMS and SPMS) exhibit a striking reduction in the capacity for repair at the lesion site (Patrikios et al., 2006). A number of groups have identified the presence of OPCs in and around those lesions that remain demyelinated, suggesting that the inability to repair is less likely the result of a depleted pool of precursor cells or a migration defect, but rather impedance in the differentiation of these OPCs into mature OLs (Chang et al., 2002; Wolswijk, 1998).

In MS, lesions that are resistant to remyelination are associated with the accumulation of a number of factors that are inhibitory to OL development as well as the process of myelination; this includes the presence of dysregulated miRs, myelin debris, netrin-1 and CSPGs, among others (Bin et al., 2013; Clarner et al., 2012; Galloway, Gowing, Setayeshgar, & Kothary, 2020; Junker et al., 2009; Lau et al., 2012; Sobel & Ahmed, 2001). The inhibitory milieu of the MS lesion is thought to be a significant contributor to the aforementioned OL differentiation block and subsequent lack of repair. CSPGs are components of the ECM that are ubiquitously expressed, with the lectican subfamily of CSPGs (aggrecan, versican, neurocan and brevican)

most prevalent in the CNS (Ruoslahti, 1996; Yamaguchi, 2000). They play important roles during CNS development but are detrimental to repair when upregulated in both an MS lesion context, as well as in spinal cord injury (SCI) (Bradbury et al., 2002; Golding et al., 1999; L. L. Jones, R. U. Margolis, & M. H. Tuszynski, 2003; Lau et al., 2012; Masuda et al., 2004). Most notably, CSPGs inhibit OL branching, thus limiting its ability to extend a process towards a denuded axon for myelination (Lau et al., 2012; Siebert & Osterhout, 2011; Y. Sun et al., 2017). Investigation of the CSPG-OL dynamic has led to the identification of protein tyrosine phosphatase sigma ($PTP\sigma$) as a cell surface receptor involved in mediating the CSPG signal in OLs (Pendleton et al., 2013). Furthermore, inhibition of rho-associated kinase (ROCK) has been associated with improvements in process extension and branching in OLs on CSPGs *in vitro*, possibly representative of a role for ROCK in mediating the inhibitory CSPG signal in OLs (Kippert et al., 2009; Pendleton et al., 2013). Interestingly, neurons respond to CSPGs *in vitro* and *in vivo* with similar morphological defects (McKeon, Schreiber, Rudge, & Silver, 1991; Snow, Lemmon, Carrino, Caplan, & Silver, 1990). This is perhaps unsurprising given the many similarities between neuron and OL growth cones, or growing tips, in terms of cytoskeletal composition. The CSPG-neuron relationship has been explored fairly extensively because of the applicability to spinal cord injury (SCI) and the accumulation of CSPGs at the injury site (L. Jones, R. Margolis, & M. Tuszynski, 2003). In the neuron, $PTP\sigma$ has also been identified as a receptor for CSPGs similar to the OL context, though additional receptors have been identified in the neuron as well including leukocyte common antigen-related receptor, Nogo1 and Nogo3 (Dickendesher et al., 2012; Fisher et al., 2011; Shen et al., 2009). Neuronal systems have also implicated a number of signaling changes associated with CSPG exposure including the inactivation of Akt kinase (Akt) and the activation of protein kinase C (PKC), GSK3 β and RhoA

(Dill, Wang, Zhou, & Li, 2008; Fisher et al., 2011; Jain, Brady-Kalnay, & Bellamkonda, 2004; Monnier, Sierra, Schwab, Henke-Fahle, & Mueller, 2003; Sivasankaran et al., 2004). Further understanding of the mechanisms driving CSPG-mediated inhibition of OL differentiation is necessary as this may lead to the identification of therapeutic avenues for encouraging OLs to differentiate in the inhibitory lesion microenvironment.

Current therapeutic strategies for MS are limited to those that target the autoimmune aspect of the disease. These strategies are effective in many, but not all, patients with relapsing-remitting forms of the disease, but have little benefit to those with progressive MS (Antel et al., 2012). Alternatively, many groups are working towards the goal of identifying strategies to overcome the aforementioned OPC differentiation block in hopes of encouraging endogenous OPCs to remyelinate. High-throughput screening platforms have identified a number of candidates with the potential to promote remyelination in the context of MS (Buckley et al., 2010; Deshmukh et al., 2013; Lariosa-Willingham et al., 2016; Mei et al., 2014; Peppard et al., 2015). Unfortunately, success in their translation to clinical use remains limited. In part, this is due to the limitations of the pre-clinical model systems available. Experimental autoimmune encephalomyelitis (EAE) has classically been considered the standard model for MS, as it recapitulates elements of the disease's immunology and has been an essential model for the development of immunomodulatory therapies (Baker & Amor, 2014). Its utility is limited to that of immunological exploration as the areas of demyelination, or formation of lesions, in this model are not highly characteristic of what we know to be true of MS (Behan & Chaudhuri, 2014). For the study of remyelination potential, a number of demyelination models have been developed including lysophosphatidylcholine (LPC), ethidium bromide (EtBr) or acute cuprizone-induced demyelination (Graca & Blakemore, 1986; Hall, 1972; Ludwin, 1978;

Woodruff & Franklin, 1999). In all of these models, a demyelination event is followed by a spontaneous recovery by means of remyelination once the toxin is removed. This spontaneous recovery is one feature that remains to distance these models from an accurate depiction of an MS lesion, particularly those that are resistant to remyelination – the ultimate targets of regenerative therapies. Additionally, the dysregulated microenvironment of the lesion is not well recapitulated in these models. One step closer to emulating the environment of lesions resistant to remyelination is the chronic cuprizone model. The copper chelator, cuprizone, is administered in the same way as the classic acute model, but for an extended time course of 12 weeks compared to the four- to six-week time course typically seen. Not only is remyelination incomplete in this model following removal of cuprizone, but also demyelinated regions are burdened with astrogliosis, deposition of ECM components including CSPGs, and dysregulation of miRs (Armstrong, Le, Flint, Vana, & Zhou, 2006; Hibbits et al., 2012; Kornfeld, Unpublished; Mason et al., 2004).

The importance of model selection and consideration of the inhibitory MS lesion environment is exemplified by the history of LINGO-1 antibody therapy - the first remyelination therapy put forward for clinical trials on human MS patients. Extensive pre-clinical testing demonstrated that anti-LINGO-1 was able to improve OL differentiation *in vitro*, as well as remyelination in select *in vivo* models including EAE, EtBr and LPC (Mi et al., 2007; Mi et al., 2005; Mi et al., 2009; Yongjie Zhang et al., 2015). This drug ultimately failed to meet its primary endpoints in clinical trial, indicating that it is not sufficient to promote remyelination in a human disease context (Cadavid et al., 2017). This unfortunate clinical trial failure can be explained in part by the fact that pre-clinical testing of this drug was conducted using experimental paradigms that do not accurately represent the lesion environment in MS. Recently, Keough and colleagues

sought to address this disconnect by testing top candidates from the latest pro-myelination screens for their ability to promote OL differentiation in the presence of inhibitory CSPGs *in vitro* (Keough et al., 2016). All of these compounds, as well as 245 Health Canada-approved compounds, were shown to have little to no benefit in this context. It is possible that these findings indicate that none of these compounds would be suitable for repair in MS as it suggests an inability to promote OL differentiation in the face of a prevalent inhibitory cue. Alternatively, the *in vitro* CSPG model may be a poor system to assess the *in vivo* applicability of potential pro-myelinating compounds. This notion is supported by the discrepancy between the inability of clemastine to overcome CSPG-mediated inhibition *in vitro*, while demonstrating early promise of remyelination in the human context from phase two clinical trial data in MS (Green et al., 2017; Keough et al., 2016). Based on this dichotomy, and the importance of not discounting potentially viable therapeutic targets, we sought to further explore the standard *in vitro* CSPG model and determine if it represents a suitable means of identifying therapeutic targets.

5.2 Results

5.2.1 Validation of CSPG effects in mouse and rat OL primary cultures

The inhibitory effects of CSPGs on various aspects of OL maturation have been reported previously (Lau et al., 2012; Siebert & Osterhout, 2011). We first sought to validate the effects of CSPGs in our primary OL culture system. OL cultures were derived from neonatal mice or rats, seeded on either a mixed ECM control substrate or a combination of mixed ECM with CSPGs and allowed to differentiate. We have recapitulated the inhibitory effects of CSPG exposure in mouse-derived OLs whereby molecular maturation appeared largely unaffected, but cell branching and myelin membrane production were significantly stunted (Figure 5.1 A, B, E, F). It is important to note that OLs allowed to differentiate on CSPGs for a number of days are

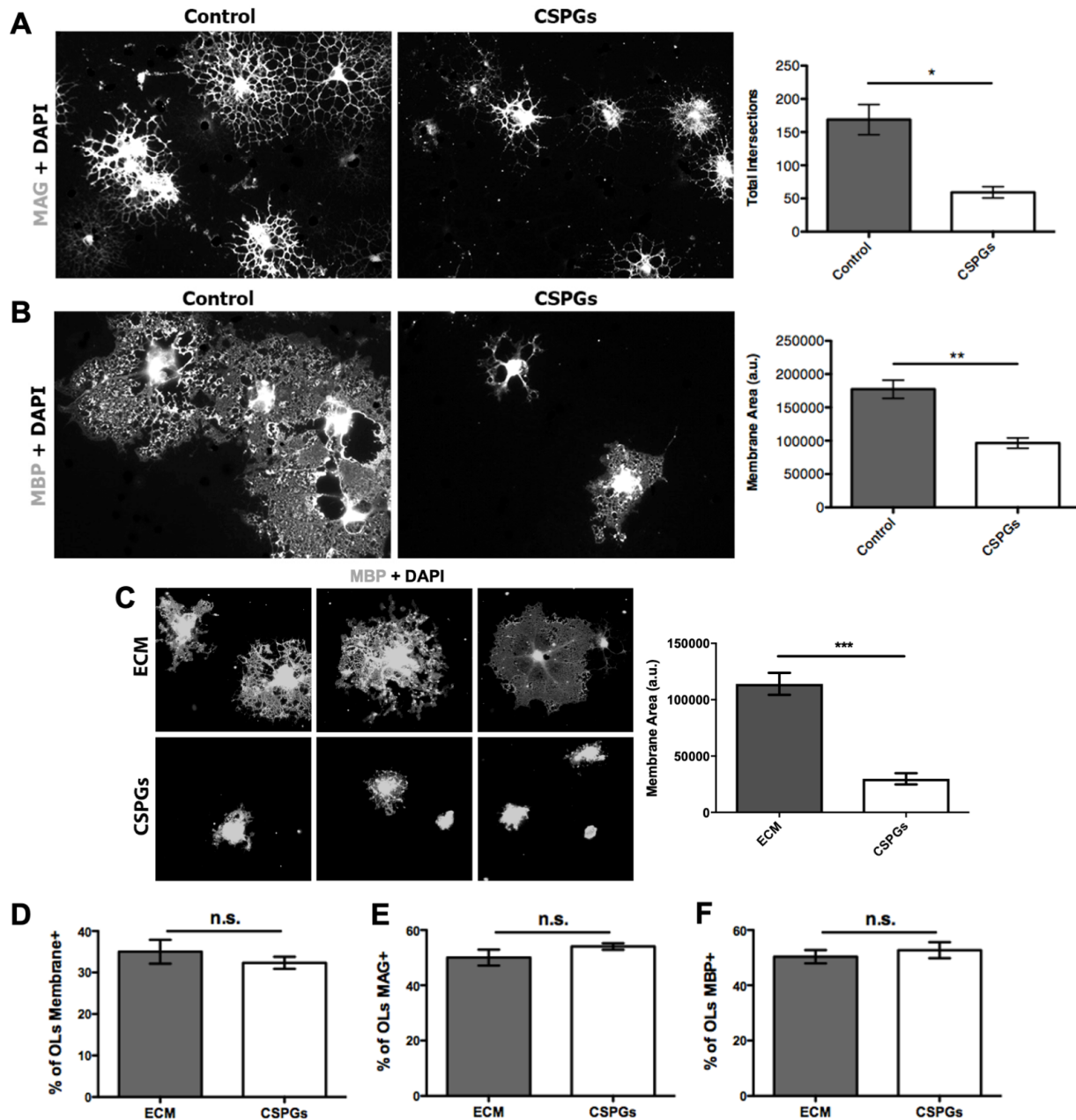


Figure 5.1 Exposure to CSPGs impairs OL morphological differentiation (branching and myelin sheet elaboration) but does not impact their ability to progress through molecular differentiation milestones. (A) Immunofluorescence micrographs of DIV3 mouse-derived OLs demonstrating the effects of CSPGs on process extension. Quantification of DIV3 MAG+ OLs by Sholl analysis; Concentric rings overlaid 10 μ m apart; Number of OL process intersections

per ring quantified. Data represents the sum of intersections across all rings per OL and is reduced in the CSPG-exposed condition relative to control ECM substrate. **(B)**

