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The Role of Matrix Metalloproteinases in Oligodendroglial Development

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Cindy Viviana Leiton Aguirre

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Abstract of the Dissertation

The Role of Matrix Metalloproteinases in Oligodendroglial Development

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Oligodendrocyte progenitor cells (OPCs) are characterized by their capacity for proliferation, migration, and maturation into myelinating oligodendrocytes. Among the known molecular mechanisms that regulate OPC development are 1) the extracellular matrix (ECM) protein laminin that is known to regulate OPC development as it is required for timely oligodendrocyte differentiation and myelination, and 2) an oligodendroglial laminin receptor, dystroglycan, which regulates oligodendrocyte morphology and maturation. ECM proteins and receptors can be remodeled by proteolytic enzymes known as the matrix metalloproteinases (MMPs), but little is known regarding these events and their consequent functions in CNS development and regeneration. While the laminin-dystroglycan adhesion complex appears to be important for oligodendrocyte development, it remains unknown whether proteolytic remodeling of this complex regulates its function and if it influences oligodendroglial development. Several MMPs have been shown to cleave laminins and dystroglycan in other cell types, suggesting that these proteins may undergo MMP-mediated proteolysis. Furthermore, oligodendroglia express at least 17 different members of the MMP family, and their patterns of expression change during

differentiation, suggesting that oligodendroglia utilize these enzymes during development. In this thesis project, I investigated whether oligodendroglial dystroglycan is proteolytically processed by MMPs in the developing brain and whether such processing plays a role in oligodendroglial development. I found that dystroglycan is cleaved in the postnatal cerebral cortex and that decreasing levels of dystroglycan cleavage correlate with developmental myelination. Similarly, OPCs in culture exhibit dystroglycan cleavage, which substantially decreases with oligodendroglial differentiation and appears to be promoted by laminin-211 but not laminin-111. Furthermore, while dystroglycan processing is due to metalloproteinase activity, it is not mediated by the metalloproteinases that cleave dystroglycan in other tissues (MMPs-2 and -9). Blocking dystroglycan cleavage using metalloproteinase inhibitors resulted in decreased OPC proliferation, where a transient G0/G1 arrest in cell cycle progression correlated with decreased expression of cyclin-cdk complex proteins. Inversely, the expression of a dystroglycan construct designed to mimic cleaved dystroglycan resulted in an increased trend in OPC proliferation. These results suggest that dystroglycan cleavage is involved in OPC proliferation and that appropriate dystroglycan remodeling may contribute to generating appropriate numbers of OPCs required for normal myelination. In other tissues, it is known that MMP-7 cleaves the dystroglycan ligand laminin. I found that exogenous MMP-7 impedes normal oligodendrocyte differentiation while MMP-7 inhibition promotes it, suggesting that MMP-7 is an inhibitor of myelination. Together, these studies demonstrate that MMP activity is important for multiple steps in oligodendroglial development, including OPC proliferation and oligodendrocyte maturation.

To the Aguirre and Leiton families, both of whom inspired my path into science.

My grandfather Juan DeJesus Aguirre
My Mother Ana Ines Aguirre
My grandmother Helena Leiton
My father German Leiton
My brother Juan Kevin Leiton

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List of Abbreviations

β-DG	Beta-Dystroglycan
ADAM	A Disintegrin and Metalloproteinase domain containing protein
BiPS	(2R)-[(4-Biphenylylsulfonyl)amino]-N-hydroxy-3-phenylpropionamide
BL	Basal Lamina
cKO	conditional knock out
Cdk	Cyclin dependent kinase
CNP	2',3'-cyclic-nucleotide 3'-phosphodiesterase
CNS	Central Nervous System
CTRL	Control
dy/dy	α2-laminin-deficient mouse
DG	Dystroglycan
DGC	Dystrophin-Glycoprotein Complex
EAE	Experimental Autoimmune Encephalomyelitis
EGFP	Enhanced Green Fluorescent Protein
F-actin	filamentous actin
Fn	Fibronectin
GFAP	Glial Fibrillary Acidic Protein
GM6001	Galardin; Broad spectrum MMP inhibitor
hESC	human Embryonic Stem Cell
HI	Hypoxia-ischemia
ICD	Intracellular domain
IGF	Insulin Growth Factor

LAMA2-/-	laminin α2 knock out mice
LM	Laminin
MMP	Matrix Metalloproteinase
MBP	Myelin Basic Protein
MCI	Mild Cognitive Impairment
MGE	Medial Ganglionic Eminence
MS	Multiple Sclerosis
NG2	Neural/Glial Antigen 2
NSC	Neural Stem Cells
NMDA-R	N-methyl-D-aspartate receptor
OPC	Oligodendrocyte Progenitor Cell
OL	Oligodendrocyte
PBMCs	Peripheral Blood Mononuclear Cells
PDB	Phorbol Dibutyrate
PDL	Poly-D-Lysine
PKC	Protein Kinase C
PMA	Phorbol Myristate Acetate,
PNS	Peripheral Nervous System
PVL	Periventricular Leukomalasia
Rb	Retinoblastoma
SDS	Sodium Dodecyl Sulfate
SEA	Sea urchin, Enterokinase, Agrin
SVZ	Subventricular Zone

TH3	Thyroid Hormone 3
Tn	Tenascin
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
Vn	Vitronectin
VLCFA	Very Long Chain Fatty Acids

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Thanks to my thesis committee for their patience and support throughout my growth as a student and that of the projects. Thanks for being willing to share reagents and experiment suggestion, which were very helpful when I had more negative data than we would have liked.

Thanks to my labmates, current and past. Iva Tzvetanova kept me sane during my qualifier, and taught me to be rigorous and disciplined to get somewhere in science. Her tough love and her criticism were always appreciated. Thanks to Chris Eyermann and Freyja McClenahan for sharing advice, complaints, and laughs. I'm sure we all benefitted from knowing that someone else was there to listen and relate to our technical troubles, and what seemed at the time, to be mindless experiments. ***Thanks*** to my advisor, Holly Colognato, for her wholehearted support from the moment I arrived in her lab before I was even a graduate student. Thanks for your wise advice, your toughness, and above of all, *your patience*.

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Nina Maung-Gaona, Kathryne Piazzola, and Toni Sperzel at the Center for Inclusive Education for their support on so many levels during my time here at Stony Brook and for recruiting me here! I especially thank Nina for keeping an eye out for me and caring about my success and well-being. I will always be grateful to you for acknowledging my efforts,

accomplishments and talents, and you should know that you inspire me to do my best as a person and in science, every day.

I am also grateful to my dear colleague, best friend, mentor and heart-sister, Luisa Fernanda Escobar-Hoyos, who kept me encouraged and motivated during the last few years of my doctoral process. As my house-mate and friend, she listened to me without judging, provided comfort and care when I needed it most, and built with me a home away from home.

I thank every person who has taken care of me and maintained my health and well-being, both physically and mentally, while I went through the doctoral process. While there are too many people to name, I will say that the Totong family was one of the kindest and most endearing to me. They welcomed me to Long Island and into their home with loving arms. ***Thank you*** for your unconditional care and love to me and your respect for my decisions.

To be the first in both of my families to earn a doctorate degree is probably the most important accomplishment of my life. *Both* of my families have gone through many, many troubles, sacrifices, hard work and labor to get me to where I am today. I am sincerely most thankful to them, for providing everything they ever have and allowing me to pursue my dream.

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Tzvetanova, I.D., McClenahan, F., Leiton, CV, Osaka, M., Schmedt, C., and Nave, K.-A., and Colognato, H. **C-terminal Src Kinase regulates oligodendrocyte progenitor development.** *(In revisions)*

Kuo E, Park DK, Tzvetanova ID, Leiton CV, Cho BS, Colognato H. **Tyrosine phosphatases Shp1 and Shp2 have unique and opposing roles in oligodendrocyte development.** J Neurochem. 2010 Apr;113(1):200-12. PMID: 20132481

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- Annual Conference, American Society for Neurochemistry, Baltimore, MD, March, 2012.
- Society for Neuroscience Annual Meeting, Washington, D.C, November, 2011.
- Gordon Research Conference on Matrix Metalloproteinases, Smithfield, RI, August, 2011.
- Celebrating Academic Excellence in Graduate Education at Stony Brook University, Stony Brook, NY, May, 2011.
- STEM Diversity Summit, Farmingdale State College, Farmingdale, NY, March, 2011.
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- Attendee. Glia in Health and Disease Meeting, Cold Spring Harbor Laboratories, Cold Spring Harbor, NY, July, 2008 & 2010.
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Chapter 1

General Introduction

1. Overview of myelin associated pathologies and areas of possible intervention

Myelination, the ensheathment of neuronal axons by a specialized glial plasma membrane, is essential for normal mammalian nervous system function. Myelin provides increased electrical transmission, fidelity, and speed over long distances, as well as protection from toxicity and architectural and metabolic support to the neuronal circuitry [1]. Central nervous system myelination is carried out by oligodendrocytes (OLs), a type of specialized glial cells which arise from a pool of neural stem cells early in brain postnatal development (Fig. 1-1).

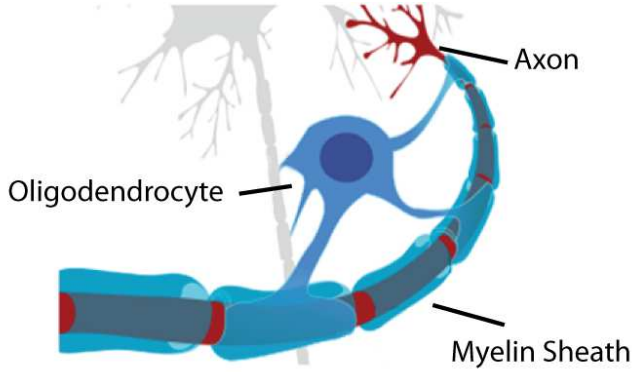


Figure 1-1: Oligodendrocytes form myelin sheaths on CNS axons.

Timely myelination and maintenance of myelin sheaths are both aspects of myelination that are necessary for proper brain function. These aspects of myelination are highlighted in conditions where myelination timing or maintenance are impaired, such as in perinatal hypoxia-ischemia and in a group of white matter pathologies collectively referred to as "leukodystrophies." Hypoxia-ischemia (HI) in preterm infants is the primary cause for white matter lesion formation and injury (known as Periventricular Leukomalasia, PVL). This condition is characterized by cell death at the ischemic core and selectively affects pre-myelinating oligodendrocytes due to their susceptibility to death at this point during their development [2]. Ischemic cell death requires repair and the generation

of new oligodendrocytes to continue the myelination program. The injury event and recovery presents a major interruption in normal developmental myelination, and can have consequences later in development [3]. Children who suffered such an insult are at high risk for developing neurological deficits that range from cerebral palsy to learning disabilities. This condition demonstrates the importance of proper myelination timing.

The leukodystrophies are characterized by mutations that occur in genes that directly or indirectly affect oligodendrocytes. X-linked adrenoleukodystrophy is the most common of these inherited disorders (1:17,000) in which fatty acid metabolism is altered due to a mutation in *ABCD1*, a peroxisomal enzyme involved in the beta-oxidation of Very Long Chain Fatty Acids (VLCFA) which include gangliosides, sphingomyelin and other lipids known to be incorporated at high levels into the myelin sheath [4]. Developmentally, patients present no issues until about 4-8 years of age, suggesting myelination is sufficiently well developed. However, while the exact cause of the disease trigger is unknown, it is known that the accumulation of VLCFAs in the patient brains correlate with clinical symptoms and disease progression, suggesting that VLCFA accumulation leads to brain toxicity [5]. Given that developmental myelination appears to carry out normally, this disease suggests that maintenance of the formed myelin sheath is also important in proper brain function.

Maintenance of the myelin sheath in the adult brain is also crucial for healthy brain function. In a CNS pathology known as multiple sclerosis (MS), white and gray matter lesions are a consequence of repeated autoimmune attacks to proteins that make up the myelin membrane. While the exact cause of why and how autoimmunity initiates in MS patients is unknown, the disease pathology is well characterized and includes such hallmarks as progressive inflammation, demyelination, axon loss, and gliosis that altogether can result in significant of

loss of vision and motor coordination (and may eventually lead to paralysis) [6]. In the first stages of the disease, the inflammatory and demyelinating pathologies predominate. It is proposed that during this phase, the brain is able to keep up with the necessary repair, i.e. generation of new oligodendrocytes and remyelination, until a threshold is reached where repair can no longer keep up with autoimmune attack and damage. However, the first phase of the disease can occur for years before any severe symptoms manifest. The transition into the secondary, or progressive, phase of MS, the most difficult one to treat and prevent, is marked by axon loss, gliosis and subsequent neurodegeneration that occurs as the increased severity of neurodegeneration with axon loss and more severe and irreversible symptoms.

In MS, neurodegeneration correlates with oligodendrocyte dysfunction and their inability to carry out repair. This can be characterized by 1) the depletion of the oligodendrocyte progenitor cell (OPC) pool that normally gives rise to mature oligodendrocytes for repair of damaged myelin, and/or 2) the ability of the available OPCs to differentiate once they've reached their target sites for repair [7-10]. At least 2 main treatment strategies could therefore be harnessed to treat MS: targeting the immune response to weaken its debilitating effects and promoting the proliferative capacity and differentiation of endogenous oligodendroglia. The first strategy is already in place, and can lessen immune attack frequency and severity. The latter strategy presents a significant challenge because there aren't any available drugs that target oligodendroglia and remyelination [11]. The current available lines of treatment that suppress the autoimmune response do not lessen the neurodegeneration that occurs in the progressive phase of the disease [12]. Therefore it is widely accepted by physicians and researchers that this treatment regimen is not sufficient, and that immune suppression should be complemented by oligodendrocyte repair agonists.

One of the strategies to identify modulators of remyelination and potential therapy targets is to understand the functions of molecular regulators during oligodendrocyte development. By targeting these effectors, the process through which oligodendrocytes are generated in development could be recapitulated in the adult brain and perhaps be pharmacologically accelerated in remyelination during disease.

2. Oligodendrocyte development and CNS myelination: regulation in time and space

Oligodendrocyte progenitor cells (OPCs) are cells characterized by their expression of the cell surface antigens NG2 and PDGFR-α (Fig. 1-2). OPCs are progeny of the neural stem cell populations in the medial ganglionic eminence (MGE) and the subventricular zone (SVZ), located in the brain's lateral walls of the ventricles [13-15]. NG2+PDGFR-α+ OPCs proliferate, migrate and populate the CNS in the late embryo and early post birth. Through extrinsic and intrinsic mechanisms (discussed below), it is thought that once reaching their target sites, many OPCs exit the cell cycle and begin the differentiation program. This is a process that is characterized by the transition of NG2+PDGFR-α+ OPCs to NG2-PDGFR-α$^-$CNP+. These CNP positive cells are termed "early pre-myelinating" oligodendrocytes whose primary role is to identify unmyelinated axons and form myelin sheaths around them. During the transition process from OPC to early-premyelinating oligodendrocytes, it is thought that the loss of NG2 as well as that of PDGFR-α is permissive for differentiation, and the upregulation of CNP (2',3'-cyclic-nucleotide 3'-

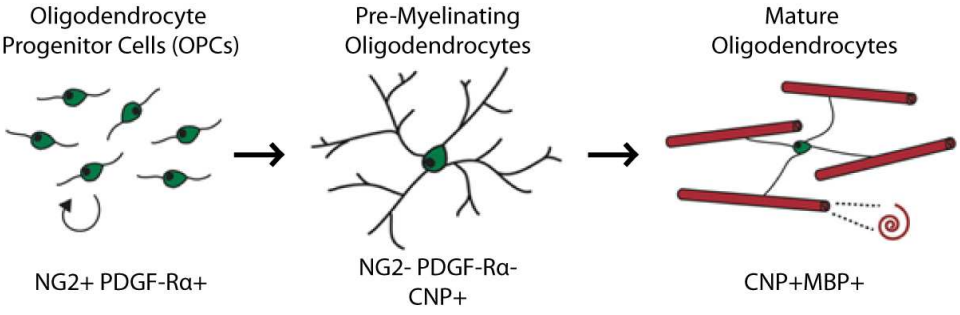


Figure 1-2: The stages of oligodendrocyte development (top) and cellular markers (bottom) used to identify oligodendrocyte differentiation.

phosphodiesterase), a myelin associated enzyme, demonstrates the cell's commitment to the morphological changes it is undergoing. In the pre-myelination stage, newly-formed oligodendrocytes extend process from their cell body and search for available axons. Upon axon contact, oligodendrocytes begin to express myelin associated proteins such as the Myelin Basic Protein (MBP) and wrapping will take place. Oligodendrocytes that express both CNP and MBP are considered mature oligodendrocytes in that they are myelination-competent.