Immunofluorescence micrographs of DIV5 mouse-derived OLs demonstrating the effects of CSPGs on myelin membrane production. Membrane area quantified by tracing the outline of membrane-producing OLs at DIV5. Membrane area is represented as arbitrary units. **(C)** Effects of CSPGs on rat-derived OLs are apparent as well, depicted in immunofluorescence micrographs of DIV5 rat-derived OLs. Membrane area quantification reveals a significant reduction in myelin membrane area from rat-derived OLs on CSPGs relative to control ECM substrate. **(D)** The proportion of OLs producing myelin membrane (initiation of myelination) at DIV5 was not affected by CSPG exposure as no difference was detected by this metric between ECM control and CSPG-differentiated OLs. **(E, F)** The ability to progress through the molecular differentiation paradigm was assessed by the percentage of OLs expressing myelin-associated glycoprotein at DIV3 **(E)** and the percentage of OLs expressing myelin-basic protein at DIV5 **(F)**. Data represent the mean $\pm$ SEM, N=3. Unpaired two-tailed Student's *t*-test; n.s.= not significant, * $p < 0.05$, ** $p < 0.01$.

capable of producing myelin sheets in culture, though the size of these sheets per cell is significantly reduced (Figure 5.1 D, E, F). We also confirmed that rat-derived OL cultures would respond similarly to this inhibitory cue, as is demonstrated by decreased membrane area at DIV5 (Figure 5.1 C). Altogether, we have validated that in our primary OL culture systems derived from both mouse and rat, the primary defect of cells grown on CSPGs is an impairment in morphological differentiation including process extension and elaboration of myelin membrane.

5.2.2 Manipulating miR-145 and miR-219 to assess *in vitro* CSPG model

MicroRNAs (miRs) are small, non-coding RNAs that play instrumental roles in many systems through post-transcriptional regulation of gene expression. A small number of miRs are currently being explored in the context of MS for their potential to promote OL differentiation and ultimately improve remyelination, namely miR-145 and miR-219 (Dugas et al., 2010; S. Kornfeld et al., 2020; H. Wang et al., 2017; Zhao et al., 2010). These two miRs have opposing effects on OL differentiation such that high levels of miR-219 and low levels of miR-145 promote OL differentiation, while the inverse expression of either miR is refractory to OL development. The therapeutic potential of both miR-219 upregulation and miR-145 knockout have been assessed individually in the *chronic cuprizone* model – the model most closely representing the microenvironmental changes seen in MS lesions resistant to remyelination, including deposition and impaired clearance of CSPGs (Hibbits et al., 2012). Either miR-219 overexpression or miR-145 deletion were associated with improvements in repair following demyelination, indicating that these manipulations in miR expression are sufficient to overcome the inhibitory lesion environment *in vivo* (H.-B. Fan et al., 2017; Kornfeld, Unpublished). We sought to investigate whether these miR manipulations in the *in vitro* CSPG model would demonstrate similar benefit, ultimately providing support for its use and applicability in

identifying compounds that could translate successfully to *in vivo* models that appropriately depict an inhibitory lesion environment.

Primary cultures were first established from miR-145 knockout mice to assess the benefit of miR-145 loss on OL differentiation on CSPGs relative to wildtype OLs. Assessment of these cultures on DIV5 reveals that the morphology of miR-145 knockout OLs is unchanged relative to wildtype. *In vitro*, OLs from both genotypes present similar reductions in MBP+ myelin membrane area when cultured on CSPGs (Figure 5.2A). Similarly, transfection of a miR219 mimic to overexpress miR-219 in primary rat OL cultures did not improve the morphological phenotype seen *in vitro* at DIV5. While the expected reduction in myelin membrane area in OLs differentiating on CSPGs was seen in the scrambled control group, membrane area was not significantly different between this group and those with miR-219 overexpression (Figure 5.2B). Validation of the upregulation of miR-219 in this system is also demonstrated (Figure 5.2C). Interestingly, exploration of miR-219 expression over time reveals that rat OLs *in vitro* exhibit higher miR-219 expression on CSPGs across all time points studied (DIV1, DIV3, DIV5) compared to those on control ECM (Figure 5.2D). This upregulation of miR-219 in a CSPG-laden environment is likely a mechanism of compensation employed by the OL in an effort to promote growth in this context. It is clear from our results that further overexpression of miR-219 does not provide additional benefit in promoting morphology of these cells. Taken together, these findings demonstrate that despite the benefits of miR manipulation in mouse models of demyelination, these same manipulations are not capable of overcoming the CSPG-mediated block in morphological differentiation *in vitro*. Given the *in vivo* experimental data on these miRs, the lack of effect of miR manipulation in these cells is perhaps less indicative of their clinical potential but rather suggests that the *in vitro* CSPG system is not representative of a

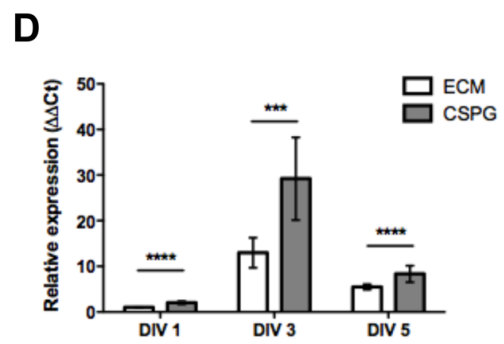
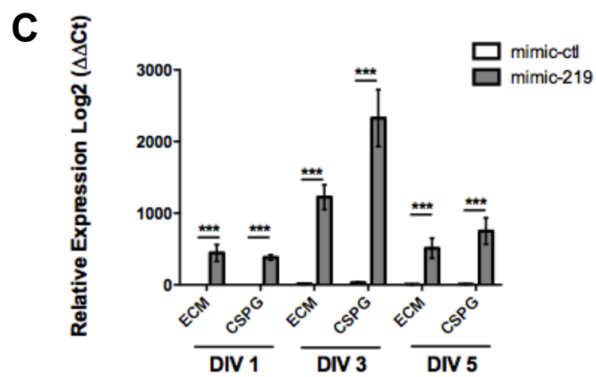
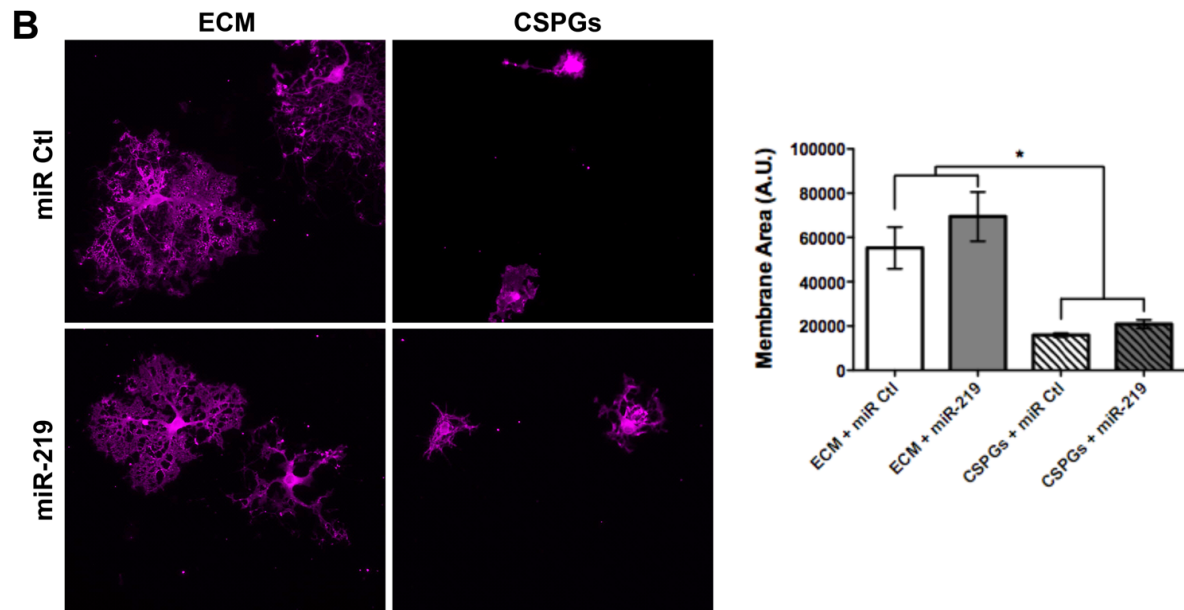
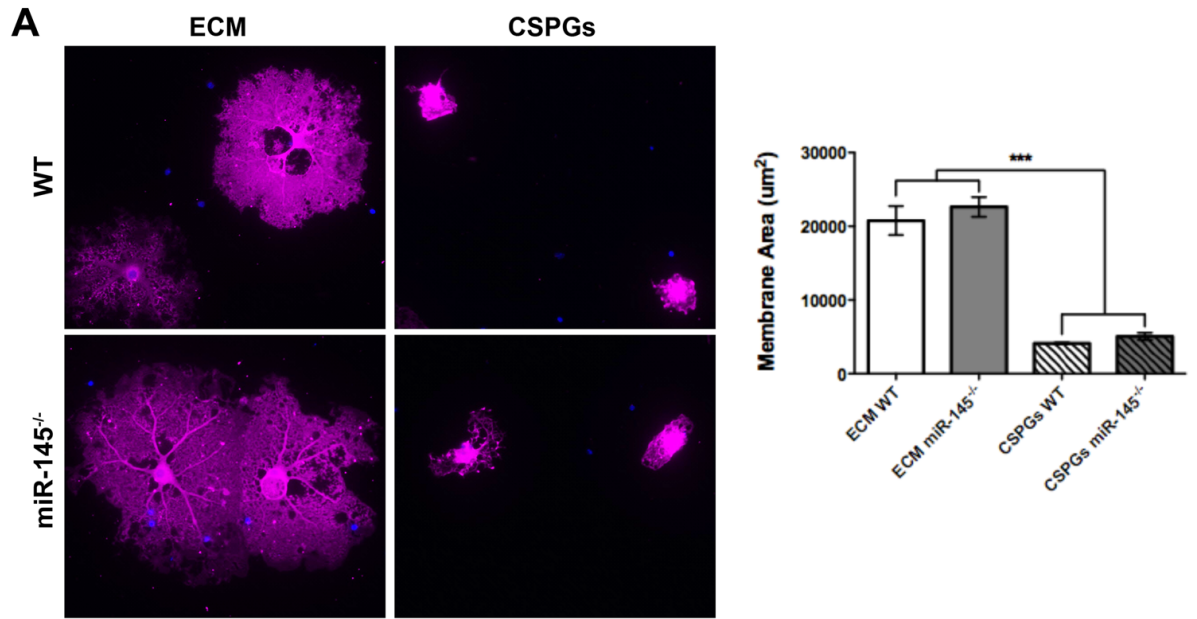


Figure 5.2 Neither inhibition of miR-145 nor overexpression of miR-219 in OLs was sufficient to improve morphological metrics on CSPGs. (A) Immunofluorescence micrographs of wildtype and miR-145 knockout OLs differentiating on CSPGs or control ECM substrate. Deletion of miR-145 is not sufficient to improve the CSPG-induced morphological impairment as quantified by myelin membrane area. Data represent the mean $\pm$ SEM, N = 3. One-way ANOVA with Tukey's post-hoc test. *** $p < 0.001$ (B) Overexpression of miR219 in rat OLs does not improve the reduction in myelin membrane area associated with CSPG exposure. Immunofluorescence micrographs depict primary rat OLs at DIV5 on ECM or CSPG substrate, transfected with miR-219 mimic or scrambled control. Quantification of myelin membrane area in graphical depiction. Data represent the mean $\pm$ SEM, N = 3. One-way ANOVA with Tukey's post-hoc test. * $p < 0.05$ (C, D) Relative expression of miR-219-5p in rat OLs by qRT-PCR using the $\Delta\Delta C_t$ method and normalized to snU6. *** $p < 0.001$, Student's t-test adjusted for multiple propagations. (C) Confirmation of mir-219-5p overexpression in transfected rat OLs on both ECM and CSPG substrates. (D) Consistent upregulation in miR-219-5p in non-transfected rat OLs differentiated on CSPGs relative to ECM at all time points assessed.

biologically relevant environment, even one with extensive production and accumulation of CSPGs.

5.2.3 Translating ROCK inhibition from *in vitro* to *in vivo* modeling

Thus far in OLs, the ROCK pathway is the only pathway whose manipulation has been shown to improve OL morphology on CSPGs *in vitro*. ROCK has also been implicated in the OL response to other cues that affect their morphology and is considered a key regulator in OL process extension and branching given its role as a cytoskeleton effector (Baer et al., 2009; Liang et al., 2004; Rajasekharan et al., 2009). In our system, in which primary OLs are exposed to a heterogeneous mixture of CSPGs, we sought to confirm the beneficial effects of ROCK inhibition on morphology. OLs were treated with a ROCK inhibitor (InSolution Rho Kinase Inhibitor, Calbiochem) on DIV1 and allowed to differentiate for an additional 24 h. As is evident from the sholl analysis quantification on DIV2 OLs, inhibition of ROCK resulted in a partial rescue in branching complexity relative to vehicle treatment (Figure 5.3).

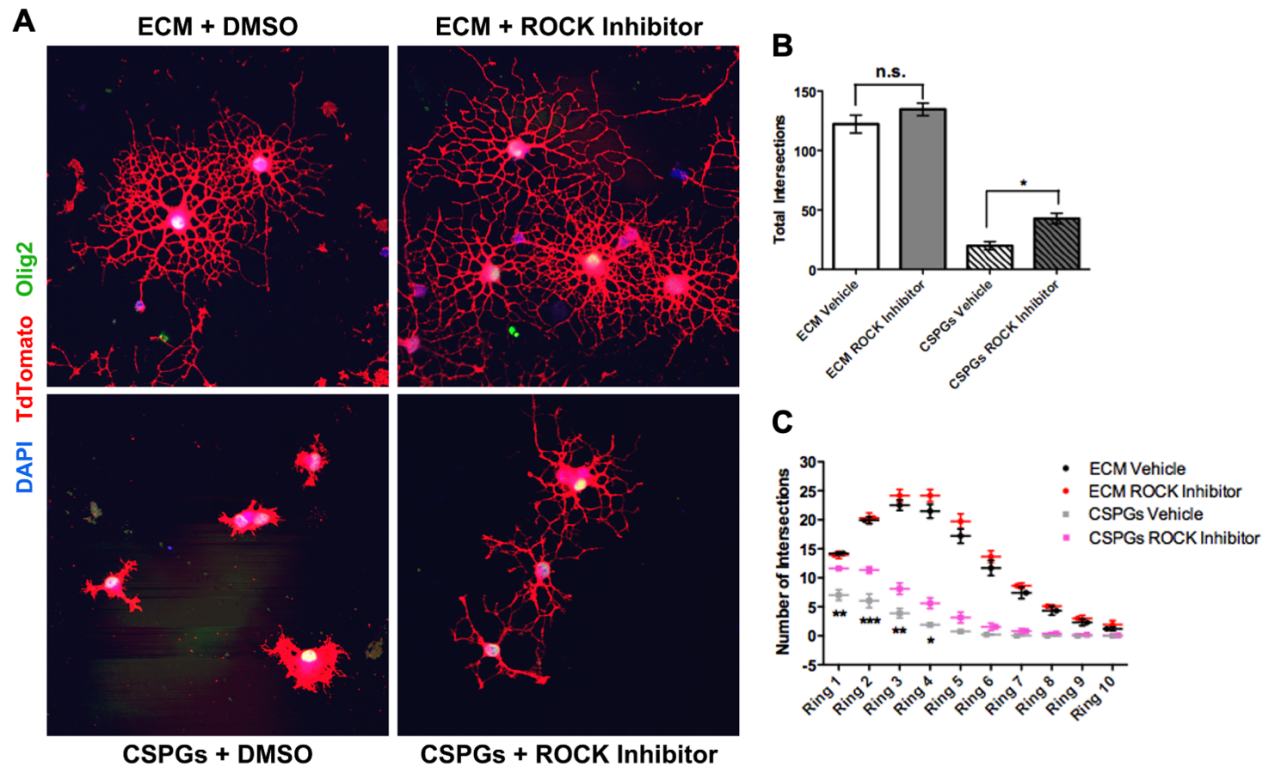


Figure 5.3 Inhibition of Rho-associated kinase (ROCK) in OLs on CSPGs improves branching measures. (A) Immunofluorescence micrographs of DIV2 OLs demonstrating the effects of CSPGs on process extension and the partial recovery induced with ROCK inhibition. (B, C) Quantification of DIV2 OLs by Sholl analysis; Concentric rings overlaid 10 μ m apart; Number of OL process intersections per ring quantified. (B) Data represents the sum of intersections across all rings per OL, and the partial rescue with the ROCK inhibitor is depicted. Data represent the mean $\pm$ SEM, N = 3. One-way ANOVA with Tukey post-hoc tests to compare drug effect within each substrate condition. *p<0.05, n.s. indicates no significant difference. (C) Data demonstrates the number of intersections with each concentric ring, with ring 1 representing the ring most proximal to the cell body while ring 10 is the most distal. Data represent the mean $\pm$ SEM, N=3. Two-way ANOVA with Bonferroni post-hoc tests. *p<0.05,

****p<0.01 and ***p<0.001.** Significance is shown for CSPG-ROCK inhibitor group relative to CSPG-Vehicle.

ROCK activation can also lead to a number of downstream changes through a chain of phosphorylation events, including the activation of NMII (Vartanian, Li, Zhao, & Stefansson, 1995). NMII inhibition has also been explored in context of CSPGs and here we sought to confirm those findings in our system (Kippert et al., 2009). Treatment with blebbistatin, an inhibitor of NMII activity, in the same treatment paradigm as the rho kinase inhibitor resulted in similar partial restoration of OL branching in the presence of CSPGs (Figure 5.4).

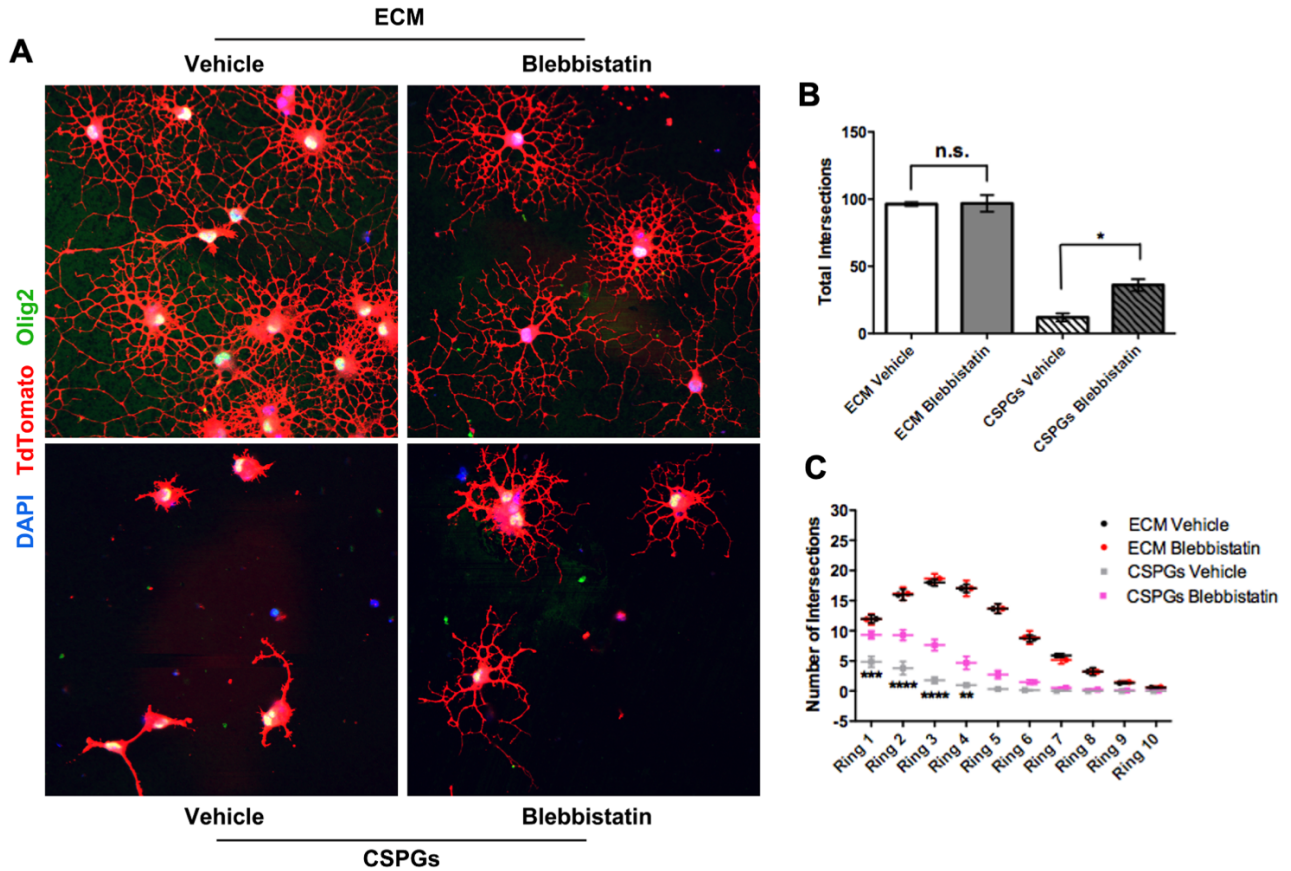


Figure 5.4 Inhibition of Non-Muscle Myosin II (NMII) with Blebbistatin improves branching measures. (A) Immunofluorescence micrographs of DIV2 OLs demonstrating the effects of CSPGs on process extension and the partial recovery of branching with NMII inhibition. (B, C) Quantification of DIV2 OLs by Sholl analysis; Concentric rings overlaid 10 μ m apart; Number of OL process intersections per ring quantified. (B) Data represents the sum of intersections across all rings per OL, and the partial rescue with Blebbistatin is depicted. Data represent the mean $\pm$ SEM, N = 3. One-way ANOVA with Tukey post-hoc tests to compare drug effect within each substrate condition. * p <0.05, n.s. indicates no significant difference. (C) Data demonstrates the number of intersections with each concentric ring. Data represent the mean $\pm$ SEM, N=3. Two-way ANOVA with Bonferroni post-hoc tests. ** p <0.01, *** p <0.001

and **** $p < 0.0001$. Significance is shown for CSPG Blebbistatin group relative to CSPG Vehicle.