Oligodendrocyte development is an elaborate developmental process that, in the rodent brain, largely occurs postnatally. Myelination in the brain follows a caudal to rostral direction, where the earliest myelinated areas can be identified at postnatal day 4. By postnatal day 21, most of the murine brain will be myelinated, or have begun to be myelinated, although the full completion occurs at about 4 weeks post-birth. Comparing the murine time points to that of human reveals the increased complexity of that of the human brain. While myelination occurs in a similar directional pattern (caudal to rostral), the timing of the events are different. The pre-frontal cortex is the last area to be myelinated and this occurs post-puberty during the later teenage years.

In the adult brain, it is known that the germinal niches that maintain neural stem cells (NSC) are the subventricular zone (neurogenic and gliogenic) and the sub-granular zone of the hippocampus (mostly neurogenic) [16]. In response to injury events in the brain, these niches are "activated" in which NSC proliferation yields new progeny [14, 16]. However, another mechanism for injury involves the activation of adult NG2+ cells, which are most commonly known as adult oligodendrocyte progenitor cells, are also termed "polydendrocytes" and are considered the fourth major type of glia in the CNS [17]. The reason behind this alternative name came from studies in which polydendrocytes were found to be the most abundant proliferating

cell in the adult brain (where approximately 70% of BrdU incorporating cells in the adult brain were NG2+) [18]. While polydendrocytes give rise to oligodendrocytes during development, more recent studies suggest that their role doesn't stop there. Polydendrocytes have been characterized to "tile" various areas of the brain where they occupy a restricted and tightly regulated density and area. Using lesion models, it was discovered that these cells are the first to respond to injury, in which they migrate to the lesioned area, proliferate, secrete factors that recruit microglia to phagocytose damaged cell components and debris, and quickly "re-tile" the recovering area [17].

Polydendrocyte multipotency has been proposed, however, it is still hotly debated. While some studies have claimed that polydendrocytes can give rise to neurons, more recent studies using more defined and elegant tools for fate mapping have failed to identify neurons as a direct progeny. However, polydendrocytes in the ventral forebrain clearly can give rise to astrocytes, suggesting that dorsal and ventral polydendrocytes have different astroglial potential and may also respond to differences to regional cues [19].

Furthermore, polydendrocytes may also contribute to synaptic maintenance in that they can associate with neuronal synapses, respond to AMPA and NMDA via expression of receptors for these neurotransmitters and also modulate GABAergic activity [20]. While the functions of these neuro-glial interactions are still under investigation, it is clear that they are important neuromodulatory mechanisms that implicate glia in yet another aspect of brain function.

In summary, while OPCs present a pool of cells that can generate oligodendrocytes during development, their transition into polydendrocytes in the adult brain may allow them to repair and respond to injury in more intricate ways than once thought. This presents an opportunity to

understand the factors that regulate OPC development and whether these mechanisms can be reactivated in polydendrocytes during remyelination and repair.

3. The intrinsic and extrinsic molecular regulation of oligodendrocyte development

The OPC clock, cell cycle dynamics and regulators

Oligodendrocyte development is a finely tuned process that is regulated by many factors. Clonal studies performed in which OPCs were found to count the time and number of divisions before exiting the cell cycle to differentiate established the theory of the "OPC intrinsic timer" [21-24]. The conclusions of these studies are summarized in figure 1-3, where it is known that OPC behavior can be modified by both the mitogen PDGF and the thyroid hormone TH3 as follows: 1) OPCs, in the absence of PDGF and the presence of TH3, will complete the committed cell cycle and will exit to differentiate, 2) in the presence of both PDGF and TH3, OPCs will divide a maximum of 8 times before exiting the cell cycle to differentiate, and 3) in the presence of PDGF and the absence of TH3, OPCs will undergo at least 8 or more cell cycles until they reach either a stimulant that promotes differentiation (like TH3) or they will themselves exit cell cycle initiation by a mechanism that is currently not fully understood [25].

	PDGF	TH3	Outcome
Scenario 1:	-	-	Differentiation with no division
Scenario 2:	+	+	OPCs will divide for a maximum of 8 times and then differentiate
Scenario 3:	+	-	OPCs will continue dividing indefinitely until TH3 is added

Figure 1-3: Conditions and modulators of the OPC timer in vitro.

The cell cycle is regulated by various molecular inducers and inhibitors. Figure 1-4 shows the cell cycle process in which the Gap1 (G1) phase is characterized by a time when the cell concentrates on growth and makes decisions about its continuing fate. During G1, cells can enter a G0 phase known as quiescence where cells appear to be in a resting state and are not

considered to be in either active differentiation or proliferation. If the cells do not enter G0, the more common alternate choices are to exit and differentiate, or they will reach a "restriction point" later in the G1 phase that once past, will irreversibly commit them to continuing through the rest of the cell cycle. There are groups of key proteins involved in progression from G1 to S phase. The cyclins (cyclins A-L, and T) are a group of proteins that through binding the cyclin dependent kinases (cdks 1-11) form a complex that phosphorylate cell cycle inhibitor proteins (like Retinoblastoma, Rb) to induce their degradation, and in this way, promote cell cycle progression [26]. There are two families of cell cycle inhibitors: the *cip/kip* family includes the genes p21, p27 and p57, and the INK4a/ARF family includes p16(INK4a), p15(INK4b), p18(INK4c), and p19(INK4d)). Members of both cell cycle inhibitor families can prevent cell cycle progression by inhibiting the formation if cyclin-cdk complexes [27]. These events are summarized in figure 1-4.

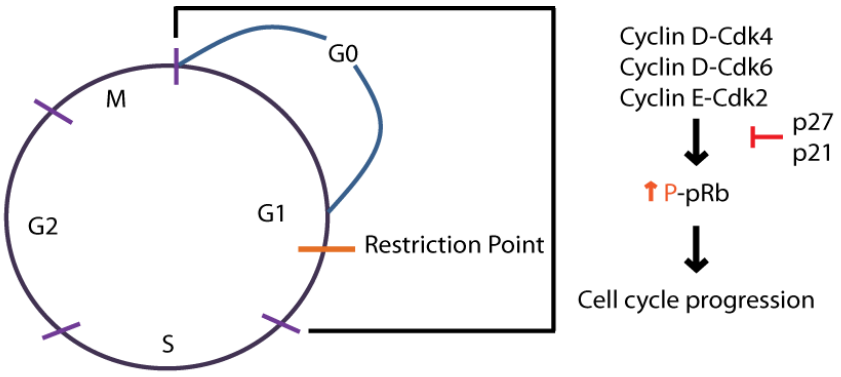


Figure 1-4: Cell cycle phases (G1, S, G2, M) and molecular regulators. The restriction point (RP) is the cell's commitment to proceed past G1. Prior to RP, "exit" into G0 (differentiation, quiesence or senescence) can occur. Cyclin-Cdk complexes promote Retinoblastoma (pRb) phosphorylation, resulting in its degradation, and therefore, cell cycle progression. Cell cycle inhibitors (i.e. p27 and p21) prevent pRb phosphorylation, and inhibit G1 progression.

Some of these cell cycle regulators have been explored to understand how the OPC clock works and how it can be modulated. The cip/kip family of cell cycle inhibitors are expressed in OPCs and oligodendrocytes. In particular, p27 protein levels increase throughout cell divisions and it is associated with the ability of OPCs to "time" their cell cycle exit [28, 29]. Similarly, p57

protein levels increase from one cell division to the next, regulating how many times OPCs divide before exiting the cell cycle [30]. On the other hand, p21 is not necessary for cell cycle exit, but it is required for differentiation [31]. Cyclin-cdk complex proteins (cyclin D1-cdk4, cyclin D1-cdk6 and cyclin E-cdk2) are expressed in OPCs and promote G1 progression as expected. As p27 levels rise in proliferating cells, p27 has been shown to progressively associate with cyclin E-cdk2 complexes pushing G1 arrest and eventually cell cycle exit in OPCs [32]. Expression of these cell cycle regulators in OPCs are explored in Chapter 2.

An overview of extracellular factors involved in oligodendroglial development

Extrinsic regulators include a slew of proteins ranging from ECM proteins and proteinases (described in more detail below), to morphogens, growth factors and hormones. Examples include the Wnts, Insulin Growth Factors (IGFs), and thyroid hormone.

The Wnt/β-catenin signaling pathway appears to regulate several different stages in oligodendrocyte development. Over-expression of Wnt or constitute activation of β-catenin in early stages of neural development both result in decreased expression of oligodendrocyte maturation markers with no changes in proliferation [33]. A later study, however, showed that inhibiting Wnt prevents expression of myelin markers and myelination [34-36]. The current explanation for these contrasting results is that Wnt may function in earlier time points as an inhibitor of myelination where it focuses on promoting neurogenesis versus oligodendrogenesis, and later, it switches to promote the maturation of oligodendrocytes. Ongoing studies are aimed at resolving these discrepancies.

IGF-1 null and IGF-1 overexpressing transgenic mice have provided insights into the importance of the IGFs in brain development and function. IGF-1 null mice display reduced

brain size and hypomyelination, whereas IGF-1 overexpressing mice have 55% larger brains and increased myelin content [37]. IGF-1 is an important regulator of OPC proliferation and survival, where it promotes cell cycle progression by activating the MAPK pathway, preventing cyclin D1 degradation and promoting cyclinD1 nuclear translocation [38]. IGF-1 also enhances OPC survival by acting through the PI3K pathway and inhibiting Bax translocation, cytochrome c release and caspase-3 activation [39]. IGF-1-mediated effects appear to be critical for normal oligodendrocyte differentiation *in vivo*, where IGF-1 knockout mice display reductions in mature oligodendrocytes and accompanied by OPC cell death [40]. Remyelination studies have shown that IGF-1 receptor nulls fare worse than WT littermates due to decreased OPC proliferation and accumulation at the site of injury, in addition to increased cell death [41]. Current studies are underway to determine the best methods to deliver IGF-1 during remyelination, which could aid in the myelin repair process.

Finally, the thyroid hormone 3 (TH3) has a profound role in promoting oligodendrocyte differentiation and myelination. It appears that TH3 acts through nuclear receptors to influence the expression of myelin associated proteins and lipids, where hypothyroidism is associated with decreased levels of these components and has been shown to results in hypomyelination, while hyperthyroidism results in the inverse situation [42, 43]. Similarly to IGF-1, ongoing studies are seeking to address whether TH3 can promote repair in demyelinated lesions in humans.

4. ECM proteins and ECM receptors that regulate oligodendrocyte biology

In the adult brain, there are only 2 areas that contain formal basement membrane structures: at the pial basal lamina on the brain surface and the vascular basement membranes at the blood brain barrier. However, ECM exists in other forms in different cell-associated areas of

the brain such as perineuronal nets that surround neurons, laminin containing "speckles" that are present at the ventricular surface, and at laminin-containing structures termed "fractones" and "bulbs" that appear to be limited to the subventricular zone germinal niche [44, 45]. While we know that the ECM changes from being broadly expressed in the embryonic brain to regionally-confined and less widely expressed in the adult, we don't yet know how the change in ECM levels and location occurs. While oligodendrocytes don't directly interact with basement membrane-like structures, human disease and ECM loss of function studies have provided us with clues about the importance of the ECM during their development and maintenance. A select few are discussed below.

Insights into ECM protein functions in developing and repairing oligodendrocytes

Some ECM proteins have been studied in the context of oligodendrocyte development and myelination. The tenascins are glycoproteins known for their expression in vertebrate embryos and in actively remodeling adult tissues. Four members make up the tenascin family: tenascin-C, R, X and W. Tenascin-C (Tn-C) is the most studied, and it is expressed in the brain in various regions during development, but in the adult, it is restricted to the hippocampus and borders of the subventricular zone. OPCs express Tn-C, but downregulate Tn-C expression as they differentiate. OPCs cultured on Tn-C displayed reduced membrane expansion, decreased migration and delayed maturation. Inversely, OPCs isolated from Tn-C knockout mice revealed that OPC proliferation was attenuated, and that OPCs were more susceptible to death presumably through their loss of responsiveness to PDGF [46]. However, Tn-C knockout OPCs showed enhanced myelin membrane formation, increased migration along the optic nerve during time points leading up to myelination of this nerve tract, and matured earlier. These results indicate that Tn-C is a suppressor of oligodendrocyte differentiation. However, Tn-R appears to have an

inverse role, in that OPCs upregulate its expression as they begin differentiation [47]. These findings for both Tn-C and Tn-R were confirmed in a more recent study performed with various *in vitro* and *in vivo* techniques [48].

Another inhibitory ligand for OPC differentiation is Fibronectin (Fn), which exists in 2 major variants: plasma Fn, secreted into the circulation by hepatocytes, and cellular Fn, which is produced by resident cells. While Fn is scarcely found in the adult brain, Fn (both plasma and cellular-secreted) is upregulated in MS lesions and is secreted by astrocytes, microglia, and endothelial cells, but not oligodendrocytes or OPCs [49]. Furthermore, Fn in lesions forms aggregates that are promoted by persistent inflammation, and inhibits oligodendrocyte differentiation. Developmentally, Fn appears to be inhibitory to oligodendrocyte differentiation in that OPCs plated on Fn substrate fail to reach normal MBP+ cell numbers and display altered membrane morphology [50]. This is supported by a study in which MMP-9, a matrix metalloproteinase required for oligodendrocyte process outgrowth (described in more detail below), is mislocalized when OPCs are plated on Fn substrates (associating with the cell soma instead of translocating to distal processes) [51]. This suggests that Fn can inhibit oligodendrocyte differentiation by preventing proper localization of proteins (like MMP-9) that promote process extension. Another mechanism that Fn uses to prevent OPC differentiation is by acting through its integrin receptor, which leads to the phosphorylation of MARCKS, a modulator of cortical actin cytoskeleton involved in vesicular trafficking that in turn can prevent proteins required at the membrane to form myelin sheets [52]. In summary, Fn prevents oligodendrocyte differentiation through mechanisms that affect protein localization and proper membrane formation and extension.

Vitronectin (Vn) has been linked to promoting proliferation where a rat oligodendrocyte cell line (CG-4) stably expressing β3 integrin showed enhanced proliferation in response to Vn [53]. Interruption of the ligand-receptor interaction through β3 blocking antibodies reversed the effect, suggesting that vitronectin may promote proliferation in OPCs. A later study showed that even though OPCs attach to Vn, they require PDGF stimulation for Vn to promote OPC proliferation through a mechanism that involves αvβ3 integrin to potentiate the mitogen response [54]. A more recent study, however, reported that Purkinje cells in the cerebellum stimulate oligodendrocyte *differentiation* through increased expression of Vn [55]. The difference in these results may be explained by different mechanisms that regulate OPC development *in vivo* where stimulation by PDGF may potentiate a proliferation response, but a pro-differentiation factor could switch Vn response to promote differentiation. Interestingly, current technologies being developed for methods to promote hESC (human Embryonic Stem Cell) differentiation into OPCs has shown that Vn promotes oligodendrogenesis, suggesting that Vn promotes oligodendrocyte specification [56]. Further studies will be required to better understand the complete function of Vn on oligodendrocyte development *in vivo*.

The laminins are a group of trimeric proteins that are formed upon the assembly of 1 of each type of 3 independently expressed protein products: the α, β, and γ subunits. There are 5 α, 3 β, and 3 γ subunit types whose combination compose 15 known types of laminins identified *in vivo*. Among these, Laminin-α2 (a component of laminin-211: α2,β1,γ1) appears to be expressed embryonically (E18.5) during the time points of myelination in the brain stem suggest that this laminin may play an important role in myelination. Although it is unknown whether this laminin is neuronal- or glial-derived, one can speculate that the presence of this laminin may act as a mechanism to capture OPCs or oligodendrocytes that express receptors for laminin-α2 (discussed

in more detail below). In vitro studies of laminin's role in oligodendrocyte development have shed light on this possibility. Early studies on ECM substrates that promote or inhibit differentiation identified laminin-211 as a factor that promoted myelin membrane sheet formation *in vitro* [50]. This study also found that blocking the β1 subunit of the integrin receptors exclusively abolished the effect induced by laminin-211 (more details on the integrin function in oligodendrocytes below). This suggested that laminin-211 aids in oligodendrocyte membrane expansion and may contribute to myelination *in vivo*.

Further studies using in vivo models of laminin-211 loss-of-function have confirmed aspects of the *in vitro* work and revealed additional roles for this protein in oligodendrocyte biology and myelination. In the first study, mice deficient in the expression of the laminin α2 subunit (termed *dy*/*dy*) due to a spontaneous and unknown mutation in the non-coding region of the laminin α2 gene, displayed a delay in oligodendrocyte development where oligodendrocyte progenitors accumulate and have an impaired ability to reach the pre-myelinating stages of development [57]. As a result, myelinated axons in the corpus callosum (major myelinated tract in the cerebral cortex), brain stem and cerebellum, displayed decreased myelination. However, studies in these mice are restricted because 1) the site of the laminin mutation is unknown in these mice, making genotyping impossible. Therefore, they can only be distinguished phenotypically, as *dy*/*dy* mice develop severe muscular dystrophy with hindlimb paralysis by ~15 days post-birth. Because these mice generally do not live past 6 weeks of age, the window of study is restricted (~P15-6weeks of age). A second mouse model, the laminin α2 knock out, was subsequently used to determine if the generation of OPCs is affected in the absence of laminin-2.

The study using laminin α2 knock out (LAMA2-/-) mice supported the initial findings from the *dy*/*dy* mice [58]. First, an accumulation of OPCs occurred at later time points in brain development that correlated with the time points studied in the *dy*/*dy.* In addition, it was found that oligodendrogenesis was altered in that reductions in OPCs in the germinal niche, the subventricular zone, were accompanied by increased cell death, suggesting that laminin-2 also plays a role in OPC survival. Finally, myelination was also altered in these mice, with both fewer myelinated axons and thinner myelin. However, an important function that was established in this study was that of the role of laminin alpha2 in the subventricular zone, a germinal niche in which a specialized ECM has previously been described to "trap" growth factors to make them available to nearby cells. This suggests that these laminin containing structures may be important for oligodendrogenesis, and further studies will be required to describe what the exact roles of laminin and these structures are during gliogenic development.

Laminin receptors expressed in oligodendroglia regulate important developmental functions

Two types of receptors mediate laminin binding in oligodendroglia: the α6β1 integrin and dystroglycan. Integrins are ECM receptors with a repertoire of 24 different combinations by virtue of the coupling of an α and β subunit. It is thought that the α subunit specifies the type of ligand the receptor can bind to, for example, α6 specifies the β1 subunit toward a laminin preference, and together, they compose a laminin receptor. Oligodendroglia express integrins αvβ1, α6β1, αvβ3, αvβ5, αvβ5, and αvβ8. Of these, α6β1 is the only laminin receptor.

During oligodendrocyte development, α6β1 integrin has been shown to regulate growth factor mediated survival where mice deficient in α6β1 display increased oligodendroglial apoptosis [59-62]. Integrin-based adhesion alone does not appear to promote oligodendrocyte

survival *in vitro*; instead, mitogenic stimulation by growth factors (i.e. PDGF, neuregulin-1, IGF-1, etc.) synergizes with laminin-integrin adhesion to enhance survival. This is thought to occur through growth factor receptor co-localization with integrins at lipid rafts [63]. However, the role of α6β1 integrin in myelination in incompletely understood due to the differing degree of results obtained by mouse models that studied ablation or expression of dominant negative forms of the β1 integrin subunit. In some cases, β1 integrin ablation has not shown any significant changes in myelination, where as in others, phenotypes range from decreased myelination of small caliber axons to decreased numbers of mature oligodendrocytes. Taken together, these studies show that loss of β1 integrin can result in myelin abnormalities. However, a few unanswered questions that apply to all of these studies are: 1) whether the second laminin receptor, dystroglycan, compensates for the loss of β1 integrin in oligodendroglia, 2) if so, to what extent and how does compensation occur, and 3) what are the functions that dystroglycan independently regulates in developing oligodendrocytes?

A few early studies into the function of dystroglycan in the CNS demonstrated its role in the development of the brain's cortical plate [64]. Because laminins and other ECM proteins are part of the pial basal lamina (BL), radial glial cells express dystroglycan to bind the pial BL during cortical development. Studies in which loss of dystroglycan was genetically altered in either neuronal or glial populations revealed that neuronal dystroglycan plays a role in synaptic

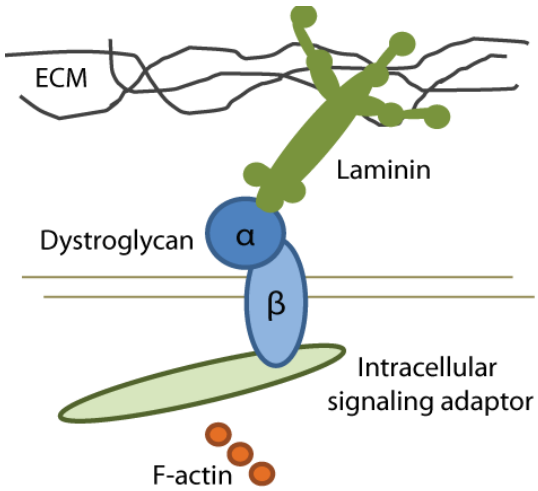


Figure 1-5: The DG-LM adhesion complex binds intracellular adaptor proteins to mediate cell signaling and support the actin cytoskeleton.

plasticity and that radial-glial dystroglycan is involved in forebrain development via regulating radial glial attachment to the pial BL. Dystroglycan is also expressed by astrocytes at the blood brain barrier basement membrane. Astrocytic morphology and blood brain barrier integrity is compromised in mice lacking neural dystroglycan (unpublished data, Michael Menezes). It is thought that dystroglycan also contributes to water uptake by astrocytes as dystroglycan forms a complex with aquaporin-4 at the astrocytic endfeet [65]. Dystroglycan has also been found to assemble on the surface of photoreceptor synaptic terminals where it is involved in normal retinal electrophysiology, although in a manner independent of laminin, and dependent on pikachurin, another dystroglycan ligand [66].

More recently, dystroglycan function has been explored in oligodendrocytes where it was found that blocking its binding site to laminin via blocking antibodies results in fewer CNP+ and MBP+ cells, decreased membrane expansion, and shorter and less complex processes [61, 67]. In a co-culture of dorsal root ganglion neurons and oligodendrocytes, blocking laminin binding to dystroglycan also resulted in the inability of OLs to initiate myelin segments [61]. At least one downstream signaling mechanism is known to contribute to the aforementioned effects mediated by dystroglycan. IGF-1 is known to promote oligodendrocyte differentiation through the activation of the MAPK signaling cascade, and it was found that siRNA-mediated knockdown of dystroglycan in oligodendrocytes leads to a blunted IGF-1 response in which MAPK activity is decreased [68]. Recent studies in our lab where dystroglycan has been genetically deleted from neural stem cells revealed increased gliogenesis but delayed myelination. The mechanisms behind this phenotype are a work in progress but suggest that dystroglycan has a role in oligodendrocyte maturation *in vivo* (unpublished results, Freyja McClenahan). Indeed, the laminin-dystroglycan signaling complex appears to be important for oligodendrocyte

development. However, how does this complex (Fig. 1-5) result in these effects on oligodendroglial development? The hypothesis studied in this dissertation suggests at least one way in which this could occur.

Dystroglycan post-translational modifications generate a functional matrix receptor

Dystroglycan is an ECM receptor comprised of two subunits. The beta subunit is transmembrane with cytosolic, transmembrane and extracellular domains, whereas the alpha subunit is entirely extracellular but remains tightly associated with the beta subunit to comprise a functional receptor unit. The alpha subunit of dystroglycan contains the extracellular ligand binding domain and binds extracellular matrix proteins such as laminin, perlecan, and agrin [69], while the beta subunit binds intracellular signaling mediators and adaptors such as dystrophin, utrophin, focal adhesion kinase, Grb-2 and caveolin-3 [70]. Forming a signaling axis that connects the ECM to signaling pathways and the cytoskeleton, dystroglycan plays an important role in cell adhesion, structural organization and cell survival. In some tissues, dystroglycan has been found to be a part of a group of proteins known as the Dystrophin-Glycoprotein Complex (DGC), which is composed of dystroglycan, dystrophin, α-, β-, γ- and δ-sargoclycans, syntrophin, dystrobrevin, and sarscospan [70]. In the DGC in skeletal muscle, for example, dystroglycan supports the structural organization of myofibrils by supporting cell survival and maintenance of the muscle tissue upon expansion and contraction

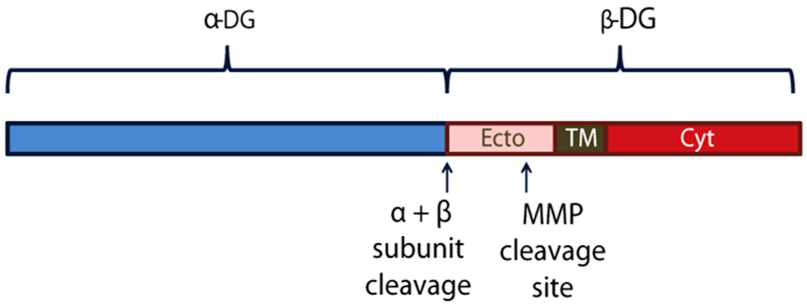


Figure 1-6: Dystroglycan undergoes post-translational auto-proteolysis into the α and β subunits. β-DG has extracellular, transmembrane and intra-cellular domains. Site of MMP-2 and 9 cleavage is located closely to the transmembrane region on the ectodomain.

[71]. In non-muscle cells such as Schwann cells, it has been shown that the DGC is required for the proper formation of Schwann cell cytoplasmic compartments around axons that regulate the appropriate length of myelin segments [72]. Furthermore, other studies have elucidated the DGC as an important signaling axis with roles in brain diseases and developmental disorders such as Mild Cognitive Impairment (MCI) and neuronal migration disorders [70]. Interestingly, some types of muscular dystrophies, which are caused by alterations in dystroglycan function, are also associated with MCI and cortical lamination defects, suggesting a role for the DGC in brain development and function [73].

The *DAG1* gene codes for a single protein product referred to as the "Dystroglycan precursor," which is post-translationally cleaved into 2 subunits: the extracellular α subunit, and the transmembrane β subunit (Fig. 1-6). The Dystroglycan precursor contains sequence similarities to secondary structures formed in SEA (Sea urchin, Enterokinase, Agrin) domains previously shown to result in autoproteolysis [74], such as that of highly glycosylated proteins known as mucins [75]. Interestingly, while SEA domains do not have exact sequence conservation among even the most related of the mucin proteins, the secondary structures as well as the cleavage site in this domain are well conserved and align between some of the mucin proteins and dystroglycan. This suggests that dystroglycan undergoes autoproteolytic cleavage post-translationally, and it is through this mechanism that the α and β subunits are separated.

Following the autoproteolytic separation of the 2 dystroglycan subunits, they enter the secretory compartments of the endoplasmic reticulum where the β subunit undergoes N-glycosylation, and the α subunit, extensive O- and N- glycosylations [69]. It is notable that the α-dystroglycan subunit is so heavily glycosylated that, although its predicted weight is 80 KDa, the apparent protein weight ranges from 120 kDa (brain) to 200 kDa (muscle), in a tissue specific

manner [76]. Glycosylation of dystroglycan remains incompletely characterized due to the many enzymes involved in and the complexity of its glycosylation process. Furthermore, the exact glycan composition and structure that is required for ligand binding is still undetermined, however, it is known that the O-mannose modifications on α-dystroglycan are essential to its ability to bind ECM ligands once it reaches the extracellular space [76] . Interestingly, 6 known putative glycosyltransferases that synthesize O-mannosyl glycans (POMT1, POMT2, POMGnT1, Fukutin, FKRP, and LARGE) all carry mutations that result in dystroglycanopathies, as many aspects of these diseases are recapitulated by dystroglycan loss-of-function in mouse models [77]. This highlights the importance of α-dystroglycan glycosylation for its proper function.

Further post-translational modification of dystroglycan can occur at the α subunit where it has been shown that furin pro-protein convertases cleave this subunit and result in loss of ECM adhesion in cancer cells [78]. It is currently unknown whether this cleavage product has any function, although it has been found in human patient serum, suggesting that it occurs in humans and that it is released systemically. Furthermore, the dystroglycan β subunit can undergo cleavage processing, an event that was investigated in detail in this dissertation and is further described in detail below.

5. Matrix Metalloproteinases in CNS development and oligodendrocyte biology

An overview of the matrix metalloproteinases

Twenty-four different enzymes constitute the matrix metalloproteinase (MMP) family (Fig. 1-7). They are divided into subgroups based on their shared protein domains or substrate preference (i.e collagenases, stromelysis, etc), although the latter property is known to vary

depending on the tissue context and expression pattern [79]. All MMPs share in common a zinc binding catalytic domain that is kept in an inactive conformation by a pro-peptide region that once removed (through mechanisms that include proteolysis, oxidation, s-nitrosylation, s-glutathiolation, alkylation, allosteric modulation and heavy metals) allows the active site to access substrates for activity [80]. Furthermore, with the exception of MMP-7, 26 and 23 (minimal domain MMPs), all MMPs share a linker hinge region and a hemopexin domain. The hemopexin domain is known to provide MMPs the ability to bind receptors and other proteins at the cell surface to localize MMPs at the sites of action, and thereby, modulate cell behaviors such as proliferation and migration [81-83]. While most MMPs are secreted, some are membrane tethered, either through the expression of transmembrane domains, GPI anchors, or Ig-like domain. Other MMPs share complimentary functions through the expression of domains that provide a similar capability, for example, the immunoglobulin-like domain for MMP-23 allows it to dock to proteins in the same way that the hemopexin domain allows other MMPs to bind other membrane proteins.

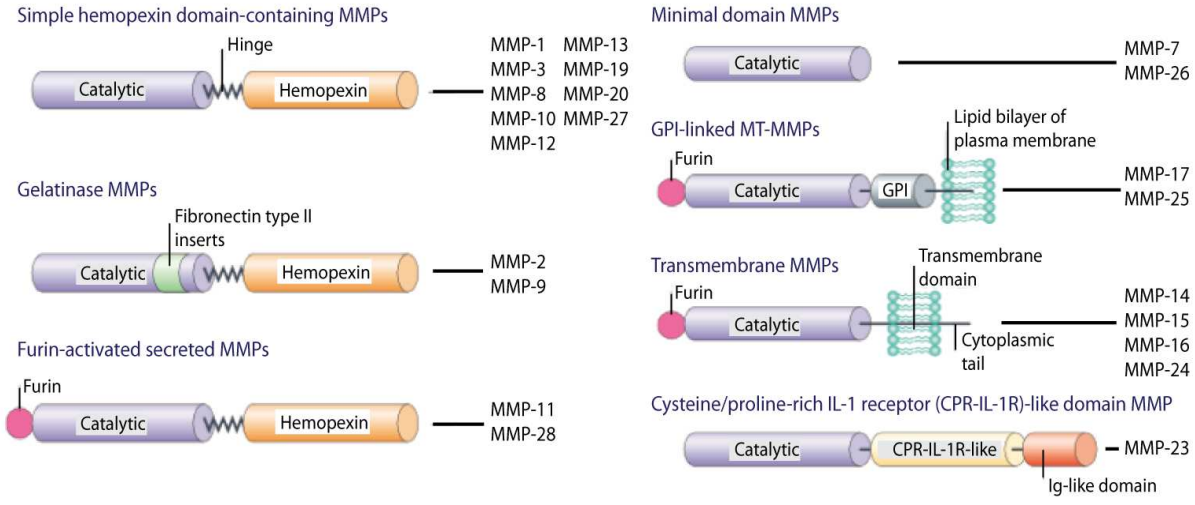


Figure 1-7: Matrix Metalloproteinase domain structure. (Adapted from Yong et al., 2005)

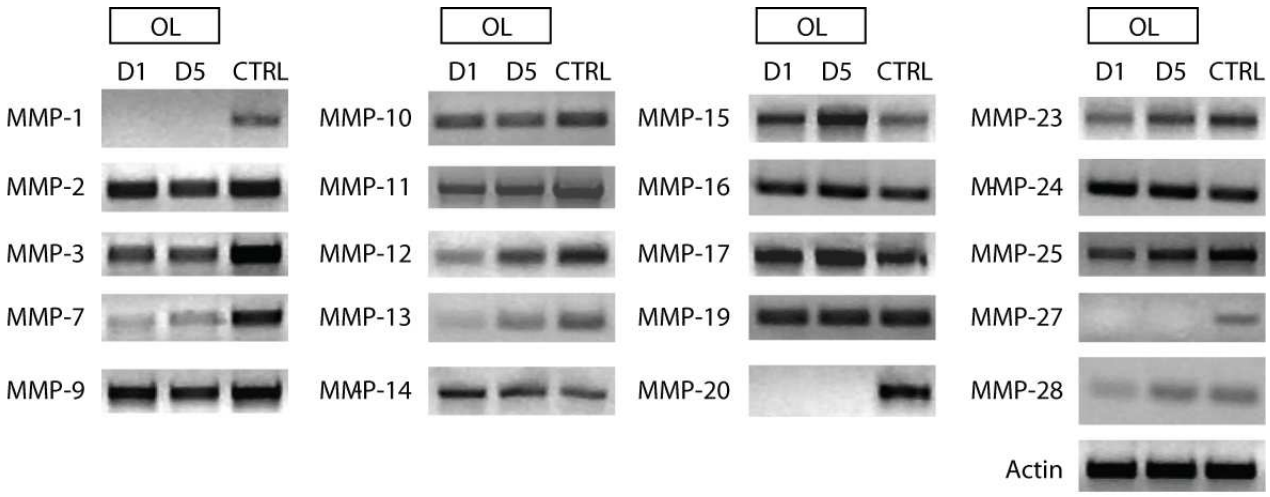


Figure 1-8: MMP gene transcripts expressed in differentiating oligodendrocytes. OPCs (OL D1) and mature oligodendrocytes (OL D5) were analyzed MMP expression. MMP-8 and 26 were excluded due to inadequate signal in oligodendroglial and positive control samples. MMP-1, 20 and 27 do not appear to be expressed by oligodendroglia. MMP-2, 9, 10, 11, 14, 19, and 24 appear to have little or no change in expression between time points, while MMP- 7, 12, 13, 15, 16, 17, 23, 25, and 28 appear to show some pattern of increased expression. Positive control: Rat uterus 1 day post birth.