Given the demonstrated ability of ROCK pathway inhibition to promote branching of OLs in the *in vitro* CSPG model, we sought to test the translational potential of ROCK inhibition in a relevant *in vivo* model. The chronic cuprizone model was employed for this purpose. Cuprizone, which induces demyelination through its copper chelation effects, was delivered to mice in their diet for 12 weeks at which point animals were returned to a diet of normal chow for a five-week recovery period. In this extended cuprizone model, compared to the standard acute delivery of 6 weeks, remyelination at the end of the recovery period is not expected to be complete due, at least in part, to the presence of substrates that are inhibitory to OL differentiation including CSPGs.

A striking loss of the myelin marker MBP is evident in the animals that had been maintained on the cuprizone diet for 12 weeks (Figure 5.5A). In our model, once the animals were returned to normal chow for a five week recovery period, a water-soluble ROCK inhibitor (Fasudil) was dosed into the animal's drinking water at 30 mg/kg based on standard protocol for treatment (Takata et al., 2013). In those given five weeks on normal chow to recover, the MBP expression clearly demonstrates the lack of complete remyelination with this model. In our samples, cuprizone-induced demyelination was visually evident in the cortex through the use of MBP staining in coronal brain sections (Figure 5.5A). The effects of Fasudil on remyelination were quantified in these sections using an ImageJ plugin to trace MBP staining and quantify the extent of its expression within a set area. The loss of MBP in the cortex of cuprizone-fed animals is clear using this quantification method, but those treated with the ROCK inhibitor during recovery did not show a significant difference in MBP compared to those in the control group (Figure 5.5B). Additionally, animals from all experimental groups completed behavioural testing, using the rotarod apparatus to assess motor coordination, balance and endurance. Success

in the rotarod task is measured by the amount of time the animal is able to remain on the rotating rod as it increases rotation speed. This metric can also be expressed as a function of distance (in meters) that the animal “traveled” while the rod rotated. It was evident that cuprizone administration for 12 weeks reduced the ability of the mice to remain on the rotarod as all cuprizone-fed mice traveled shorter distances than those fed normal chow (Figure 5.5C). Further, following 5 weeks of recovery with either Fasudil or vehicle treatment, no improvements were detected in the ability of animals in these groups to remain on the rotating rod, particularly when compared to those animals fed normal chow for the entirety of the experiment. Taken together, these findings highlight that the only pathway identified thus far with the ability to enhance OL morphology in the *in vitro* system did not translate to benefit in a relevant *in vivo* demyelination model.

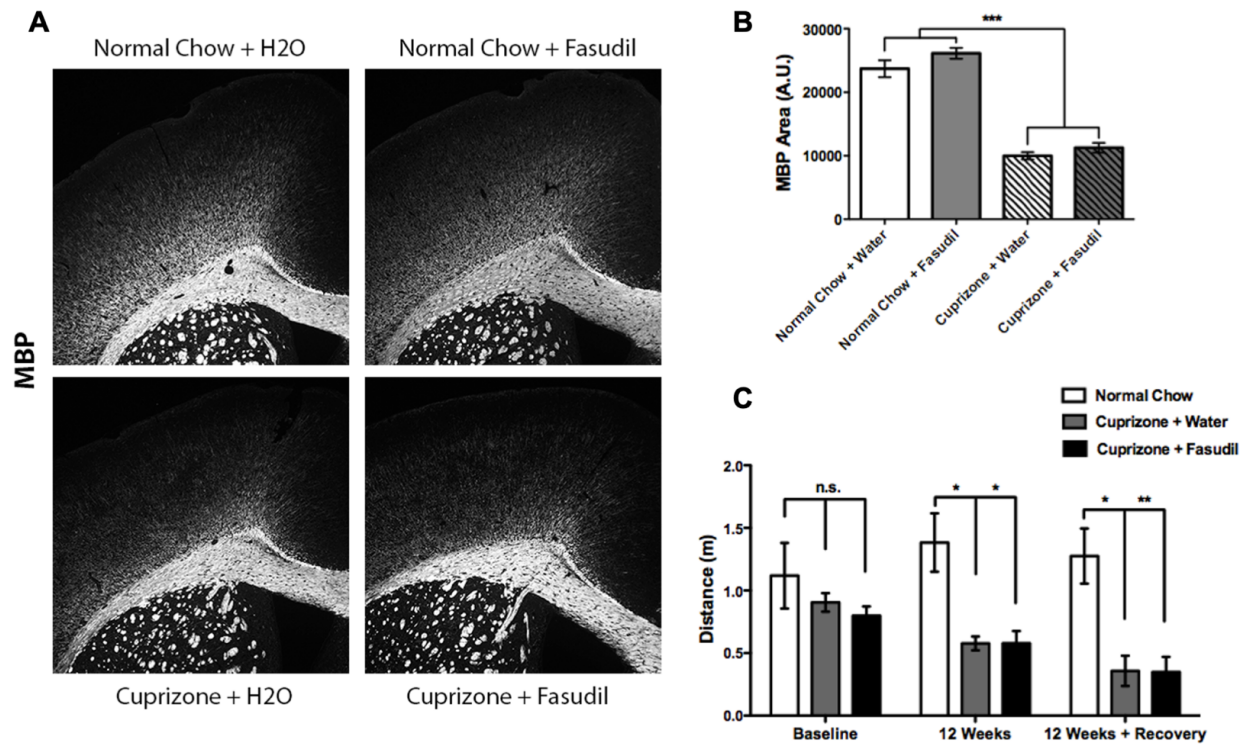


Figure 5.5 ROCK inhibition (Fasudil treatment) did not improve remyelination in the cortex of cuprizone-fed mice. Eight-week old mice were either maintained on normal chow or introduced to cuprizone-supplemented chow for 12 weeks to induce demyelination in the *chronic cuprizone* model. Following 12 weeks, cuprizone-fed animals were returned to a normal diet and mice were either maintained on normal drinking water or given Fasudil (ROCK inhibitor)-supplemented drinking water for 5 weeks. **(A)** Immunofluorescence micrographs of tissue collected from mice of each treatment group at the end of the paradigm demonstrate not only an evident loss of MBP, particularly in the cortex, of mice on the cuprizone diet relative to those on normal chow, but also a lack of improvement in this MBP expression in the animals treated with Fasudil. **(B)** MBP area was quantified using defined area parameters in the cortex of these animals to quantify the extent of MBP expression, and thus, remyelination. The visually evident loss of MBP is depicted, as is the lack of improvement by Fasudil. Data represent the mean $\pm$ SEM, N = 3 or 4. One-way ANOVA with Tukey's post-hoc test. *** $p < 0.001$. **(C)** Assessment of

motor coordination and balance through rotarod behaviour testing supports an inability of Fasudil treatment to improve remyelination following chronic delivery of cuprizone. The distance traveled on the rotarod as depicted here inversely correlates with the latency to fall from the rod. Prior to cuprizone diet implementation (baseline) distance traveled did not differ among groups. However, in mice fed cuprizone for 12 weeks, the distance traveled was significantly lower than mice fed normal chow. Further, after 5 weeks of return to normal chow and treatment with either vehicle or Fasudil, significant decreases in distance were still detected relative to those mice fed normal chow throughout but vehicle-treated and Fasudil-treated groups did not differ from each other. Depicted here are distances traveled in the first trial on the second day of testing. Data represent the mean $\pm$ SEM, N = 4 or 5. Two-way ANOVA with Bonferroni post-hoc tests. * $p < 0.05$, ** $p < 0.01$ and n.s. indicates no significant difference.

5.2.4 Testing the involvement of GSK3 β in the CSPG/OL interaction

The incongruencies presented here between the *in vitro* CSPG model and relevant *in vivo* demyelination models, including the inability of miR manipulations to improve OLs in the *in vitro* model despite their benefit *in vivo*, as well as the benefit of ROCK inhibition *in vitro* with unsuccessful translation *in vivo*, suggests that CSPGs *in vitro* may not be a suitable model to directly test potential pro-myelinating therapeutics. However, using the *in vitro* system to discover more about the mechanisms driving CSPG-mediated morphological impairments in OLs may be useful from an OL biology standpoint where we can learn more about the major mechanistic players involved in establishing, as well as maintaining, the exquisitely complex cytoskeletal architecture of this cell type. In addition, the *in vitro* system may also be useful for uncovering pathways directly affected in the OL by interactions with CSPGs, thereby indirectly discovering potential therapeutic targets that could ultimately be tested in a more disease-relevant system. As described previously, GSK3 β signaling has been implicated in the inhibitory CSPG interaction in neurons (Dill et al., 2008). Given the similarities in neuron and OL growing tips, as well as the pathways that govern dendrite extension and process extension respectively, we sought to characterize GSK3 β activity in OLs exposed to CSPGs. One measure of GSK3 β activity levels is through phosphorylation status, such that decreased levels of phosphorylation at serine 9 of GSK3 β (p-GSK3 β) correlate with increased GSK3 β activity. Assessment by western blot indicated that GSK3 β phosphorylation status did not change in OLs either five or 10 minutes following CSPG treatment (Figure 5.6A). Levels were also assessed one hour and one day following CSPG treatment and again did not differ from those treated with ECM (data not shown). There is evidence that GSK3 β phosphorylation events in a neuron triggered by the presence of certain ECM molecules may be localized to the growing tip as opposed to large-scale