MMP-9 is involved in oligodendrocyte development, myelination and remyelination

MMPs are known to be involved in the processing of extracellular matrix proteins, membrane-bound receptors, cytokines, growth factors, and even other proteases, indicating that they do more than just remodel ECM in that they are also activating signaling modulators that mediate cellular responses and behaviors, and initiating complex proteolytic cascades [84]. MMP-9 has been implicated in some of these aspects that coordinate oligodendrocyte function, and currently, it is one of the most well-characterized MMPs in oligodendrocyte development, myelination, and remyelination. Two studies have described the role of MMP-9 in oligodendrocyte process extension, a step that is crucial in allowing oligodendrocytes to maximize the number of axons they can myelinate. First, a gain of function approach utilized a PKC agonist (phorbol myristate acetate, PMA) as a pharmacological approach to induce MMP-9

expression in OPCs. Upon PMA treatment, a concomitant increase in MMP-9 is accompanied by increased process outgrowth [85]. These effects are blocked by PKC and MMP inhibitors, respectively, suggesting that PKC (a known regulator of oligodendrocyte process outgrowth [86]) may act through upregulation of MMP-9 activity to promote process outgrowth in oligodendrocytes. The second study employed 2 loss of function approaches: process outgrowth was characterized in OPCs isolated from MMP-9 null mice as well as in OPCs cultured in the presence of TIMP-1, an endogenous MMP inhibitor with high affinity toward MMP-9. In both cases, process outgrowth was decreased in OPCs compared to controls [87]. Together, these studies suggested that oligodendrocytes require MMP-9 for normal process outgrowth.

Subsequent studies demonstrated that MMP-9 regulates oligodendrocyte development and myelination *in vivo*. In one study, a major myelin tract in the brain, the corpus callosum, displayed a myelination delay at p7 in MMP-9 null mice that by p21 is comparable to that of the wild type littermate [88]. The study further demonstrated that MMP-9 normally cleaves IGFBP-6 (insulin growth factor binding proteins), a protein that normally sequesters IGFs until MMPs release them from inhibitory binding, during brain development. Because IGFs regulate the differentiation and survival of developing oligodendrocytes, in the absence of MMP-9, IGFs are kept inactive, and development is delayed.

Furthermore, through studies of lysolecithin-induced demyelination in the cerebral cortex, MMP-9 was shown to aid in the remyelination process by cleaving the inhibitory chondroitin sulfate proteoglycan (NG2), which normally accumulates at the injury site [89]. Two weeks after demyelination NG2 is cleared and remyelination begins in the wild type. This is in contrast to the MMP-9 null injury site in which NG2 deposition is accompanied by OPCs that

Table 1: Matrix Metalloproteinases implicated in oligodendrocyte development, myelination and remyelination

MMP	Implication	Mechanism/Proof of Point	Reference
3	Identified in forebrain germinal matrix cells (source of neurons and glial cells) and mature OLs via in situ hybridization		Del Bigio et al., 1995 [90]
	In a model of ischemia/reperfusion, MMP-3 was upregulated in OLs. In culture, it was identified in OLs but not OPCs.	Agrin clearance was correlated with MMP-3 expression in vivo. In vitro, MMP-3 cleaved agrin, suggesting that MMP-3 could be degrading agrin during injury.	Solé et al., 2004 [91]
	Correlated with the onset of demyelination in a spontaneously demyelinating mouse model (ND4)	Crossing the ND4 with transgenic-overexpressing TIMP-1 mice attenuated demyelination	D'Souza et al., 2002 [92]
	Expression in and around human MS lesions, primarily by immune cells.		Maeda et al., 1996 [93]
9	Expressed by OPCs at growing processes; required for OL process extension		Uhm et al., 1998 [85]
	Expressed in the optic nerve during myelination, OPCs isolated from MMP-9 null display decreased process extension		Oh et al., 1999 [87]
	MMP-9 null shows delayed myelination similar to that of MMP-12 null	Same as MMP-12 null (above)	Larsen et al., 2006 [88]
	Involved in promoting remyelination in a lysolecithin-induced demyelination in spinal cord model in MMP-9 null mice	Accumulation of NG2 at the lesion site prevents OPCs from migrating in to remyelinate	Larsen et al., 2003[89]
	Supporting evidence for MMP-9 promoting oligodendrocyte process outgrowth	Fibronectin, a substrate known to inhibit oligodendrocyte process outgrowth, mislocalizes MMP-9 on processes, preventing proper extension	Sisková, et al., 2009 [51]
	Acute MMP-9 Inhibition via SB-3CT in a model of spinal cord injury increased OPC proliferation and OL differentiation, and improves immunopathology of myelin	Cleavage of NG2 and NMDA-R in OPCs *in vivo*	Liu et al., 2011 [94]

	MMP-9 secreted by oligodendrocytes has an angiogenic effect	*in vitro*: OLs treated with IL-1β induce endothelial tube formation. *In vivo*: increased IL-1β in damaged white matter and increased MMP-9 expression associated with MBP+ OLs after Lysophosphatidylcholine induced demyelination.	Pham et al., 2012 [95]
	In response to a white matter injury model (prolonged cerebral hypoperfusion stress), OPCs located near the blood brain barrier (BBB) upregulated MMP-9 expression	MMP-9 secreted by OPCs weakened endothelial barrier *in vitro*, suggesting that the MMP-9 expressed by these cells in the disease model contribute to BBB breakdown	Seo et al., 2013 [96]
12	Expressed by OPCs and oligodendrocytes. MMP-12 null OPCs exhibited decreased process outgrowth, rescued by rMMP-12		Larsen et al., 2004 [97]
	Upregulated during brain myelination	Increased IGFBP-6 that is normally cleaved by MMP-12 to release IGF-1. MMP-12 null OLs treated with IGF-1 are rescued from maturation delay *in vitro*	Larsen et al., 2006 [88]
	Necessary for timely CNS myelination		
	MMP-12 null displays normal OPC numbers but decreased mature oligodendrocytes		
	Neonatal Hypoxic-ischemia induced brain injury shows MMP-12 upregulation in neurons, microglia and oligodendrocytes, suggesting that it contributes to brain injury		Svedin et al., 2009 [98]
	Highest upregulated MMP in a virus-induced demyelination model. Same model induced in an MMP-12 null showed reduced demyelination.	Injecting MMP-12 in white matter results in focal demyelination and decreased oligodendrocytes, suggesting it mediates demyelination	Hansmann et al., 2012 [99]
13	Produced by oligodendrocytes in a model of cerebral ischemia; Observed intra-nuclear localization in all brain cells where MMP-13 was upregulated.		Cuadrado et al., 2009 [100]
28	Cleaves myelin proteins		Werner et al., 2007 [101]
	Upregulated in EAE and MS demyelinated lesions.		Werner et al., 2008 [102]
	EAE treatment with blocking antibodies enhances myelination	Increased MAPK and pErbB2 and 3	

cannot enter the lesion site to remyelinate, suggesting that MMP-9 is required for clearance of NG2 in lesioned sites prior to re-infiltration of OPCs to remyelinate. Together, these studies identified roles for MMP-9 in process outgrowth, myelination and remyelination. However, because MMPs act through various different substrates to mediate their effects, it is important to understand what other substrates MMP-9 targets may exist that regulate myelination.

Other functions have been attributed to MMP-9, and other MMPs have been implicated in oligodendrocyte development, myelination and remyelination. These have been summarized in table 1. While one study describes the expression of some of the MMPs during oligodendrocyte differentiation [97], we took the opportunity to determine the expression pattern of the MMPs in cultured OPCs and oligodendrocytes. The results from this initial screening are illustrated in figure 1-8 and suggest that oligodendroglia are major expressors of MMPs during their differentiation.

MMP-7 is upregulated in mouse models of MS and in MS lesions in humans

An understudied MMP in the context of brain development is one of the minimal domain MMPs, MMP-7. This is the smallest (Pro-form: 28 kDa, Active: 19 kDa) member of the MMP family, but is a known mediator of various cellular behaviors and alterations that mediate disease progression. In particular, several studies have implicated its role in mediating the immune response in MS and Experimental Autoimmune Encephalomyelitis (EAE), a mouse model of MS in which an inflammatory attack is induced through injection of a myelin peptide fragment. Several days later, MS like symptoms are followed by demyelination and remyelination [103]. The first studies in which MMP-7 was found to be upregulated in EAE came in the late nineties when blocking MMP activity was being explored to treat neurodegenerative disease. MMP-9 and

MMP-7 were found to be expressed in the cerebrospinal fluid and spinal cords of EAE animals, and treatment with BB-1101, a broad spectrum MMP inhibitor, decreased disease severity in induced animals [104]. However, it was described that MMP-7 was primarily expressed by immune cells, which suggested that inhibiting their access to myelinated tissues could prevent the most prevalent damage associated with degeneration and poor disease prognosis. A subsequent study then confirmed that MMP-9 and 7 (via mRNA analysis) were expressed in all types of MS lesions [105]. However, this study did not identify which cells were associated with these enzymes, and did not characterize specifically whether immune cells alone or other cell types in the lesion expressed these MMPs. A study that followed looked at the expression of MMPs in subsets of peripheral blood mononuclear cells (PBMCs) (i.e. B, T lymphocytes, and monocytes) from normal individuals and found no differences in the expression of MMP-7 between these cell types. In MS patients, there were significant increases in MMP-2, 14 and Timp-2 in monocytes [106]. This suggests that PBMCs may not normally express MMP-7 until reaching the site of infiltration, whereby MMP-7 allows them to break down blood brain barrier molecules that allow them entry into the CNS. This is confirmed by a more recent study in which EAE induced in MMP-7 null mice showed protection from leukocyte infiltration and therefore no disease symptoms were observed [107]. While much work in EAE and MS has characterized the role of MMP-7 during the disease, it is not clear what the role of this enzyme is during the remyelination events that occur after the onset of injury, in addition to whether MMP-7 has any role during brain development, particularly of oligodendrocytes.

MMP-7 substrates relevant to oligodendrocyte development

One protein targeted for proteolysis by MMP-7 is the N-methyl-D-aspartate receptor (NMDA-R), which is an ionotropic glutamate receptor that is primarily known to control

synaptic plasticity and memory function [108]. NMDA-R is a heterotetramer composed of GluN1 and GluN2 or GluN3 (also denoted as NR1-3). Two subunits of either two combinations assemble to form a functional receptor: 2 GluN1 subunits + 2 GluN2 or 2 GluN1 + 1 GluN2 and 1 GluN3. Eight different isoforms of GluN1 and differential expression of GluN2 and GluN3 make up multiple receptor types that contain different functional properties. The extracellular domains of the subunits contain modulatory and ligand-binding domains, while intracellularly, they can bind various kinases, actin architecture regulators, and signaling proteins that coordinate cellular responses. NMDA-R activity is required for the formation of dendritic extensions that mature to form dendritic spines, the major sites of information processing in the brain.

Dendritic spines are formed by extensions of thin, motile filopodia that appear like hair-like extensions at the dendrite [109]. Through subsequent signaling and physiological responses, the filamentous actin (F-actin) becomes concentrated at these extensions and mature into functional dendritic spines which express NMDA-R. NMDA-R has actin binding activity in its intracellular portion, and thereby stabilizes dendritic spines through various mechanism reviewed in [110]. It is known that changes in the ECM surrounding dendritic spines affects their response to stimuli, maintenance and overall health. In an effort to understand what mechanisms lead to dendritic spine alteration in disease, Bilusova *et al* treated mature hippocampal neuron cultures (in which dendritic spine formation is completed) with recombinant MMP-7, one of the MMPs known to be upregulated during injury. MMP-7 treatment resulted in drastic changes in dendritic spine morphology where normal, mature dendritic spines underwent a major transformation into long thin filopodia reminiscent of immature spines. Furthermore, actin rearrangements were described in which filopodia were redistributed into rope-like structures in the dendritic shaft, a characteristic of premature dendrites. This suggested that MMP-7 severely altered dendritic spine

morphology. Interestingly, NMDA treatment induced the same effect on dendritic spines, suggesting that NMDA-R was involved. Indeed, treatment of cultures with MMP-7 and an NMDA-R antagonist (MK-801) abolished the effect induced by MMP-7. While cleavage of the NMDA-R receptor was explored in this study, it was not found to occur after MMP-7 treatment, suggesting that MMP-7 modulates dendritic spine dynamics through a different mechanism that affects the NMDA-R receptor but induces major changes in actin cytoskeletal dynamics.

The GluN1 subunit has been shown to be targeted for proteolytic cleavage by MMP-7 in hippocampal neurons, where cleavage of the GluN1 (and speculatively) GLuN2 subunits that can result in impaired ligand binding [111]. While this study characterized in the detail the site of GluN1 cleavage induced by MMP-7 in cortical slices, the functional significance of this cleavage event was not explored.

These studies raise intriguing questions about what the role of MMP-7 may be during oligodendrocyte development given that OPCs express NMDA-Rs. In OPCs, NMDA-Rs function in vivo to form synapses with neurons, are involved in mediating response to NMDA in vitro which promotes myelin protein synthesis, and are downregulated during oligodendrocyte differentiation [112, 113]. Although NMDA-Rs are not required for myelination or oligodendrocyte development, they appear to mediate an important aspect of oligodendroglial function in the brain, that of connectivity to and fine tuning of neuronal activity [114]. Therefore it will be interesting to explore the effects that MMP-7 may have on NMDA-R expressing OPCs in vitro and in vivo.

Another MMP-7 ligand that was first established in a pancreatic cancer model is the Notch signaling receptor [115]. Its canonical ligands, Jagged and Delta, are normally expressed

on axons and astrocytes, and function to induce notch cleavage by metalloproteinases (ADAM-17 or MMP-7). This cleavage event results in ectodomain shedding, and activates the next cleavage modification at the membrane where a different, membrane tethered metalloproteinase (ADAM-10; γ-secretase), cleaves and releases the Notch Intracellular Domain (ICD). The ICD translocates to the nucleus where it goes on to activate genes that repress OPC differentiation [116]. However, it is now known that "non-canonical" notch ligands (F3/Contactin) serve to promote OPC differentiation [117]. Because MMP-7 can cleave Notch in other cell types, it would be interesting to determine if this action of MMP-7 can lead to the repression or the promotion of OPC differentiation.

In summary, while the roles of MMPs have been established in many disease and injury models, it remains unknown whether they play a role in oligodendrocyte development, myelination, and myelin repair. From studies on MMPs-3, 9, 12 and 28, we have obtained clues that MMPs can contribute to proper development and recovery from injury. However, these are only 4 of 24 MMPs, of which the rest remain poorly characterized in the context of CNS function. However, we now also know that there are substrates that we can predict to be targeted by MMP activity and hypothesize to modulate specific functions relevant to oligodendrocyte development and myelination.