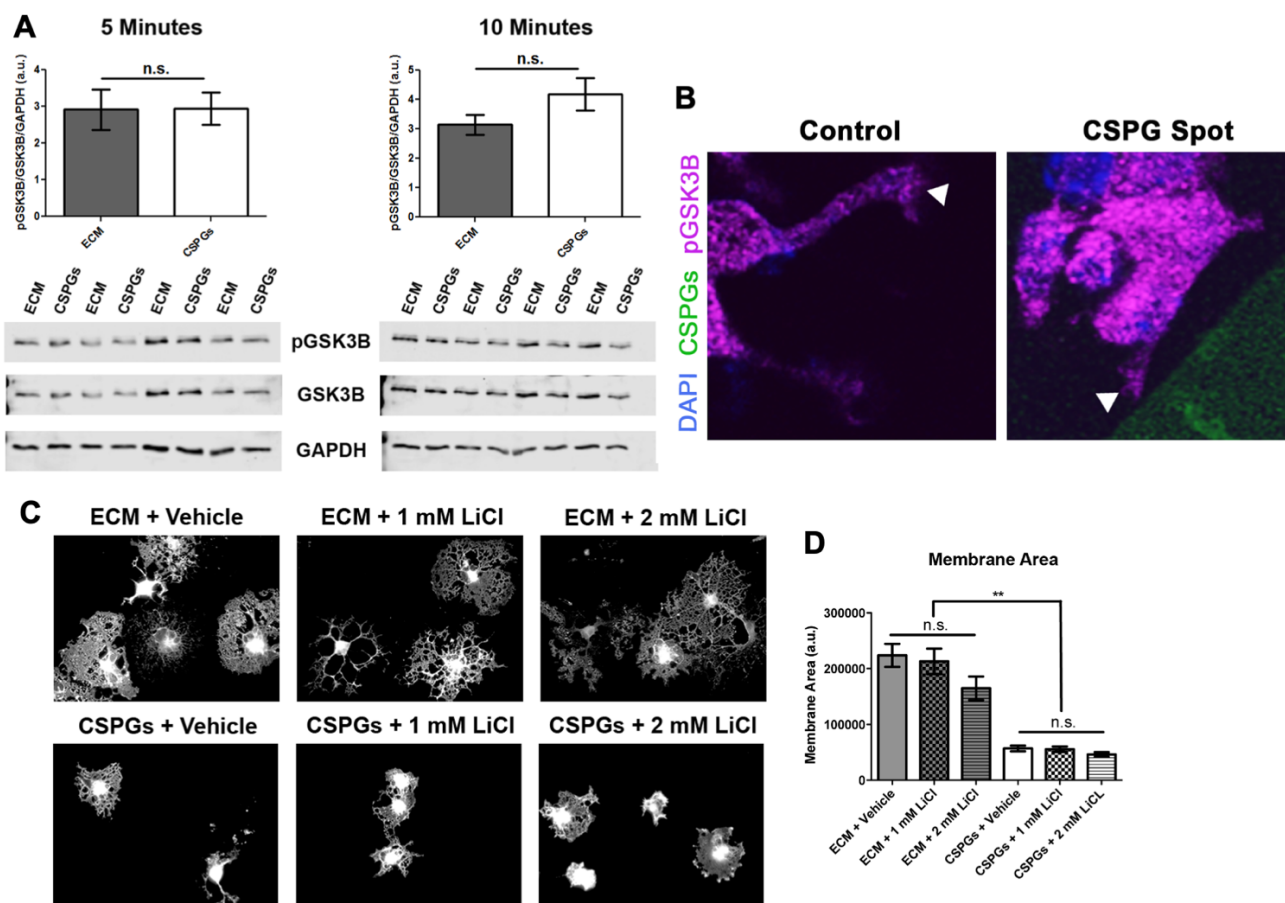


Figure 5.6 GSK3 β does not play a role in mediating the inhibitory CSPG signal in OLs. (A) Immunofluorescent western blots indicate levels of GSK3 β and phosphorylated GSK3 β (Ser 9; inactive form) in lysates from OLs plated on CSPGs or ECM for 5 or 10 minutes. Data represent the mean $\pm$ SEM, N=4. Unpaired two-tailed Student's *t*-test; n.s. indicates no significant difference. (B) Immunofluorescent micrographs depicting intensity and distribution of phosphorylated GSK-3 β in OL cultures 5 hours post-seeding in the presence or absence of CSPG substrate. No apparent difference in GSK-3 β activity in cells approaching the inhibitory CSPG rim. (C, D) OLs differentiated for 5 days on ECM or CSPGs and treated with LiCl or vehicle control. (C) Immunofluorescence micrographs of MBP $^{+}$ OLs depict morphology of each treatment group at DIV5. (D) Quantification of the area of myelin membrane produced (arbitrary

units). LiCl treatment did not affect the extent of membrane production in either substrate group.

Data represent the mean $\pm$ SEM, N=4. One-way ANOVA with Tukey's post-tests; ** $p < 0.01$,

n.s. indicates no significant difference.

changes to phosphorylation status throughout the entirety of the cell (Eickholt, Walsh, & Doherty, 2002). Acknowledging that this may also be true in the OL, thus rendering it difficult to detect phosphorylation changes by western blot, we employed a CSPG spot assay and used immunofluorescent labelling to assess p-GSK3 β in the growing tip approaching the CSPG barrier (Figure 5.6B). There were no overt differences in the staining pattern of p-GSK3 β in OLs growing near or through the CSPG barrier compared to cells on control substrate, though this was not quantified.

Studies identifying GSK3 β as a crucial signaling effector in neurons on CSPGs demonstrated that treatment with a GSK3 β inhibitor was sufficient to rescue the morphology of the cells (Dill et al., 2008). Following a similar paradigm, we sought to treat OLs differentiating in the presence of CSPGs with a known GSK3 β inhibitor – lithium chloride (LiCl). The vehicle treated OLs on CSPGs demonstrated the expected decrease in morphological metrics, myelin membrane area in this case. OLs differentiating on CSPGs but treated with LiCl were not significantly different than vehicle-treated cells, demonstrating a similar reduction in morphological differentiation (Figure 5.6C, D). Importantly, given the lack of effect of GSK3 β inhibition, the efficacy of LiCl treatment in altering p-GSK3 β was validated in Oli-neu cells treated at the same dose (Supplementary Figure 1). Taken together, these findings suggest that GSK3 β activity is not an important effector in CSPG-mediated inhibition of OL morphological development, distinguishing the response of OLs to this cue from that of the neuron.

5.2.5 Using the *in vitro* CSPG model to identify process retraction

Our GSK3 β results suggest that the pathways translating the CSPG signal into cytoskeletal effects may not be common between neurons and OLs, despite structural similarities in their growing tips. We next sought to use the *in vitro* CSPG system to take a broader approach

to identifying mediators of OL process extension inhibition. To do so, we first wanted to investigate the morphological impact of CSPGs more deeply and determine what effects CSPG exposure would have at a different stage of the cell's development. Thus far, the impact of CSPGs on OL morphology has largely been explored in cells which have been exposed to CSPGs early in the differentiation process, usually at seeding stage as OPCs, but it remains unknown what effect CSPG exposure would have on OLs that were already morphologically complex. One hypothesis was that CSPG addition would act simply as a physical barrier to further branching and process extension, leaving cells similarly complex after the addition of CSPGs relative to before. Alternatively, we speculated that CSPGs added to morphologically complex OLs would induce a more active process of branch retraction, reflected by more simple morphologies after CSPG addition relative to before. To this end, OLs were differentiated on a permissive ECM control substrate for two days (DIV2), reaching an intermediate stage of differentiation. At this point, either CSPGs or the control ECM substrate were added to the cultures, which were allowed to differentiate for an additional 8 or 24 h and were then compared back to the DIV2-fixed cultures to assess for changes in morphological complexity (Figure 5.7A). The cells exposed to CSPGs for 24 h were not only less morphologically complex than those treated with ECM control substrate for the same length of time, but also appeared less complex than the DIV2 baseline, as indicated by sholl analysis quantification (Figure 5.7B). This indicates that the addition of CSPGs at a later time point likely leads to active retraction of processes as opposed to simply blocking further extension of branching ultimately stunting growth.

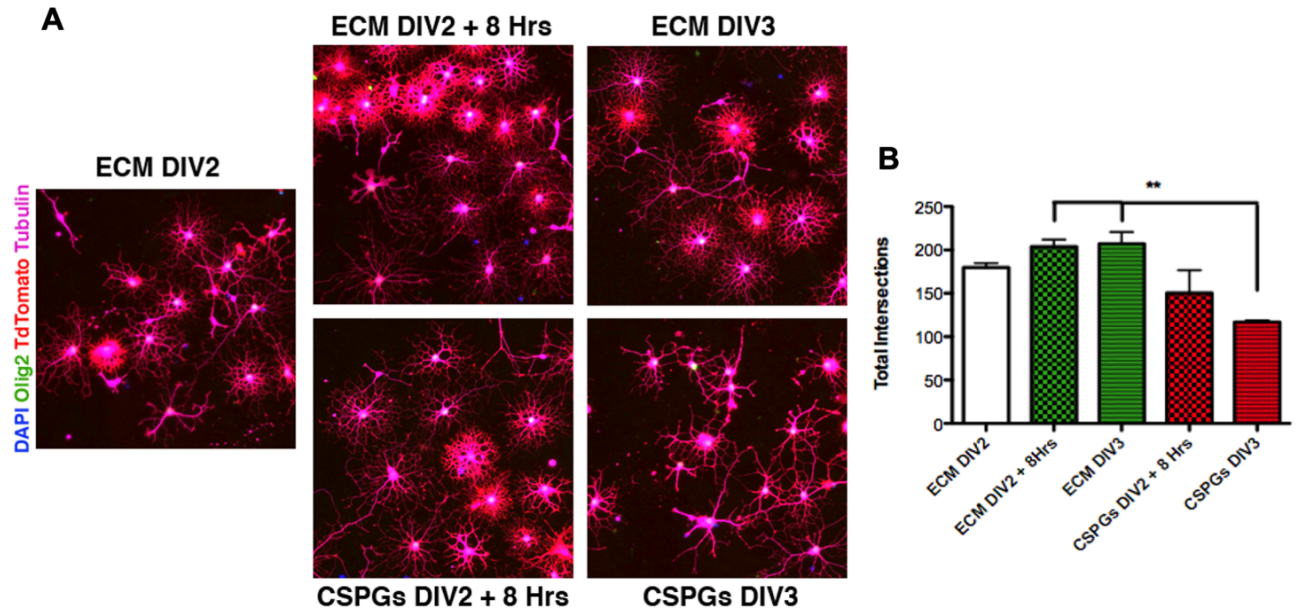


Figure 5.7 Addition of CSPGs to differentiating OLs results in process retraction. (A)

Immunofluorescence images depicting the response to late CSPG addition. All OLs were seeded on ECM substrate and allowed to differentiate until DIV2, at which point they were fixed as the DIV2 baseline (left panel) or treated with CSPGs or control ECM and allowed to differentiate for another 8 or 24 hours (middle and right panels, respectively). **(B)** Quantification of OL complexity by Sholl analysis; Concentric rings overlaid 10 μm apart; Number of OL process intersections per ring quantified. Data represents the sum of intersections across all rings per OL, and the reduction in complexity following CSPG addition is clearly depicted. Data represent the mean $\pm$ SEM, N=3. One-way ANOVA with Tukey's post-hoc, ** $p < 0.01$

5.2.6 Identification of cytoskeleton effectors involved in CSPG-mediated process retraction

The retraction of processes in OLs following CSPG exposure presents an interesting facet of OL biology to explore in this model. The implication that CSPGs induce a more active retraction process in the cells as opposed to a more passive physical blockade suggests that there is more to be uncovered with regards to the CSPG signaling effectors, but also to identify the cytoskeletal effectors associated with process retraction. In the hopes of identifying cytoskeletal changes involved in the retraction of processes following CSPG exposure, we employed a cytoskeleton themed qPCR array using RNA collected from cells treated with CSPGs compared to those treated with ECM as a control. The full list of cytoskeleton-related genes assessed in these arrays is provided in Appendix 1. The experiment was conducted with five biological replicates of each test group. Mitogen-activated protein kinase 13 (*Mapk13*) was the only gene identified meeting the criteria of a 1.5 or greater fold change between the two groups and a significance value less than 0.05 (Figure 5.8). More specifically, *Mapk13* expression was 2.16-fold higher in intermediate OLs treated with CSPGs relative to those treated with ECM. This finding that only one gene in a cytoskeleton-focused array was altered following CSPG addition to OLs is surprising given the dramatic morphological impact associated with their presence. Taken in this context, the OL response to CSPGs, either mechanistic or compensatory, appears to be driven by other means of regulation and less so by transcriptional changes in key cytoskeleton regulators.

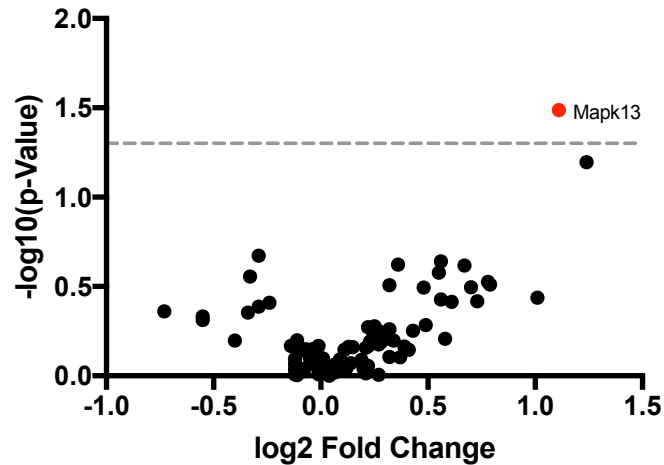


Figure 5.8 Lack of transcriptional changes in cytoskeletal effectors to account for CSPG-induced process retraction. Volcano plot presentation of genes surveyed in a focused cytoskeleton PCR array using primary mouse OLs collected 8 hours after treatment with CSPGs on DIV2 compared to those treated with control ECM substrate with the same paradigm. Cutoff line represents $p < 0.05$. *Mapk13* is depicted in red and is the only gene sufficiently changed to exceed the cutoff criteria (fold change $> 1.5x$, $p < 0.05$). $N = 5$ for each treatment group.

5.3 Discussion of Results

Remyelination therapy for MS is a promising avenue currently being pursued by many research groups in an effort to fill the void of non-immunomodulatory treatment strategies for this disease. The exploration of remyelination and identification of therapeutic angles to promote this process is still in its infancy and has had limited translational success to date. We speculate that a significant barrier to the successful development of a remyelination strategy is the lack of model systems that accurately recapitulate the complexities of the MS lesion environment, which is known to accumulate factors that impair OL differentiation. CSPGs have been recognised as a key inhibitory component of MS lesions that are resistant to repair, prompting the use of CSPGs *in vitro* to test the efficacy of potential pro-myelinating compounds. In this study, we explore the applicability of the *in vitro* CSPG modeling system for this use, as well as expand on the current knowledge of the CSPG-OL interaction.

Taken together with the history of remyelination therapy development to date, our results, particularly those exploring the ability of miR manipulations to benefit OLs on CSPGs as well as those assessing the translation potential of rho kinase inhibition, emphasize the importance of model selection and suggest that *in vitro* CSPG modelling is not appropriate for testing the potential success for pro-myelinating therapeutics. Our lab, and others, have explored the effects of miR manipulations on OL differentiation and remyelination. Both the inhibition of miR-145 and the upregulation of miR-219 improve OL differentiation *in vitro*, but more importantly, both have demonstrated improved remyelination in the chronic cuprizone model - an *in vivo* model that exhibits the accumulation of factors inhibitory to OL differentiation similarly to the progressive MS lesion environment (H.-B. Fan et al., 2017; Hibbits et al., 2012; Kornfeld, Unpublished). In our hands, neither overexpression of miR-219 nor knockout of miR-145 was

sufficient to promote OL differentiation in an *in vitro* CSPG model. Oppositely, ROCK inhibition, which has been demonstrated by others to promote OL branching on CSPGs *in vitro* and supported here, was not successful in promoting remyelination in the chronic cuprizone mouse model. This disparity is further supported by other examples in the literature. Most notably, clemastine is currently being pursued in clinical trials for MS and is showing early promise as a pro-myelinating therapeutic, despite the fact that it was unable to promote OL branching in an *in vitro* CSPG model (Green et al., 2017; Keough et al., 2016). It is not entirely clear why the findings of the *in vitro* CSPG model struggle to translate into *in vivo* relevance, but one could speculate that it is simply too difficult to distill the three-dimensional process of OL differentiation in the presence of a complex set of ECM molecules into a two-dimensional model. Ultimately, it is clear that taking the inhibitory components of the MS lesion environment into account when developing pro-myelinating therapy options is critical, but these discrepancies suggest that the *in vitro* CSPG model is not the best method to employ.

While the *in vitro* CSPG system may not be functional as a remyelination drug screening platform, it remains essential that we understand the CSPG-OL dynamic on a deeper level as CSPG-mediated inhibition of OL differentiation likely plays an important role in preventing repair in progressive MS lesions. Thus far, the response of OLs to CSPGs *in vitro* has only been assessed when CSPGs are present at the time of seeding OPCs for differentiation, or very shortly after. Here, we explored how OLs would respond to CSPG addition at a later stage of differentiation. Interestingly, CSPG addition to OLs at DIV2 (characterized by an intermediate level of arborization) resulted in a process retraction response, as opposed to simply blocking extension of processes further. In an effort to interrogate the process retraction response to CSPGs further, a cytoskeleton-themed qPCR array was employed using RNA collected from

intermediate OLs treated with CSPGs and revealed only one transcriptional change. Mitogen-activated protein kinase 13 (MAPK13, also termed SAPK4, p38 δ), was upregulated in OLs treated with CSPGs relative to those treated with control ECM substrate. In other cell types, MAPK13 activation is seen in response to inflammatory cytokine exposure and certain forms of cell stress, including osmotic stress, and has been shown to exert effects on the cell through direct phosphorylation of stathmin, a key regulator of microtubule dynamics (Jiang et al., 1997; Parker et al., 1998). In the OL, an essential role for stathmin has been identified in the morphological differentiation process (Aixiao Liu, Muggironi, Marin-Husstege, & Casaccia-Bonnel, 2003; A. Liu et al., 2005). Given the MAPK13 link to regulation of cytoskeleton dynamics through stathmin, the increase in expression identified in OLs following CSPG treatment seemed to be a noteworthy potential compensatory mechanism employed by the OL under stress. However, databases have shown that expression of MAPK13 in OLs (and OPCs) is nearly negligible, which brings into question the biological relevance of this change in our assay (Marques et al., 2016; Y. Zhang et al., 2014).

Alternatively, it is interesting to consider the overall lack of cytoskeleton-related transcriptional changes in the OL while it is undergoing such a dramatic morphological response. Although we had expected to detect relevant pathological or compensatory transcriptional changes using this assay, we propose that this may not be the most effective means to capture drivers of the cytoskeletal response to this cue. The activity levels of many actin and tubulin regulatory proteins are often controlled by phosphorylation status rather than altered expression levels. This is particularly notable for the CSPG/OL interaction, in which activation of PTP σ has already been identified (Pendleton et al., 2013). In non-OL cell types, CSPG engagement with PTP σ has been shown to prevent its dimerization, rendering it active (Coles et al., 2011). Its

activation, among other changes in the cell, is associated with the dephosphorylation and subsequent inactivation of major signaling effectors Akt and Erk1/2 (Sapieha et al., 2005). The inactivation of these effectors can lead to activity changes, regulated by phosphorylation and dephosphorylation events, in a number of proteins that directly interact with the cytoskeleton including MAP1B and stathmin (Y Ohtake, Saito, & Li, 2018). To address this, the future directions of this work will include establishing the phospho-proteome for OLs treated with CSPGs or control substrate from which we can identify phospho-proteomic changes in OLs undergoing process retraction following the addition of CSPGs.

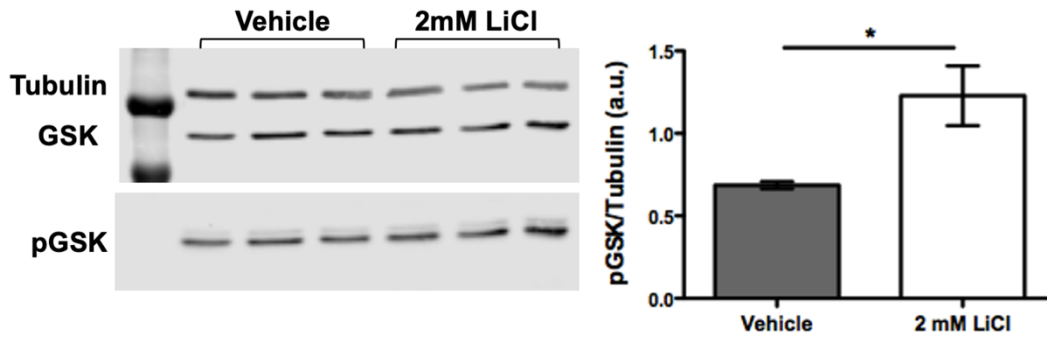
The data presented in this work is not only informative of the OL-CSPG relationship, but also provides insights into mechanistic differences between the fairly well-characterized neuronal growth cone and the newly appreciated “growth cone-like” tip of OL processes. Morphologically, the organization of cytoskeletal components in the two structures are reminiscent of one another; this includes a central region consisting primarily of bundled microtubules forming the bulk of the newly forming process and out from which the F-actin rich lamellipodia and filopodia radiate (Michalski et al., 2016; Miller et al., 2005). It is this growing tip of the cell that responds to ECM molecules, among other factors, in the cell’s environment while it differentiates. The structural similarities between neuronal and OL growth cones, along with shared morphological responses to certain guidance cues and substrates including CSPGs, led to the hypothesis that pathways involved in mediating the CSPG interaction would be shared between the two cell types as well (Bagnard, Lohrum, Uziel, Puschel, & Bolz, 1998; Manthorpe et al., 1983; Relucio, Tzvetanova, Ao, Lindquist, & Colognato, 2009; Siebert & Osterhout, 2011; Snow et al., 1990; Spassky et al., 2002). This hypothesis was supported by the identification of the shared receptor, PTP σ , in mediating the CSPG signal both in OLs and neurons (Pendleton et

al., 2013; Shen et al., 2009). Rho kinase inhibition on CSPGs resulting in improved morphology metrics in both cell types is yet another similarity, though specific overactivation in OLs exposed to CSPGs remains to be determined (Kippert et al., 2009; Yosuke Ohtake, Wong, Abdul-Muneer, Selzer, & Li, 2016; Pendleton et al., 2013; Shen et al., 2009). It is possible that rho kinase is simply a highly influential effector of cytoskeleton dynamics in the OL, that its inhibition in these cells on CSPGs promotes process extension in a nonspecific manner rather than correcting for a pathological overactivation of rho kinase induced by CSPGs. Given the lack of translation for rho kinase inhibition in our *in vivo* demyelination studies, this is an important distinction to be made. Contrary to the hypothesis that neurons and OLs would mechanistically respond to CSPGs in similar fashion, GSK3 β activity was unchanged in OLs on CSPGs, despite evidence that its overactivity is involved in mediating the CSPG signal in the neuron (Dill et al., 2008). Further, inactivation of GSK3 β did not improve the morphology of OLs as has been reported in the literature for the neuron. This is particularly curious given the mechanism by which GSK3 β is thought to be involved following CSPG exposure in the neuron; activation of PTP σ reportedly leads to the inactivation of Akt, which subsequently leads to the activation of GSK3 β (Dill et al., 2008; Yosuke Ohtake et al., 2016). A number of groups have identified the activation of PTP σ in OLs when CSPGs are present, yet the lack of GSK3 β involvement suggests that this downstream pathway may be engaged differently in OLs than it is in the neuron. Taken together, we propose that while some of the mechanisms at play during CSPG-mediated inhibition of morphology may be conserved between neurons and OLs, some mechanisms are apparently distinct and warrant further investigation.

Overall, this work supports the need for consideration of the inhibitory milieu of the MS lesion when developing remyelination-based therapies. However, we propose that the *in vitro*

CSPG model is not appropriate to use as a simulation of the refractory lesion environment for testing the potential of pro-remyelinating therapeutic strategies. Rather, we suggest that it be employed solely for the purpose of understanding the mechanisms involved in CSPG-OL interaction – a relationship with a number of outstanding questions. A greater understanding of the biochemistry underlying not only the OL's interaction with this inhibitory cue, but also with others, is necessary to fully understand the OL differentiation block in chronic MS lesions.