6. ECM and receptor remodeling during CNS development and possible implications for CNS function

MMP-7 has been implicated in cleaving laminin through various in vitro cleavage assays where laminin-1 (α1,β1,γ1) and laminin-5 (α3,β3,γ2) have been identified as substrates [118-121]. Laminin-1 cleavage can result in various peptide products that can have effects on cellular

behavior. One of the known laminin-1 fragments is called P1 and further cleavage results in the release of "cryptic sites" known as IKVAV and SIKVAV [122-124]. These peptides have been characterized mostly in the context of inflammation and cancer where they stimulate the expression of proteinases (MMP-2, 9, 12 and urokinase plasminogen activator), activate the MAPK signaling cascade, and promote synthesis of prostaglandin E_2 [123-127]. However, MMP-7/laminin-1 cleavage assays did not directly identify whether these particular fragments are released upon MMP-7 cleavage, so the question still remains as to whether the cleavage of laminin-1 by MMP-7 is biologically significant and if this cleavage event occurs *in vivo*.

However, it is reasonable to speculate that if MMP-7 does release laminin-1 fragments, they may be involved in some biological context because the effects due to cleavage of laminin-5 by MMP-7 *have* been characterized in colon carcinoma. Cleavage of the laminin γ2 chain by MMP-7 releases a 90 KDa c-terminal fragment and was shown to promote cell motility of colon cancer cells *in vitro* [120]. This effect was blocked with an MMP-7 specific neutralizing antibody, suggesting that MMP-7 mediates colon cancer cell motility by cleaving laminin-5 *in vitro*. While it is clear that the release of laminin fragments can have deleterious consequences due to their role in disease, it is unknown whether these fragments are released during development, and if so, what their effects they can have.

If laminins can be cleaved and have significant effects on cellular function, could their receptors also undergo proteolytic processing as well? As mentioned in section 4 of this chapter, dystroglycan is a laminin receptor in oligodendrocytes involved in differentiation. There are various reports in which MMP-2 and MMP-9 have both been implicated in cleaving dystroglycan, resulting in loss of attachment to the ECM in various tissues that include astrocytes at the blood brain barrier basement membrane in an inflammatory model of MS (EAE), sciatic

nerves in a model of neuritis, muscle in a model of muscle dystrophy, endothelium of blood vessels in osteoarthritis, loss of ECM attachment of squamous cell carcinomas, and various cancer lines from aggressive tumors in vitro [128-133]. From these studies, it is clear that dystroglycan cleavage is metalloproteinase mediated, and that there is a role for MMP-2 and 9 in cleaving the receptor. However, is dystroglycan processing associated only with disease progression? Is it possible that this molecular event can also occur developmentally, particularly during brain development? And, can it have a positive effect on cellular development when it occurs as part of a normal physiological event?

The first and only published study to look at dystroglycan cleavage during development suggests that the answers to some of these questions are ”yes” in the context of Schwann cell development. Whereas oligodendrocytes wrap around multiple axons to form multiple short myelin segments, Schwann cells (peripheral nervous system myelinating cells) each wrap around one axon and form one long segment. To fulfill the needs of maintaining high conduction and metabolic support to both itself and the axon, they polarize their outermost cytoplasm outside the myelin membrane into 2 types of domains called patches and Cajal bands. The patches are areas where the cell membrane is juxtaposed to the myelin membrane, and Cajal bands are regions with abundant cytoplasm that form channels that help the elongation and stability of the membrane [72]. It was known since the time of Ramon Cajal himself that Schwann cells form these compartments, but their molecular characterization was never defined. In the recent study, it was found that dystroglycan is developmentally cleaved by MMP-2 and 9 and segregated into 2 pools: the cleaved receptor which associates with Cajal bands since these are areas where laminin-2 is not present, and non-cleaved receptor is associated with so-called patches, where laminin-2 is present. Interestingly, each pool of cleaved and non-cleaved dystroglycan is

associated with a different set of intracellular modulators, suggesting that it plays a different function in each form [134]. Even more interesting is the fact that this report shows an example of how dystroglycan cleavage occurs developmentally and performs an important physiological function.

Furthermore, studies of dystroglycan function in other cell types raise provocative questions about whether there is more to dystroglycan cleavage than just detachment from the ECM: dystroglycan has been found in the nucleus of HeLa, C_2C_{12}, PC12, human prostate cancer tissue, and myoblasts, suggesting that it plays a role in the nucleus [135-140]. Furthermore, dystroglycan was found to associate and localize with the nuclear envelope, splicing speckles, Cajal bodies, nucleoli, nucleoplasm and inner nuclear membrane [140]. In the nuclear envelope, it was found to associate with lamin-B1, suggesting that it could play a role in nuclear architecture. This is further supported by evidence where members of the Dystrophin-Glycoprotein-Complex (as described in section 4) were found to associate with dystroglycan in the nucleus [135-137]. Interestingly, ablation of dystroglycan in mammary epithelial cells results in S-phase cell cycle arrest, and altered centrosome-nucleus linkage in myoblasts, suggesting that dystroglycan plays a role in proliferation and in nuclear organization [140, 141]. While there is evidence that dystroglycan exists in the nucleus as a full length protein, there is a possibility that when dystroglycan is cleaved, it is transported into the nucleus where it plays functions like those described above. Evidence from in our lab supports this scenario as examination of cytoplasmic and nuclear fractions from OPCs and oligodendrocytes demonstrated that dystroglycan can be found in the nucleus of OPCs (unpublished data, Christopher Eyermann).

7. <u>Hypothesis: Matrix metalloproteinases remodel laminin-dystroglycan adhesion complexes in developing oligodendroglia</u>

ECM ligands and receptors are important for oligodendrocyte responses to environmental cues. ECM is expressed in higher amounts during development and is largely reduced by adulthood in much of the brain parenchyma. Laminin loss of function studies demonstrated that laminins are required for proper oligodendrocyte development and myelination. While one of the laminin receptors, α6β1 integrin, promotes oligodendrocyte survival and affects myelination, it doesn't appear to mediate oligodendrocyte interactions with laminin by itself. Dystroglycan loss of function studies have recently placed this receptor in the spotlight due to its role in mediating oligodendrocyte interactions with laminin that are required for proper oligodendrocyte differentiation *in vitro*. Because ECM expression changes during development, it is important to understand how oligodendrocyte ECM receptors respond to this change and whether they themselves are remodeled to reflect changes in the cell's response to the local environment.

Matrix metalloproteinases have been implicated in injury and disease, and their role during brain development is just beginning to be understood. Their substrate repertoire is vast and includes laminin and dystroglycan, but whether laminins and dystroglycan are substrates for these proteinases during brain development, specifically in developing oligodendrocytes, is not known. ***The hypothesis explored in this thesis work has therefore been to determine if matrix metalloproteinases cleave laminin and dystroglycan and regulate oligodendroglial proliferation and differentiation.***

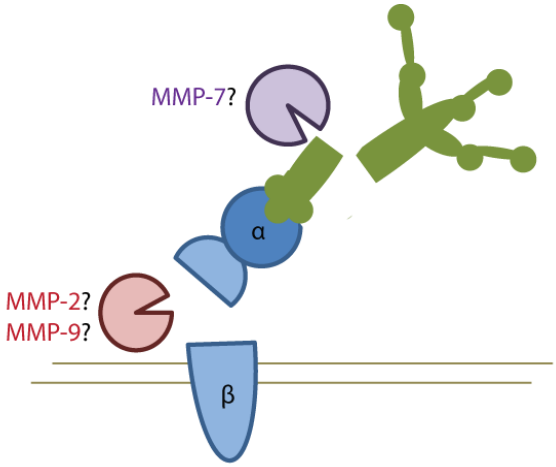


Figure 1-9: Hypothesis: MMP-2 or 9 cleave dystroglycan and MMP-7 cleaves laminin releasing the adhesion complex in developing oligodendroglia.

Chapter 2:

Experimental Procedures

Rat primary oligodendrocyte progenitor cell cultures: Cerebral cortices were dissected from postnatal day 0-2 rats and meninges were removed. Tissue was minced and digested with papain (Worthington) for 45 minutes, triturated, and resuspended in DMEM supplemented with penicillin, streptomycin, and 10% FBS. The tissue suspension from each brain was grown in a 75cm^2 flask, with media changed every 3 days. After 10-12 days in vitro, oligodendrocyte progenitor cells (OPCs) were isolated using a procedure modified from the mechanical agitation and differential adhesion method described by McCarthy and De Vellis ([142, 143]). Isolated cells were resuspended in "proliferation media" (SATO supplemented with 10 ng/ml of both PDGFα and bFGF (Peprotech)) and plated on either Poly-D-Lysine (PDL) or laminin (LM) coated dishes (for biochemistry) or 8-well Permanox chamber slides (for immunocytochemistry). Dishes and slides were coated with for 1 hour at 37°C, washed 3 times with water and dried before cell plating. Laminins 1 and 2 were resuspended in ice-cold PBS at 10 μg/ml and used for coating PDL pre-coated dishes for 4 hours at 37°C. After laminin coating, 3 washes in PBS followed and dishes were kept in the last PBS wash to prevent drying before plating cells.

Mouse primary oligodendrocyte progenitor cell cultures: Progeny from PDGFRα-Cre^{Tm} : Adam-(10 or 17)-Fl/Fl were injected with tamoxifen immediately after birth. At postnatal day 3, cerebral cortices were dissected into individual vials and tails were collected for genotyping procedures. Isolated cortices were digested with papain (Worthington) for 20 minutes, triturated, and resuspended in DMEM supplemented with penicillin, streptomycin, and 10% FBS. Each brain was plated into a PDL coated 25 cm^2 flask and media was changed 3 days after initial plating. 3 days later, insulin (5 μg/ml) was added to the second media change to stimulate

survival and proliferation. OPCs were isolated from flasks as described above for the rat oligodendrocyte progenitor cell cultures.

qRT-PCR array: RNAs were extracted and purified from differentiating OPCs using the Qiagen RNeasy mini kit (#74106): Cells were lysed in buffer RLT, and passed 10 times through 20 gauge needles using 1 ml sterile syringes. Homogenates were pre-equilibrated for RNA isolation with 70% ethanol in DEPC treated water and then passed through a Qiagen RNeasy column. Following one wash with buffer RW1, Dnase treatment (#79254, Qiagen) was performed in column for 15 minutes at room temperature. Another wash in buffer RW1 was followed by 2 washes with buffer RPE. RNAs were eluted using water provided in the kit, and concentrations per sample were quantified using a spectrophotometer (ND-1000, Nanodrop). Equal amounts of RNA from sample to sample were loaded into cDNA synthesis reactions at a 1 µg maximum load and cDNAs were synthesized using the ProtoScript II First Strand cDNA Synthesis Kit (New England Biolabs, #E6560S). 500 ngs of cDNAs were mixed with RT² qPCR Mastermix (#330502, SA Biosciences-Qiagen) and loaded onto the Extracellular Matrix & Adhesion Molecules PCR array plate (#PARN-013Z, SA Biosciences-Qiagen) using the manufacturer's instructions. PCR cycles were performed in an Opticon 2 instrument (MJ Research) at the Stony Brook University DNA Sequencing Core. Array analysis and quality control were performed as per the kit's instructions.

MMP-2 and -9 siRNA transfections: To transfect primary OPCs, cells were isolated and resuspended in proliferation media as described above and grown on uncoated 100 cm^2 petri dishes for 20-24 hours. OPCs were detached using trypsin (0.5X final) centrifuged in medium containing 10% FCS, and resuspended in proliferation medium. OPCs in suspension were rested for 1 hour at 37^0C prior to electroporation using the Rat Glial transfection reagent and

Nucleofector electroporation system (Amaxa) as per the company's protocol. Control (non-targeting for rat), MMP-2 (M-093919-00-0010), or MMP-9 siRNAs (L-093933-01-0005) were all siGENOME SMARTpool siRNAs purchased from Dharmacon, Thermo Scientific, were used to transfect cells. Post-transfection, cells were plated on laminin-coated dishes in proliferation media. 12 hours post-transfection, media was slowly pipetted out and replaced with new proliferation medium for a final volume of 2 mls in 35 mm dishes. RNA or protein lysates were prepared from transfected cells at 12 hours post-media change. MMP-2 and 9 knock down was assessed via zymography.

Preparation of conditioned media and cell lysates: Conditioned media (isolated from OPCs and oligodendrocytes to assess MMP-2 and 9 expression via zymography) was passed through a 0.45 μM filter before being aliquoted and stored at -80^{0}C. Cells were lysed in boil buffer (20mM Tris pH 7.4, 1% SDS, and protease and phosphatases inhibitor cocktails (Calbiochem)) scraped, collected, and boiled at 95^{0}C for 15 minutes.

Zymography: Conditioned media collected from cell samples were processed as described above. 0.5 -1.5 mls were concentrated using Amicon ultra 0.5 ml 30K membrane centrifugal filters (Millipore). Non-denaturing loading buffer was added to the conditioned media concentrates which were then run on 10% polyacrylamide gels embedded with 1% gelatin (Porcine Gelatin G2500, Sigma-Aldrich) until the 50 KDa bands reached the bottom of the gel. Gels were washed twice with 2.5% Triton solution for 15 minutes and incubated overnight in substrate buffer (50mM Tris-HCL, pH 7.6, 10mM $CaCl_2$). Gels were stained with coomasie blue and scanned for analysis 20-40 hours post incubation.

Recombinant MMP, TIMPs and PDB treatments: rMMP-2 (10082-HNAH) and rMMP-9 (10327-HNAH) were purchased from Sino Biological (Beijing, China). MMP-2 activation was carried out in activation buffer (50 mM Tris, 10 mM Cacl2, 150 mM Nacl, 0.05% Brij-35, pH 7.5) at 37^{0}C for 1.5 hours. rMMP-9 was activated with trypsin at 150 μg/ml in activation buffer for 30 minutes at room temperate. Activation was tested using DQ-Gelatin (EnzChek Gelatinase kit, Molecular Probes, #E-12055). Recombinant active MMP-7 (#444270, Calbiochem) was resuspended in sterile PBS at 100ng/ml, aliquoted and stored -80°C. Phorbol Dibutyrate (PDB) was purchased from LC Laboratories, solubilized in DMSO, and was used at 100 nM. Recombinant TIMPs were purchased from R&D Systems, rTIMP-1 (cat#970-TM), rTIMP-2 (cat# 971-TM), rTIMP-3 (973-TM), rTIMP-4 (974-TSF). All TIMPs were used at a 100ng/ml and were used to treat OPCs for 24 hours.

MMP Inhibitors: (2R)-[(4-Biphenylylsulfonyl)amino]-N-hydroxy-3-phenylpropionamide, BiPS (MMP-2/9 Inhibitor II, #444249, Calbiochem) was used at 12.5 μM (unless otherwise indicated). MMP-7 Inhibitor (MMP Inhibitor II, #444247, Calbiochem) was used at 2 μM. Broad spectrum MMP inhibitor, Galardin (GM6001, 364205, Calbiochem) was used at 20 μM. All were solubilized in DMSO.

Western Blotting: β-DG was detected using MANDAG2(7D11) (Developmental Studies Hybridoma Bank, University of Iowa) at 1:100 in 5% Milk in TBS at 4^{0}C overnight, after blocking in the same solution for 30 minutes at room temperate. 3 washes for 7 minutes each in TBS-T followed all antibody incubation steps. HRP-conjugated anti-mouse IgG was used at 1:2000 for ECL detection and biotinylated anti-mouse IgG followed by streptavidin bound IR-800 was used for Odyssey detection (LICOR, Lincoln, NE). For MBP and CNP, 4% BSA in

TBS-T was used for block, primary and secondary antibody incubations. Blocking was performed for 1 hour at room temperature, primary antibodies were diluted at 1:1500 and incubated overnight, and secondaries were diluted at 1:1000 and incubated for 1 hour at room temperature. All washes were done with TBS-T for 7 minutes each.

Immunocytochemistry: OPCs and oligodendrocytes were seeded on ligand coated chamber slides (as described above), fixed in either 1) 100% ice-cold methanol for 8 minutes at -20°C and washed with PBS 2 times before blocking in 2% BSA in PBS + 0.01% triton-X100 for 45 minutes at room temperature, or 2) 4% paraformaldehyde for 15 minutes at room temperature followed by 3 washes in PBS, blocking in 10% donkey serum with 0.01% triton X-100 in PBS for 1 hour, and following primary and secondary antibody incubations in blocking solution. The immunohistochemical method used for each antibody is described in Table 2. Blocking solution was used to resuspend primary antibodies (NG2, CNP and MBP) at 1:1500 and incubating overnight at 4°C. The next day, 3 washes in PBS were followed by secondary antibodies in block for 1 hour at room temperature, after which 3 washes in PBS, DAPI staining for 10 minutes and mounting in Slow Fade Gold (Life Technologies) followed.

Cell differentiation assays (NG2, CNP and MBP population counts): oligodendroglia plated in chamber slides and treated with MMP inhibitors or MMP recombinant proteins were fixed and processed for immunohistochemistry as described in table 2. Zeiss epifluorescence microscope was used to take 20 and 40x images of stained cells. DAPI density counts were followed by cell marker counts in each condition.

Cell proliferation and death assays: Ki67 antibody was used at 1:5000 to immunocytochemically label proliferating OPCs in 10% donkey serum with 0.01% Triton X-100

after ice-cold methanol fixation for 8 minutes at -20°C. BrdU was added to OPCs at the time of plating to label proliferating cells, and they were labeled for 24 hours in the presence of BiPS. Cells were then fixed in 95:5 ice-cold ethanol-acetic acid for 25 minutes at -20°C. The 5-Bromo-2′-deoxy-uridine Labeling and Detection Kit I (Roche, Cat # 11 296 736 001) was used to detect BrdU labeled cells. Apoptag Fluoroscein In Situ Apoptosis Detection Kit (Chemicon, cat # S7110) was used to detect apoptotic OPCs after Ethanol:Acetic acid (2:1) fixation for 25 minutes at -20°C as indicated on manufacturer's protocol.

Table 2: Antibodies, western blotting and immunohistochemistry conditions used

Protein target	Supplier	Catalog number	Species	Immunohistochemistry (I) or western blotting (WB) procedure
NG2	Millipore	AB5320	Rabbit	I: Methanol fix and BSA blocking solution
CNP	Sigma	C5922	Mouse	I: Methanol fix and BSA blocking solution
				WB: BSA blocking solution
MBP	Serotec	MCA-409S	Rat	I: Methanol fix and BSA blocking solution
				WB: BSA blocking solution
Nestin	Yale Sch. Of Med.		Mouse	I: PFA fix and donkey serum blocking solution
GFAP	Dako	Z0334	Rabbit	I: Methanol fix and BSA blocking solution
Iba1	Wako	019-19741	Rabbit	I: PFA fix and donkey serum blocking solution
Actin	Sigma	A5441	Mouse	WB: BSA blocking solution
p27	BD Transduction	610241	Mouse	WB: Milk blocking solution
Cdk2	Santa Cruz	sc-163	Rabbit	WB: Milk blocking solution
Ki67	Abcam	ab15580	Rabbit	I: Methanol fix and donkey serum blocking solution
MMP-7	Abcam		Rabbit	WB: BSA blocking solution
MMP-7	Vanderbilt		Rat	I: PFA fix and donkey serum blocking solution

Flow cytometry: OPCs treated with either DMSO or BiPS were trypsinized and pelleted as indicated above. Pellets were resuspended in Krishan buffer (Sodium citrate 0.1%, NP-40 0.2%,

HCl 1N 1 drop/100mL), Propidium iodide (0.05mg/mL), Rnase A (0.02mg/mL). Flow cytometry readings were analyzed via ModFit LT software.

qRT-PCR analysis of cell cycle regulators: RNA was isolated from cells using the Qiagen RNeasy kit using standard procedures indicated in the company's protocol in addition to DNase treatment using Qiagen DNase treatment. Purified RNAs were reverse transcribed using ProtoScript M-MuLV First Strand cDNA Synthesis kit (New England Biolabs, cat # E6300S). Applied Biosystems Fast SYBR Green master mix was used to amplify products using the following primer sequences: Cyclin D1: Fwd- GCGCCCTCCGTTTCTTACTT; Rev-TCGCAGACCTCTAGCATCCA. Cyclin D3: Cyclin E: Fwd-CCAGGATAGCAGTCAGCC; Rev-CTCCATCAGCCAGTCCAG. p21: Fwd- GCAGACCAGCCTAACAGATTTC; Rev-GGCACTTCAGGGCTTTCTCT. p27: Fwd-CGGCGGCAAGAGAGGC; Rev-GAAGAGGTTTCTGCAGGCGG. GAPDH: Id2, c-fos and c-jun as published in [144]. Reaction melt curves were analyzed for quality control.

β-DG expression construct: β-DG intracellular domain cDNA in tandem with EGFP flag tag is described in detail in [145] (referred to as "DGc"). This construct expresses the cytosolic domain of β-dystroglycan and EGFP backbone vector was used as control. Cells were transfected by electroporation using the Rat Oligodendrocyte Nucleofector Kit (Amaxa). OPCs were seeded into PDL-coated chamber slides or glass coverslips, and maintained 12-14 hours at 37°C in proliferation media before switching to serum-free media supplemented with BrdU for 8 hours. Proliferation was detected via indirect immunocytochemistry to visualize BrdU incorporation in GFP expressing cells (as described above). A minimum of 100 GFP-positive cells per condition were counted in 3 independent experiments.

Tissue lysates: Cerebral cortices were dissected from mice at various stages of development (postnatal days 0, 5, 8, 10, 15, and 21) and cryopreserved in liquid nitrogen and stored at -80^{0}C. Tissue pulverization was performed using a CryoGrinder (OPS Diagnostics), followed by lysis in boil buffer (20mM Tris pH 7.4, 1% SDS including protease and phosphatase inhibitor cocktails (Calbiochem)) at 95^{0}C for 15 minutes.

Tissue cryosections and immunohistochemistry: Postnatal day 1 mice were dissected and dunk-fixed in 4% paraformaldehyde for 12 hours before switching to 30% sucrose to cryoprotect tissue. Tissues were then placed in OTC cryo-embedding compound and frozen in a 2-methyl butane-crushed dry ice slurry and stored at -80°C. Equilibration to -20°C was done 12 hours prior to tissue sectioning and 18 μm sections were cut onto glass slides and stored at -80°C until immunohistochemical processing. Sections were blocked with 10% donkey serum in PBS with 0.01% triton for 1 hour in humid chambers at room temperature before diluting anti-rat MMP-7 at 1:5000. Primary incubation was done at 4°C overnight in humid chambers. Secondary anti-rat antibodies were diluted at 1:500 and incubated with tissues in humid chambers for 1 hour at room temperature. DAPI staining, mounting using Slow Fade Gold (Life Technologies), and placement of cover slips followed.

Statistical Analyses: For all statistical analysis a 2 tailed, paired, student's t-test was performed with a 0.05 confidence value. * is designated for p values ranging less than 0.05, ** for less than 0.01 and *** for less than 0.001.

Chapter 3:

Metalloproteinase-Mediated Dystroglycan Processing Regulates Proliferation in Oligodendrocyte Progenitor Cells

Introduction:

Oligodendrocyte progenitor cells (OPCs) are characterized by their proliferative and migratory capacity, and upon cell cycle exit, OPCs mature into myelinating oligodendrocytes in the central nervous system. Understanding the process of oligodendrocyte development and function has become crucial in developing therapeutic strategies for diseases like multiple sclerosis (MS) where repeated bouts of autoimmune attack lead to OPCs that cannot sufficiently remyelinate lesioned areas followed by a depletion of the OPC pool of that normally supplies remyelinating cells [12]. This eventually results in decreased regenerative capacity and subsequent neurodegeneration. Re-establishing the OPC pool as well as promoting OPC differentiation could serve as useful approaches to stimulating remyelination in MS. However, the intrinsic and extrinsic factors that regulate OPC proliferation and differentiation are not completely understood.

Among the extrinsic factors that have been shown to contribute to proper and timely oligodendrocyte development are the laminin proteins. Our lab previously reported two mouse models, the α2-laminin-deficient mouse (*dy/dy*) and the laminin α2 null mouse (*Lama2*$^{-/-}$), in which both display thinner myelin and fewer myelinated axons attributed to a delay in the generation of mature oligodendrocytes coinciding with OPC accumulation [57, 58]. These studies, in addition to others, demonstrate that laminin-2 modulates OPC survival, timely production, and differentiation [50, 60, 146]. Furthermore, one of two laminin receptors

expressed by oligodendroglia, dystroglycan, has been shown to regulate oligodendrocyte process extension and differentiation *in vitro* [61, 67]. Together, these studies suggest that the laminin-dystroglycan signaling complex plays an important role in oligodendroglial development. What remains to be understood is how this signaling complex mediates its effects in oligodendroglia during development.

Dystroglycan is composed of 2 subunits, the α subunit which binds laminin and other ECM proteins, and the β transmembrane subunit which binds the α subunit on its ectodomain and cytosolic signaling modulators on its cytoplasmic end. Together, the dystroglycan subunits link to extracellular matrix proteins and bind intracellular adaptor proteins that can affect cytoskeletal dynamics and cascade signaling pathways to influence cellular behaviors [69, 70].

A subset of matrix metalloproteinases (MMPs) have been shown to proteolytically process dystroglycan [147]. In particular, dystroglycan cleavage by MMP-2 and 9 at the β subunit results in loss of its connection to the α subunit, leaving behind the transmembrane and cytosolic portions of the β subunit at the membrane [128, 134]. Whether this cleavage event occurs during OPC development and whether it has a functional consequence is unknown.

In this study, I determined that dystroglycan is cleaved in the postnatal cerebral cortex and that decreasing levels of dystroglycan cleavage correlate with developmental myelination. A similar pattern was observed in cultured oligodendroglia, where dystroglycan cleavage was highest in OPCs and substantially decreased with oligodendrocyte differentiation. I found that dystroglycan cleavage was promoted on laminin-2, as OPCs plated on this substrate showed increased dystroglycan cleavage compared to those on poly-D-lysine and laminin-1.

Furthermore, I found that dystroglycan cleavage was dependent on metalloproteinase activity, as broad spectrum MMP inhibitors attenuated it. Through various gain- and loss-of-function approaches, I determined that MMP-2 and MMP-9 were not responsible for dystroglycan cleavage in OPCs. Metalloproteinase inhibitor treatment resulted in decreased dystroglycan cleavage in OPCs, where in parallel, proliferation was also decreased. Along with a transient cell cycle arrest at the G0/G1 phase, a trend in decreased transcripts and protein levels of the cell cycle regulator cyclin D1 were observed, in addition to significantly decreased cdk2 and a transient increase in the cell cycle inhibitor p27. These results suggest that inhibition of metalloproteinase activity correlating with decreased dystroglycan cleavage is involved in regulating OPC proliferation. The decrease in proliferation did not appear to be caused by increased apoptosis or precocious differentiation. Finally, expression of the β-DG intracellular domain mimicking β-DG cleavage resulted in a mild increase in proliferation, suggesting that β-DG cleavage is involved in regulating OPC proliferation.

Together these findings suggest that laminin-dystroglycan interactions are actively remodeled during OPC development, that dystroglycan processing may contribute to appropriate OPC proliferation, and that the loss of regulated dystroglycan cleavage may contribute to myelination abnormalities observed in α2-laminin-deficient mice.

Results:

Dystroglycan cleavage has been primarily shown to correlate with the onset and severity of injury and disease, but whether it plays a role during developmental processes remains unknown for the most part. To date, only one study has shown that dystroglycan cleavage regulates a developmental process, which is the formation of Schwann cell axonal domains in the

peripheral nervous system where the interaction between laminin and dystroglycan is crucial for proper PNS myelination [134]. However, whether dystroglycan cleavage plays a role during oligodendroglial and central nervous system development is currently unknown. To determine whether dystroglycan cleavage occurs during myelination, cerebrocortical lysates were analyzed for β-DG expression during time points before, during and after myelination (Fig. 3-1). Analysis of β-DG expression during these time points revealed the presence of the 43 kDa β-DG subunit (β-DG 43), in addition to the cleavage product (β-DG 30) (Fig. 3-1a). Quantification of the ratios of β-DG 30 over the sum of β-DG43 plus β-DG 30 revealed that β-DG cleavage was highest at postnatal day 1, and decreased thereafter (Fig. 3-1b: Percent β-DG cleavage at: P1: 21% ± 1.9%; P5: 11.8% ±2.4%; P8: 4.8% ± 0.7%; P10: 2.9% ± 0.8%; P15: 2.2% ± 0.4%; P21: 2.1% ± 0.5%; Adult: 1.5% ± 0.4%. P1 vs. P5: Student's t-test (paired, 2-tailed) p-value: not significant, n=3. P1 vs. P8: Student's t-test (paired, 2-tailed) p-value: 0.015, n=3. P1 vs. Adult: Student's t-test (paired, 2-tailed) p-value: 0.009, n=3. All comparisons between P1 and all time points after P5 were statistically significant.) These results suggest that β-DG cleavage occurs at time points preceding myelination in the cerebral cortex where much of the brain is undergoing of growth and expansion.

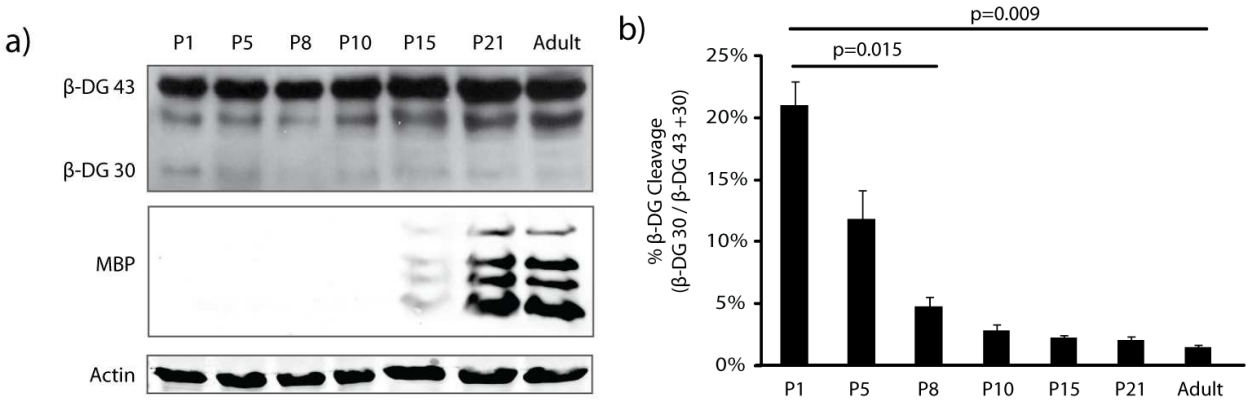


Figure 3-1: β-DG cleavage inversely correlates with myelination during cerebrocortical development. Lysates from cerebral cortices at postnatal days 1, 5, 8, 10, 15, 21, and 5 months of age were analyzed for expression of β-DG and MBP. b) Ratio of β-DG30 / β-DG30 + β-DG43 yields the percent of β-DG 30 over total.

It is known that neural cell types other than oligodendrocytes (i.e. astrocytes, neurons) express dystroglycan. It is therefore possible that the β-DG cleavage pattern observed in the cortical lysates is not representative of the oligodendrocyte population alone. To address whether β-DG cleavage occurs in oligodendroglia, OPCs were grown *in vitro* and β-DG expression and cleavage were assessed. I plated OPCs either on poly-D-lysine (PDL), a non-physiological adherent substrate, and laminin-2, a dystroglycan ligand, in addition to varying either proliferation or differentiation conditions. Cell lysates were collected after 1 day of proliferation and after 1, 3 and 5 days after differentiation. Analysis of these lysates via western blot revealed overall β-DG expression increases on both PDL and laminin-2 over time, where a peak is reached on day 3 of differentiation on laminin-2 (Fig. 3-2b: Fold change in all samples relative to PDL proliferation Day 1: PDL Diff Day 1: 1.4±0.3, PDL Diff Day 3: 2.1±0.3, PDL Diff Day 5: 4.2±2.2, LM Prolif Day 1: 1.1±0.6, LM Diff Day 1: 1.2±0.6, LM Diff Day 3: 2.7±0.6, LM Diff Day 5: 2.1±0.7. Student's t-test (paired, 2–tailed) between any of the groups was not significant, n=3).

Furthermore, I found that β-DG cleavage was apparent in OPCs and decreased sharply as the cells exited cell cycle and began the differentiation program (Fig. 3-2a and c: Percent β-DG cleavage: white bars (PDL): OPC proliferation day 1: 2.1% ± 1.8%, oligodendrocyte differentiation at day 1: 2.5% ± 0.2%, at day 3: 2.1% ± 0.4%, at day 5: 2.6% ± 0.9%. Gray bars (LM-2): OPC proliferation day 1: 13.9% ± 6.9%, oligodendrocyte differentiation at day 1: 5.5% ± 0.5%, at day 3: 3.9% ± 0.7%, at day 5: 2.8% ± 0.2%.

Furthermore, β-DG cleavage appeared to be promoted by laminin-2 in that OPCs grown on PDL, did not show β-DG cleavage when compared to those grown on laminin-2 (Fig. 3-2c:

Percent β-DG cleavage on PDL Prolif Day 1: 2.1%±0.6% versus LM Prolif Day 1: 13.9%±2.4%. Student's t-test (paired, 2–tailed) p-value: 0.011, n=8).