5.4 Supplementary Material



Supplementary Figure 5.1 Efficacy of LiCl treatment validated in Oli-Neu cells. Oli-Neu cells were treated with 2 mM LiCl on DIV1 and protein collected for western blotting on DIV2. Quantification confirms increase in phosphorylated GSK3 β (Ser 9; inactive form) following treatment. * $p < 0.05$; $N = 3$.

Chapter 6: General Discussion

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In this work, we have made the surprising discovery that OLs do not seem to require disease-associated genes *Smn* or *Snca* for essential development and functionality in the cell. Furthermore, we have demonstrated that changing the environment of the OL, specifically the addition of CSPGs, induces severe morphological defects including process retraction, and that these defects are almost insurmountable in an *in vitro* context. Looking at these results in isolation, a notable pattern emerges - the gene deletions have little to no impact on the OL, while changing the microenvironment has a profound impact. While this is of course not universal, as a number of genetic manipulations have been identified with deleterious effects on the OL, it does mirror the almost enigmatic depiction of OLs in the literature of a highly resilient cell type that is also highly vulnerable. The resiliency is apparent from the regenerative capacity of OLs and CNS myelination to the point that a model that does not spontaneously remyelinate, a desire for many in the field of progressive MS, has taken time to find. It is also evident in the number of genetic manipulations that result in myelination defects early in development, but are compensated for and result in normal or near normal myelination *in vivo* (Bartlett & Skoff, 1986; Grier et al., 2017; H. J. Kim et al., 2006; R. W. O'Meara et al., 2013; Relucio et al., 2009). Conversely, the sensitivity or vulnerability of OLs has been documented. OLs are thought to be a particularly vulnerable cell type as a result of their relatively high metabolic rates coupled with their high intracellular iron content and low anti-oxidative enzymes, rendering the OL more susceptible to oxidative damage as well as mitochondrial injury (Thorburne & Juurlink, 2002). Additionally, OLs are considered sensitive to glutamate toxicity, certain inflammatory cytokines, as well as the activity of other cells in their environment, such as reactive astrocytes and the factors they secrete (Matute, Sanchez-Gomez, Martinez-Millan, & Miledi, 1997; McDonald,

Althomsons, Hyrc, Choi, & Goldberg, 1998; McTigue & Tripathi, 2008; Selmaj & Raine, 1988; Shiow et al., 2017; Vartanian et al., 1995; Y. Wang et al., 2011). Adding to this is the notion that OLs are differentially susceptible to cell death depending on their stage of development such that in general, post-mitotic OLs are thought to be more resilient than OPCs with the exception of the OL that is actively myelinating (Butts, Houde, & Mehmet, 2008). Altogether, it becomes apparent that this simultaneous resiliency and vulnerability within the OL is not such an enigma after all. Rather, a greater understanding of the complexity of this cell type, and how stage of development combined with a multitude of genetic and environmental factors ultimately come together to contribute to the health and functionality of the OL is needed.

OL Function and Dysfunction Assessment - Limitations

Genome-wide and targeted transcriptomic analysis were used to aid in the discovery process of identifying the impact of α -syn loss in OLs and to elucidate cytoskeleton effectors involved in the response to CSPGs in OLs, respectively. In both instances, we were struck by the low number of candidate genes identified and the seemingly low biological relevance of those that were. Future directions of this work will include phosphoproteomic analysis to better address the morphological changes in OLs differentiating on CSPGs. In the field of OL biology, these modalities, in addition to proteomics more broadly, are tools readily used to gauge the differentiation status and general state of the OL. We propose that this classic gene- and protein-centric view of OL functionality is still quite limited, and that diversifying the tools and techniques used to explore OL biology is necessary to adequately address various aspects of OL biology. A growing appreciation for this need is becoming evident as the number of publications exploring lipid biology in OLs slowly increases. Lipidomics is of particular interest to the OL field given the high lipid content in this cell type and the importance of lipids in myelin (Saher &

Stumpf, 2015; Schmitt, Cantuti Castelvetti, & Simons, 2015). In addition to technical hesitation to exploring this important facet of OL biology, lipidomics are difficult to employ as a metric as currently there are inadequate baseline measures of how the lipidome changes throughout differentiation and which lipid species are essential for this process. This work has begun, with a number of groups exploring the importance of cholesterol in OL differentiation and myelination (Saher et al., 2005). We are also beginning to contribute to this area through exploration of sphingolipidome changes throughout OL differentiation *in vitro* (Appendix 2). No doubt the expansion of this area will provide additional metrics to assess the health and maturity of the OL.

A greater limitation to this work, particularly relevant in the exploration of *Smn* or *Snca* loss on OL functionality, is identifying an appropriate means to challenge mouse OLs *in vitro*. In both gene knockout studies, we did not detect any overt defects in OLs lacking these disease relevant genes. However, in both contexts we were only able to explore their involvement in the typical differentiation paradigm. We have not addressed the importance of either gene in protecting the cell from stress, including oxidative stress, or potential involvement in response to or activation of the immune system. The ability to reliably establish primary OL cultures from mice is a relatively new innovation in the field, and a great achievement to allow for OLs to be derived from knockout mouse models. In general, mouse-derived OLs are more sensitive to manipulation than their rat-derived counterparts making some cell stress assays difficult to employ in these models. Immune challenges can still be done in mouse derived OLs and are particularly relevant to our genes of interest, including experimenting with conditioned medium treatments collected from stimulated astrocytes or microglia, as has been done previously by other groups (Hattori et al., 2017; Moore et al., 2015). This is a worthy pursuit given what is known about both *Smn* and *Snca* function in other cell types. More specifically, *Smn* has been

found to protect motor neurons from cell death. Natural variation in Smn levels within healthy motor neurons was captured using single cell sorting and those motor neurons expressing lower levels of Smn protein were more prone to cell death following treatment with toxic compounds than those with naturally higher Smn levels (Rodriguez-Muela et al., 2017). Further, Smn deficiency has been shown to impede the cell's ability to form stress granules, indicating that Smn plays a role in a cell's stress response and ultimately aiding to help in cell survival in stress conditions (Zou et al., 2011). In the case of *Snca*, its presence has been shown to be protective against neurotropic viruses as well as in a bacterial sepsis model (Beatman et al., 2016; Tomlinson et al., 2017). Interestingly, RIPK3 (receptor-interacting protein kinase-3) has been demonstrated to restrict viral infection in neurons and *Ripk3* was identified in our RNA-seq experiment to be significantly downregulated in *Snca*^{-/-} OLs (Daniels et al., 2017). Further, α -syn expression in enteric neurons was shown to correlate with the density of inflammatory cells in children suffering from upper gastrointestinal distress, again suggesting a likely role for α -syn in response to immune activation in this system (Stolzenberg et al., 2017). In addition to these parallel findings in other cell types, investigation of α -syn's role in immunity and protecting against infection is warranted given the potential therapeutic angle for MSA that includes the knockdown of α -syn in OLs – conditional on the α -syn knockdown itself being well-tolerated and not associated with adverse effects on OL health and function.

Extrapolating OL-Related Function

Considering OL function and dysfunction in a broader scope allows us to extrapolate hypotheses from one application to another in an informed manner. Relevant to this thesis, expanding the view of α -syn in OLs away from its well-known pathological role in MSA allows us to draw a plausible connection between α -syn's endogenous function in OLs and the immune

system. The involvement of α -syn in MSA disease pathogenesis is clear and while its exploration in this context is essential, it does not offer many indications for α -syn's function in OLs in a non-pathological context. In our efforts to uncover endogenous functions of α -syn in OLs, our α -syn gene knockout model revealed no overt defects in the OL, though transcriptomic analysis revealed a small number of differentially expressed genes in knockout OLs that may point to a connection between α -syn in the OL and the immune system. Though the specific connection is yet to be elucidated, support for this hypothesis comes from MS pathology based on the finding that α -syn reactivity was detected diffusely in cells in MS lesions (Lu et al., 2009). First, the diffuse staining pattern of α -syn in this context indicates that its presence is more likely associated with its functional role as a protein, rather than its presence in other pathologies in aggregated form. Moreover, the details of its presence are informative of its potential function. α -syn was detected in most of the assayed active MS lesions, many of the chronic active lesions, but none of the chronic inactive lesions. In this classification system, *active* and *inactive* refer to immune cell infiltration and the associated activity of the immune response at the lesion. These findings indicate that α -syn reactivity is greater in those lesions undergoing a more active immunological response than those that have cleared the immune cell infiltration and remain damaged. Furthermore, in EAE, the mouse model dominantly used to explore MS-related inflammatory responses, α -syn was increasingly detected in neurons, OLs and astrocytes when compared to control animals (Papadopoulos, Ewans, Pham-Dinh, Knott, & Reynolds, 2006). Taken together, this cross-disciplinary view of α -syn biology supports the notion that α -syn in OLs should be interrogated for a potential role in responding to immune-related activity. This connection is one example of many that support the need for broadening our view outside of the directly related pathology, cell type or disease state in order to generate appropriate hypotheses.

OL Involvement in Classically Neuron-Centric Diseases

Consistent with the notion that a more holistic approach to OL biology is informative, including interrogating OL biology not only within the context of *primary oligodendroglipathies* but also in the context of other neurological and neuromuscular disorders, we can draw parallels from our findings to others in the literature that are interesting but also puzzling (Figure 6.1). For example, comparing what is known about OLs in ALS with our findings on OLs in SMA is curious. A number of parallels have been drawn between ALS and SMA, in part due to the motor neuron being the primary target in each (Šoltić et al., 2018). Both ALS and SMA are increasingly appreciated as multi-cellular pathologies affecting far more than just the motor neuron; in the case of ALS, this includes the identification of myelin defects in patients and OL defects following certain disease-associated gene mutations or in iPSCs derived from ALS patients (Ferraiuolo et al., 2016; Kang et al., 2013; Philips et al., 2013). Even more interesting is the finding that iPS-derived OLs from ALS patients have been found to induce motor neuron death in co-culture experiments, indicating a significant role for OLs in this disease (Ferraiuolo et al., 2016). The absence of OL pathology in our SMA studies was even more surprising given this context. Our findings highlight one of many differences between ALS and SMA pathology and could contribute to differences between the two in terms of disease presentation.