To determine if laminin-1, another dystroglycan ligand, exerted the same effect on β-DG cleavage, cells were grown on either PDL, laminin-1 or laminin-2 coated plates in proliferation conditions. I did not observe β-DG cleavage on either PDL or laminin-1 to the same extent as that of laminin-2, suggesting that this laminin type uniquely promotes dystroglycan cleavage in OPCs (Fig. 3-3a and b: Percent β-DG cleavage on PDL: 1.0%±0.4%, LM-1: 0.9%±0.5%, LM-2: 3.9%±0.7%. Student's t-test (paired, 2 –tailed) PDL vs. LM-2 p-value: 0.015, n=4; LM-1 vs. LM-2 p-value: 0.016, n=4).

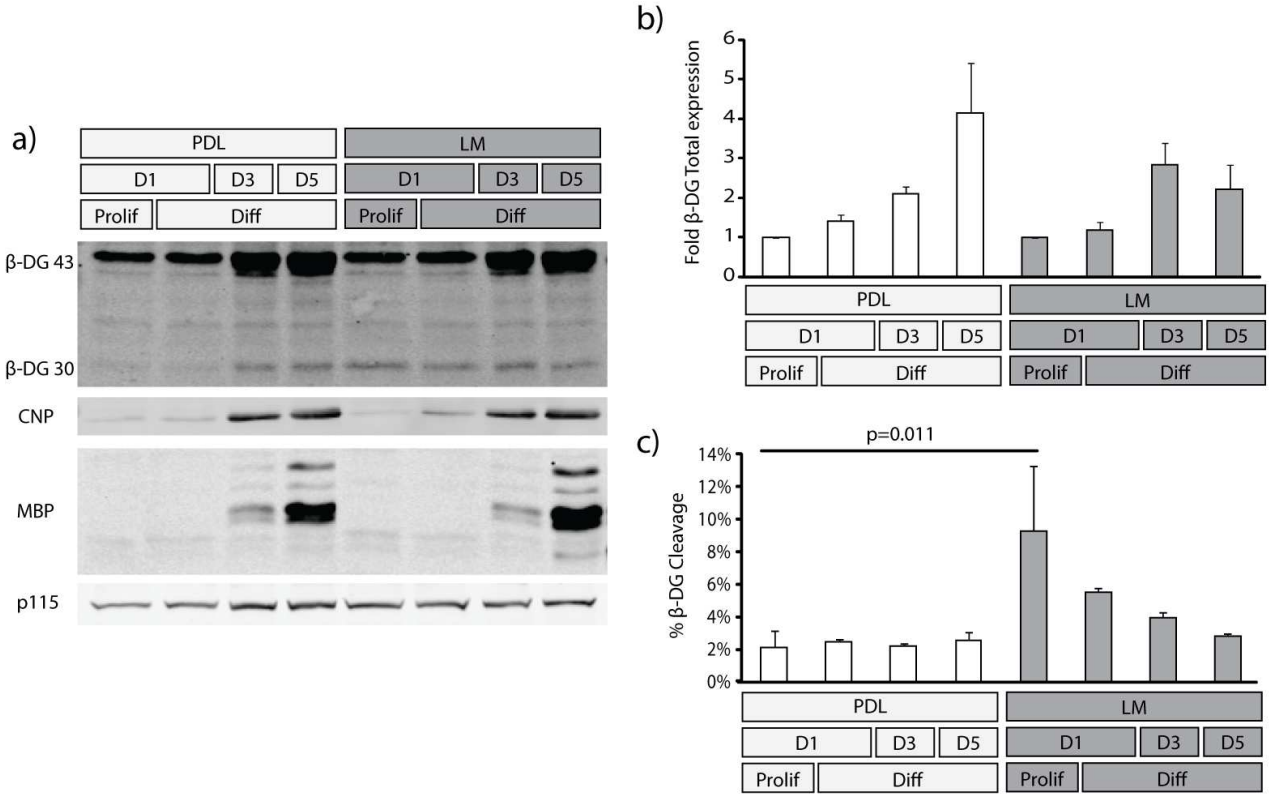


Figure 3-2: β-DG is cleaved in cultured oligodendroglia. a) Western blots assessing β-DG, CNP and MBP, were used to determine expression of these proteins in OPCs and differentiating oligodendrocytes for 1,3, and 5 days. p115 was used as a loading control. b) Quantification of total β-DG (β-DG 43 + β-DG 30 / p115) expressed in fold change relative to PDL at Day 1 of proliferation. c) Quantification of the percent of β-DG cleavage as described in figure 3-1.

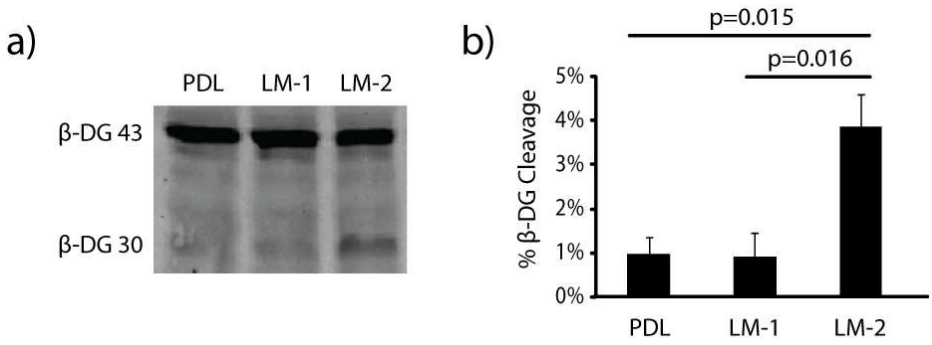


Figure 3-3: Laminin-2, but not laminin-1 or PDL, promote β-DG cleavage in OPCs. a) OPCs plated in proliferating conditions on PDL, laminin-1, or laminin-2 show that β-DG cleavage is promoted only on laminin-2. b) Quantification of the percent of β-DG cleavage as described in figure 3-1.

Matrix metalloproteinases, specifically MMP-2 and 9, have been implicated in β-DG processing in various cell types. To determine if metalloproteinase activity is involved in β-DG cleavage, I employed a broad spectrum metalloproteinase inhibitor, Galardin (GM6001, 25 μM), and an MMP-2/9 inhibitor (BiPS, 12.5 μM) to block MMP-2 and 9 activity. I found that both inhibitors blocked β-DG processing in OPCs (Fig. 3-4a and b: Percent of β-DG cleavage: DMSO: 5.4%±1.0%; Galardin: 1.2%±0.2%; BiPS: 1.0%±0.2%. Student's t-test (paired, 2–tailed) DMSO vs. Galardin p-value: 0.018, n=4. DMSO vs. BiPS p-value: 0.026, n=4), suggesting that metalloproteinase activity is involved in β-DG cleavage, and furthermore, that MMP-2 and 9 could be responsible for β-DG cleavage. However, MMP inhibitors are prominently known to lack specificity to the MMPs that they are claimed to specifically target, particularly at concentrations beyond the predetermined IC50s. To address the specificity of the MMP-2/9 inhibitor (BiPS), IC50 concentrations reported to maintain specificity for MMP-2 (17 nM) and MMP-9 (30 nM) were tested. I observed no changes in β-DG cleavage in the range of 17 nM – 1.25 μM (Fig. 3-4c and d), but again did observe attenuation of β-DG cleavage at 12.5 μM, the concentration used in fig. 3-4a (Percent β-DG cleavage: CTRL: 9.2%3.6%; 17nM: 10.2%±4.2%; 30nM: 7.1%±1.8%; 1.25μM: 6.3%±0.6%; 12.5μM: 0.9%±0.2%. Student's t-test (paired, 2 –

tailed) CTRL vs. 12.5µM p-value: 0.0012, n=7). Furthermore, since with increasing inhibitor concentrations inhibition of other metalloproteinases can occur, it is possible that other metalloproteinases are responsible for β-DG cleavage, but not MMP-2 or 9.

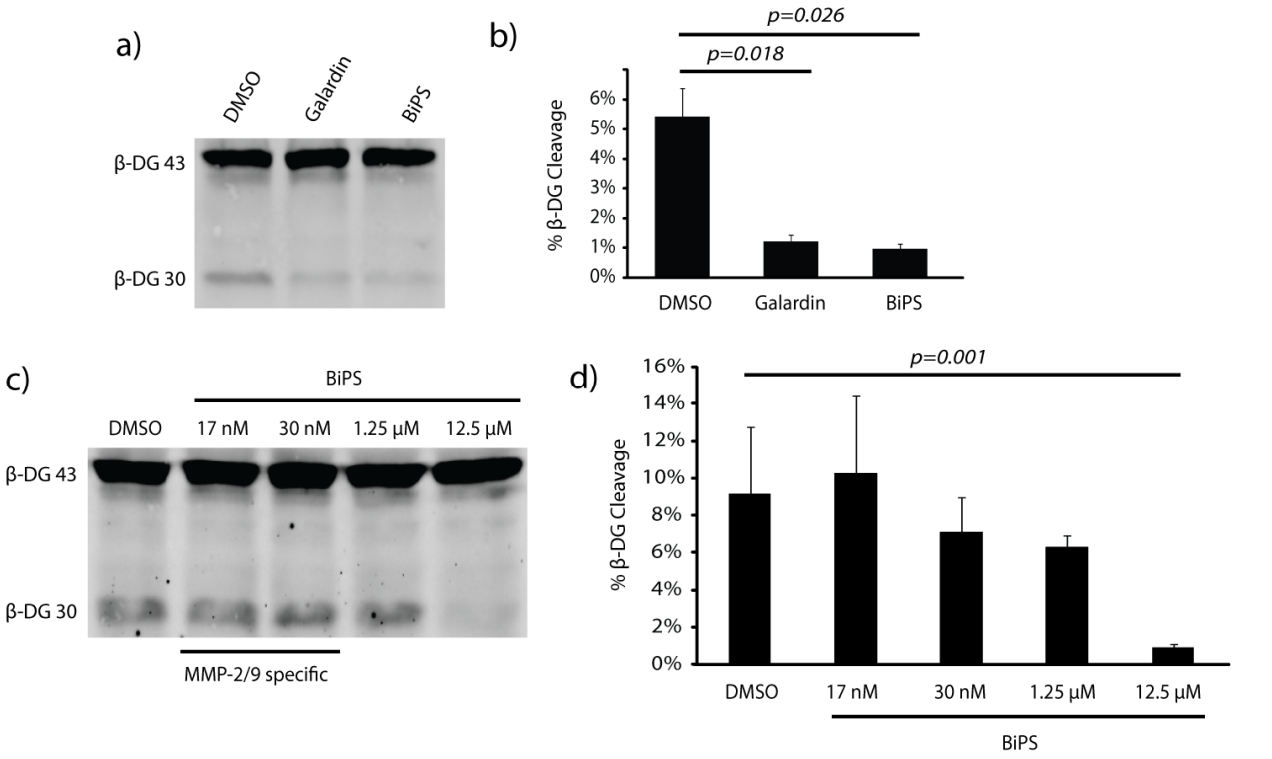


Figure 3-4: Metalloproteinase inhibitors attenuate β-DG cleavage in OPCs. a) Galardin (GM6001, 25 µM) and BiPS (MMP-2/9 inhibitor, 12.5 µM) were used to treat laminin-2 plated OPCs in proliferating conditions for 24 hours. b) Percent of β-DG cleavage post Galardin and BiPS treatment. c) OPC treatment with increasing concentrations of BiPS, including ones that target MMP-2 and 9. d) Percent of β-DG cleavage post BiPS treatment shows a statistically significant decrease at 12.5 µM.

To determine if MMP-2 or 9 are involved in β-DG shedding in oligodendroglia, conditioned media collected from proliferating and differentiating cells was analyzed via gelatin zymography. Surprisingly, I observed that MMP-2 and MMP-9 expression was lower during proliferation conditions in comparison to differentiation, inversely correlating with β-DG cleavage (Fig. 3-5a). I concentrated the media from the day 1 proliferation and differentiation conditions on both substrates, and performed zymography to determine if MMP-2 or MMP-9 is

expressed at all in OPC proliferating conditions. I then found that MMP-2 and MMP-9 are expressed during proliferation, albeit at much lower levels than that of differentiating conditions (Fig. 3-5c: Fold change in MMP-2 expression at Day 1 relative to PDL Prolif: LM Prolif: 1.0±0.1; PDL Diff: 5.0±1.3; LM Diff: 4.6±0.9. Student's t-test (paired, 2 –tailed) PDL Prolif vs. PDL Diff p-value: 0.001, n=4; PDL Prolif vs. LM Diff p-value: 0.001, n=4. Fig. 3-5d: Fold change in MMP-9 expression at Day 1 relative to PDL prolif: LM Prolif: 4.3±1.1; PDL Diff: 26.2±5.4; LM Diff: 27.9±6.9. Student's t-test (paired, 2 –tailed) PDL Prolif vs. LM Prolif p-value: 0.01, n=4; PDL Prolif vs. PDL Diff p-value: 0.002, n=4; PDL Prolif vs. LM Diff p-value: 0.01, n=4). I reasoned that it is still possible that the low expression of MMP-2 and 9 could be sufficient to result in β-DG shedding. Furthermore, MMP-9 is significantly upregulated in laminin-2 proliferating conditions when compared to PDL proliferating conditions, presenting the possibility that this small but significant upregulation could be sufficient to induce β-DG cleavage (Fig. 3-5, b and d).

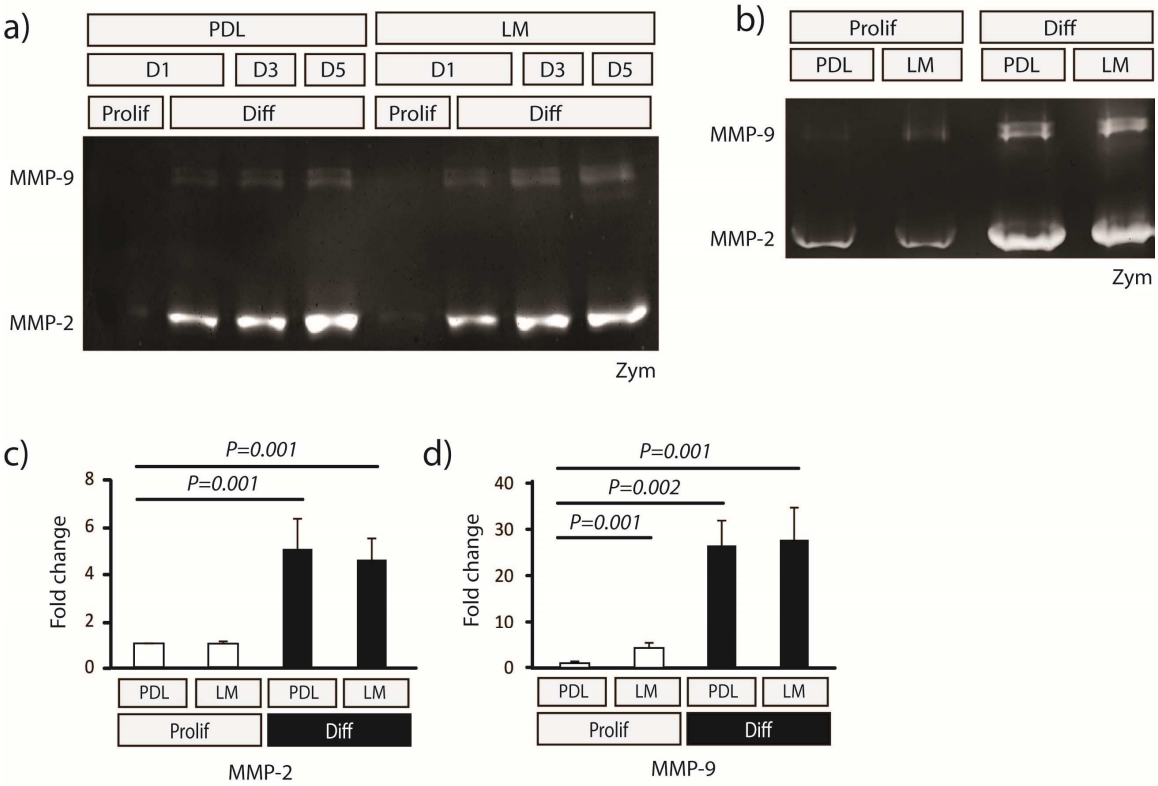


Figure 3-5: MMP-2 and 9 are expressed at lower levels in proliferating OPCs in contrast to differentiating OPCs. a) Conditioned media from proliferating (1 day) and differentiating (1,3,5 days) oligodendroglia was analyzed by gelatin zymography ("Zym"). b) OPC conditioned media from PDL or LM cells in proliferating or differentiating conditions at day 1 was analyzed via Zym. Quantification of MMP-2 and 9 expression in b are illustrated in c and d for MMP-2 and 9, respectively.