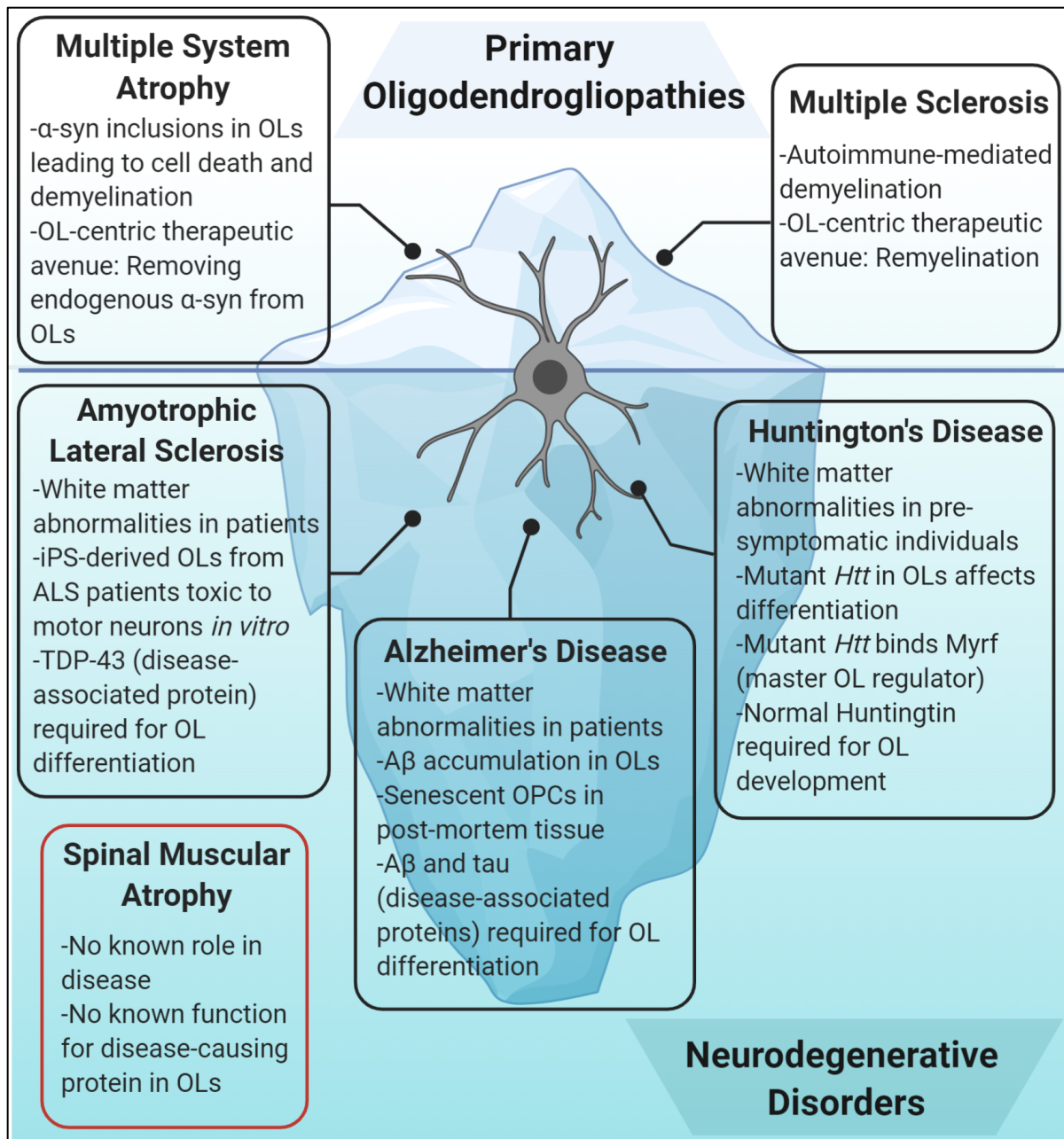


Figure 6.1 Oligodendrocyte involvement in primary oligodendroglipathies is only the tip of the iceberg. Schematic depiction of OL involvement in both primary oligodendroglipathies as well as classically neuron-centric neurodegenerative disorders. Neurodegenerative disorder details include evidence of OL involvement as well as biological roles of disease-associated proteins in OL function. The obscurity of SMA lacking a known connection to OLs is also depicted. Figure created with BioRender.com.

Similarly, our work identifying that OL differentiation and CNS myelination are unaffected in an α -syn knockout model is interesting when compared to OLs in the contexts of AD and HD. In MSA, AD and HD, the primary pathology is thought to be driven by the accumulation of aggregated proteins - α -syn, A β and tau, and huntingtin, respectively. While MSA is the only one of the three considered a primary oligodendrogliopathy, all three diseases have identified involvement of OL pathologies. Pathologically, these three disease states are all thought to be quite different, yet death of the OL in each is a notable commonality. This may suggest a more non-specific response to the accumulation of aggregated proteins as was eluded to earlier. More relevant to our reverse question on the normal function of α -syn in the OL, it should be noted that physiological roles for the other disease-associated proteins mentioned above (A β , tau and huntingtin) have been identified in the OL. Huntingtin has been shown to be essential for the development of the OL *in vivo*, as a mouse model with limited huntingtin expression exhibited myelination defects, and *in vitro* as OLs cultured from this model displayed impaired differentiation ability (Arteaga-Bracho et al., 2016). In the case of AD-associated proteins, both A β and tau deficiency in the OL is associated with significant impairments in differentiation. More specifically, both A β and tau have been shown to play roles in the transport of *Mbp* mRNA for local translation at the distal tips of OL processes and have been shown to be essential for morphological differentiation of the cell (Klein et al., 2002; Quintela-López et al., 2019; Seiberlich et al., 2015). The fact that these other aggregate-prone proteins all have clear physiological functions in the OL, as opposed to simply pathological, supports the notion that a role for α -syn in the OL is likely and further exploration must be done to uncover it.

Closing Remarks

Our work has explored OL function and dysfunction within the context of several neurodegenerative diseases. We have identified that loss of Smn, the hallmark pathology of SMA is inconsequential to OLs and suggests a unique lack of involvement for this cell type in the pathogenesis of SMA. Further, our efforts have revealed that α -syn, the protein thought to be primarily responsible for OL cell death in MSA, does not have an obvious physiological function in this cell type. Though further exploration is needed in avenues clearly identified within this thesis, our findings support the exploration of endogenous *Snca* knockdown in mature OLs as a possible therapeutic avenue for MSA to limit the accumulation of aggregated α -syn. Lastly, our work has expanded on the known effects of an MS lesion-associated inhibitory factor, CSPGs, on OL morphology. In addition to exploring the utility of an *in vitro* CSPG model system, we identify novel distinctions between OL and neuronal responses to CSPGs while also identifying an imminent need to explore phosphoproteomic changes in OLs on CSPGs to better understand this relationship.

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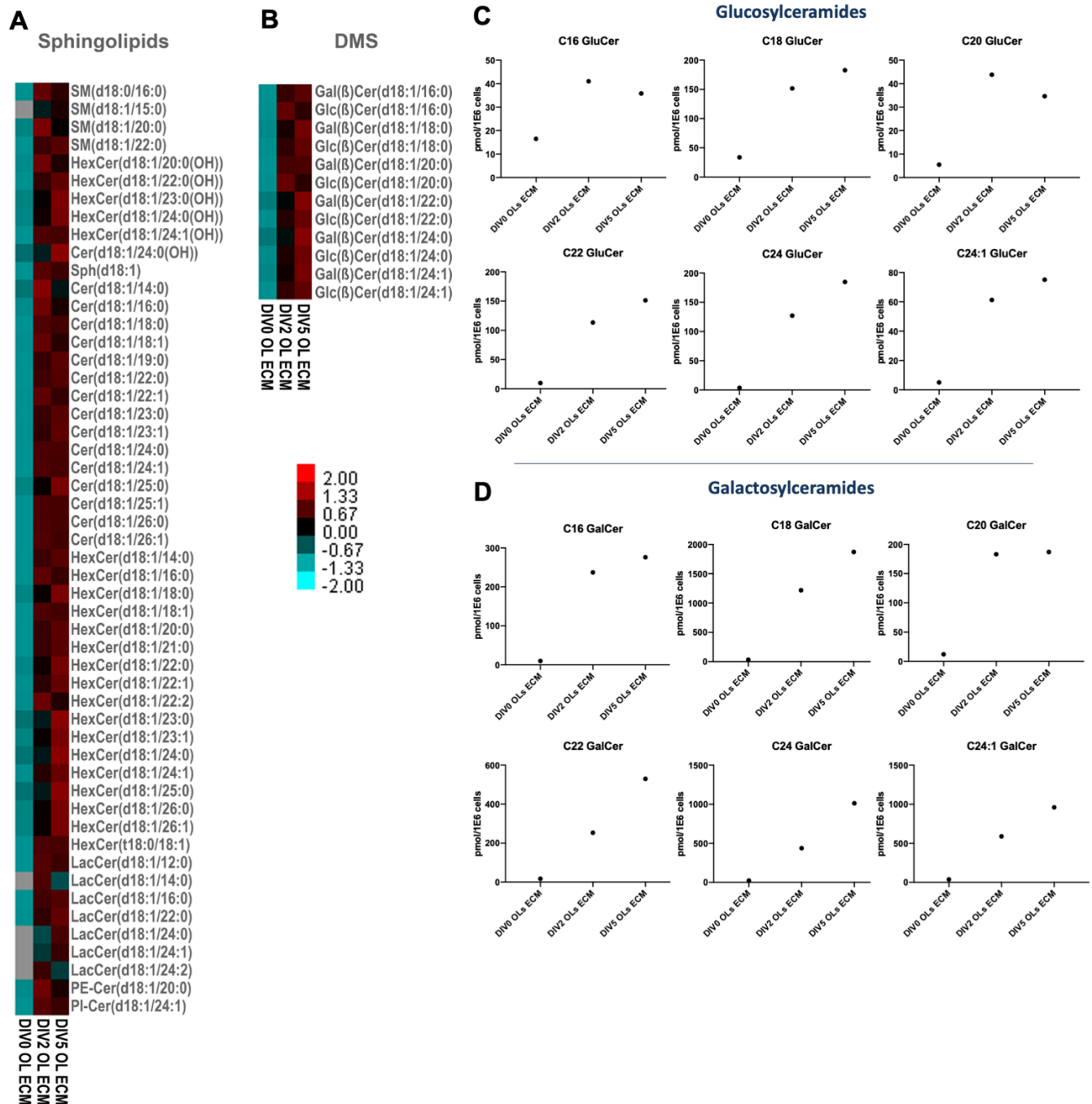
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Appendix A: Genes assayed in cytoskeleton-effector qPCR array. Genes in bold are housekeeping control genes.

Actr2	Cdc42ep3	Macf1	Racgap1
Actr3	Cdk5	Map3k11	Rdx
Arap1	Cdk5r1	Mapk13	Rhoa
Arfip2	Cfl1	Mapre1	Rock1
Arhgap6	Cit	Mapre2	Ssh1
Arhgdib	Clasp1	Mapt	Ssh2
Arhgef11	Clasp2	Mark2	Stmn1
Arpc1b	Clip1	Mid1	Tiam1
Arpc2	Clip2	Msn	Vasp
Arpc3	Crk	Mtap4	Was
Arpc4	Ctnn	Mylk	Wasf1
Arpc5	Cyfip1	Mylk2	Wasl
Aurka	Cyfip2	Nck1	Actb
Aurkb	Diap1	Nck2	B2m
Aurkc	Dstn	Pak1	Gapdh
Baiap2	Ezr	Pak4	Gusb
Cald1	Fnbp11	Pfn2	Hsp90ab1
Calm1	Fscn2	Phldb2	
Cask	Gsn	Pikfyve	
Ccna1	Iqgap1	Ppp1r12a	
Ccnb2	Iqgap2	Ppp1r12b	
Cdc42	Limk1	Ppp3ca	
Cdc42bpa	Limk2	Ppp3cb	
Cdc42ep2	Llg11	Rac1	



Appendix B: Sphingolipid profiling of OL differentiation. Lipids extracted from mouse OLs differentiating *in vitro* for 4 h (DIV0), 2 days (DIV2) or 5 days (DIV5) were separated by mass spectrometry using differential ion mobility spectrometry (DMS) coupled with liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-DMS-MS/MS). (A)

Heat maps of Z-Scores for all sphingolipid species detected across all time points of differentiation, as well as separation of cerebroside detected with DMS (**B**). (**C**, **D**) Preliminary analysis of cerebroside species abundance as detected by LC-ESI-DMS-MS/MS reveal a general shift from glucosylceramides to galactosylceramides over time. Glucosylceramide species (**C**) seem to peak in abundance at intermediate differentiation stages, while almost all galactosylceramide species (**D**) become immensely more abundant between intermediate and mature stages. Abundance of each species presented is normalized to the number of cells collected and presented in pmol per 1E6 cells.