Next, I opted for direct approaches to rule out MMP-2 and 9 involvement in β-DG cleavage in OPCs. First, a loss of function approach was employed in which siRNA mediated knock down of MMP-2 and 9 was performed. 24 hours post-transfection, MMP-2 and 9 levels were assessed by zymography, and while both MMP-2 and 9 levels are decreased upon single and double siRNA treatment, none of these conditions result in decreased β-DG cleavage (Fig. 3-6a and b: Percent β-DG cleavage in: CTRL siRNA: 15.7%±4.2%, MMP-2 siRNA: 12.1%±3.6%, MMP-9 siRNA: 14.2%±5.7%, MMP-2 and 9 siRNA: 10.8%±4.1%. No changes were statistically significant using a Student's t-test (paired, 2 –tailed) between any of the groups, n=4). As a second approach, TIMP proteins (endogenous inhibitors of MMPs *in vivo*) were used to attempt blocking MMP-2 or 9 activity. TIMP-1 is known to have a preference for MMP-9 inhibition, while TIMP-2 preferentially targets MMP-2. TIMP-3 can block MMPs in addition to ADAMS proteases, providing us a useful clue as to what other metalloproteinases could be involved. However, recombinant TIMP protein treatment did not result in decreased β-DG cleavage, supporting the notion that MMP-2 and 9 activity is not involved in β-DG cleavage (Fig.3- 6c and d: Percent β-DG cleavage: CTRL: 6.6%±2.0%, TIMP-1: 6.6%±2.4%, TIMP-2: 6.1%±1.9%, TIMP-3: 5.8%±1.6%, TIMP-4: 6.0%±1.8%. No changes were statistically significant using a Student's t-test (paired, 2 –tailed) between any of the groups, n=4), In addition, this result suggests that metalloproteinases known to be inhibited by the TIMP proteins may not be involved in β-DG cleavage.

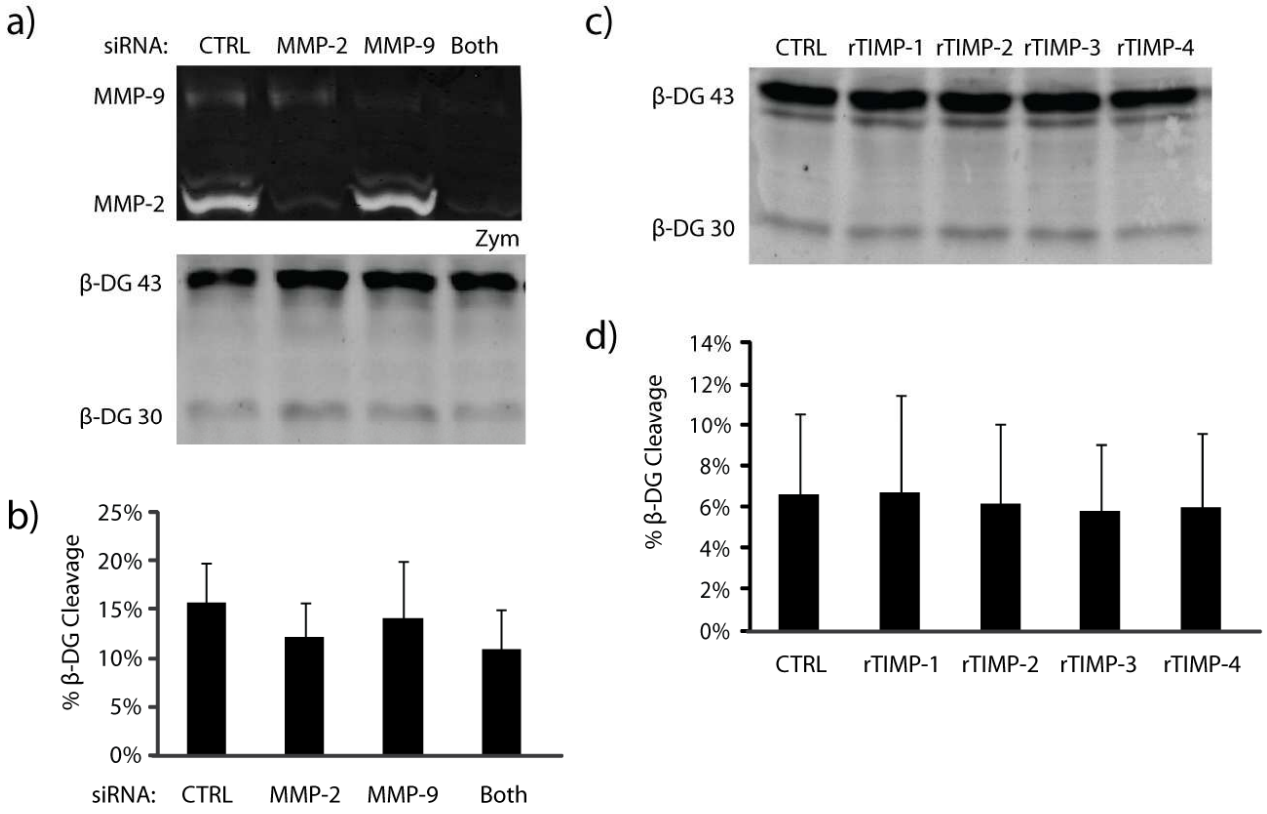


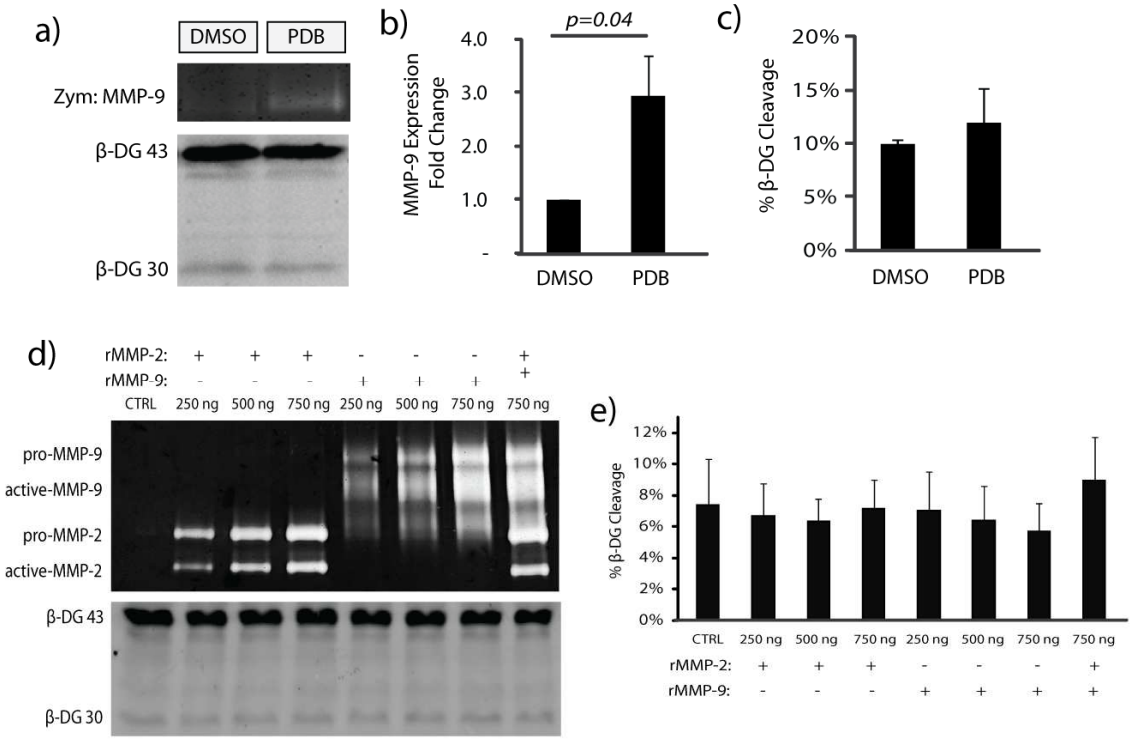
Figure 3-6: MMP-2 and 9 are not necessary for β-DG cleavage in proliferating OPCs. a) Zymography performed on conditioned media from siRNA treated OPCs followed by corresponding assessment of β-DG cleavage. b) Percent of β-DG cleavage upon siRNA treatment c) OPCs were treated with 100ng/ml rTIMPs for 24 hours. d) Percent of β-DG Cleavage upon rTIMP treatment.

Given that in figure 3-5d MMP-9 expression is higher on LM-2 than PDL, it is possible that MMP-9 is sufficient to induce β-DG cleavage. Therefore, to determine if MMP-9 is sufficient to induce β-DG cleavage, I treated OPCs with phorbol dibutyrate (PDB), a Protein Kinase C agonist that leads to upregulation of MMP-9 expression. After treating OPCs for 24hrs with PDB, I determined that while MMP-9 expression was indeed upregulated as assessed by zymography (Fig. 3-7a and b: Fold change in MMP-9 expression relative to DMSO control: PDB treatment: 2.9±0.8. Student's t-test (paired, 2 –tailed) DMSO control vs. PDB treatment p-value: 0.04, n=3), β-DG cleavage remained unchanged (Fig. 3-7a and c: Percent β-DG cleavage:

DMSO control: 9.9%±0.4%; PDB treatment: 12.0%±3.2%. No changes were statistically significant using a Student's t-test (paired, 2 –tailed) between the control and treatment groups, n=3). To determine if 24 hours was sufficient time to allow for the increased MMP-9 expression to take action, I also treated cells for 48 hours and tested upregulation of MMP-9 in addition to β-DG cleavage. Similar to the results in figure 3-7a-c, I observed increased MMP-9 expression but no changes in β-DG cleavage (data not shown). These results suggest that MMP-9 is not sufficient to induce β-DG cleavage in OPCs.

To determine if MMP-2, 9, or both are sufficient to induce β-DG cleavage, I then employed recombinant active MMP-2, 9, or both to treat proliferating OPCs on LM-2. Several doses of rMMP-2 and rMMP-9 were used to treat OPCs (250 ng, 500 ng, 750 ng, or 750 ng of both MMP-2 and 9 in combination), and after 24 hour treatments I did not observe any changes in β-DG cleavage (Fig. 3-7d and e: Percent β-DG cleavage in CTRL: 7.3%±3.0%; rMMP-2 250 ng: 6.7%±2.1%; rMMP-2 500 ng: 6.4%±1.4%; rMMP-2 750 ng: 7.1%±1.9%; rMMP-9 250 ng: 7.0%±2.5%; rMMP-9 500 ng: 6.4%±2.2%; rMMP-9 750 ng: 5.7%±1.8%; Both: 8.9%±2.8%. No changes were statistically significant using a Student's t-test (paired, 2 –tailed) between any of the groups, n=4). Similarly, as described above for the PDB experiment, I treated OPCs up to 48 hours with rMMPs and found no changes in β-DG cleavage, suggesting that MMP-2 and 9 are not sufficient to induce β-DG cleavage in proliferating OPCs.

Figure 3-7: MMP-2 and 9 are not sufficient to induce β-DG cleavage in OPCs. Phorbol Dibutyrate (PDB, 100 nM) treatment of OPCs upregulated MMP-9 expression (a, b), but no apparent change in β-DG Cleavage was observed (a,c). Recombinant MMP-2 and 9 were used to determine if β-DG cleavage could be induced. As depicted in (d) and quantified in (e), β-DG cleavage remains unchanged even when both MMP-2 and 9 are added simultaneously to OPCs.



ADAM proteinases are another family of metalloproteinases that, similar to MMPs, can cleave various matrix and receptor ligands and result in effects akin as those ascribed to MMPs. To determine if ADAM-10 or ADAM-17 are involved in β-DG cleavage in OPCs, ADAM-10 or ADAM-17 floxed mice were crossed with Tamoxifen-inducible PDGFR-α-cre recombinase expressing mice that yielded progeny of either Heterozygous flox (Het) or Homozygous flox (conditional knock out: cKO). Tail-vein injection of tamoxifen into postnatal day 1 mouse pups of the expected progenies yielded excision of the ADAM-10 or 17 loci after 2 days post-injection. At this time, cerebral cortices were dissected and mixed glial cultures were established as described in detail in chapter 2. OPCs were purified from mixed glial cultures obtained from each genotype, and plated on Laminin-2 in proliferation conditions. Figure 3-8 shows that in both Het and cKO, there are no apparent changes in β-DG cleavage, suggesting that neither ADAM-10 nor 17 are involved in β-DG cleavage in proliferating OPCs.

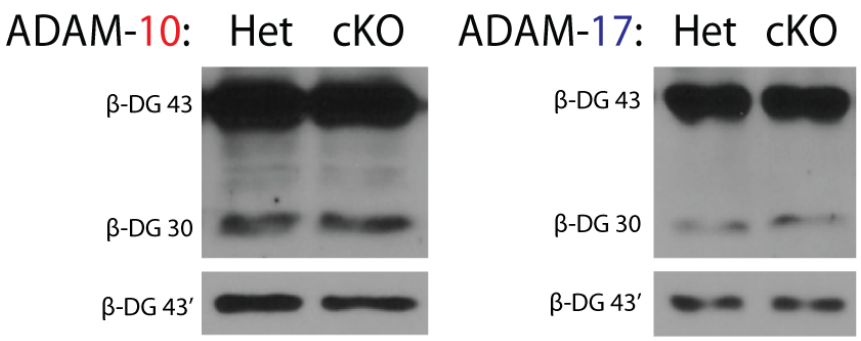


Figure 3-8: ADAM-10 and 17 are not involved in β-DG cleavage in OPCs. ADAM-10 (red) and 17 (blue). Heterozygote (het) and conditional knock out (cKO) β-DG 43' is a shorter exposure of the β-DG 43 (n=3, for each).

While I was unable to identify the protease responsible for β-DG cleavage, I reasoned that I can modulate β-DG cleavage by blocking metalloproteinase activity, and in this way, use a loss of function approach to understand what the role of cleaved β-DG is in OPCs. I therefore assessed OPC phenotypic changes upon blocking β-DG cleavage via metalloproteinase inhibition. Upon treatment of OPCs with BiPS over a 24 hour period, I found a 30% decrease in OPC proliferation as assessed by both Ki67 immunodetection and BrdU incorporation (Fig. 3-9a: Percent of Ki67+ cells: DMSO: 44.3%±7.2%; BiPS: 5.1%±1.7%. Student's t-test (paired, 2 –tailed) DMSO control vs. BiPS treatment p-value: 0.012, n=4. Fig. 3-9b: Percent of BrdU+ cells: DMSO control: 46.8%±5.7% vs. BiPS treatment 14.2%±2.5%. Student's t-test (paired, 2 –tailed) DMSO control vs. BiPS treatment p-value: 0.023, n=4). To rule out that cell death was not a reason for the decrease in OPC proliferation, a TUNEL assay was performed in which some increase in cell death was associated with inhibitor treatment (Fig. 3-9c: Percent of TUNEL+ cells: DMSO control: 2.4%±0.7%; BiPS treatment: 8.3%±1.5%. Student's t-test (paired, 2 –tailed) DMSO control vs. BiPS treatment p-value: 0.029, n=3). However, it alone cannot be the only reason for the decrease in proliferation observed. Therefore, to determine if decreased proliferation resulted in cell cycle exit and differentiation, immunocytochemistry for newly formed oligodendrocytes using the oligodendrocyte-specific differentiation marker, CNP, was

used to detect the populations of newly formed oligodendrocytes. However, I did not find a change in the number of CNP positive cells (Fig. 3-9d: Percent of CNP+ cells: DMSO control: 7.1%±3.2%; BiPS treatment: 7.1%±1.6%. No statistically significance resulted from a Student's t-test (paired, 2–tailed) comparing the groups, n=4), suggesting that while metalloproteinase inhibition decreases proliferation, this shift does not result in premature differentiation.

After ruling out cell death and differentiation as the cause for the proliferation changes observed, I turned to characterize cell cycle changes that could result in decreased OPC proliferation. Propidium iodine staining followed by flow cytometry analysis was used to determine the populations of cells existing in G0/G1, S, G2/M phases of the cell cycle upon BiPS treatment. Between 12 and 24 hours of BiPS treatment, I observed an increase in the number of OPCs at the G0/G1 phase (Fig. 3-10a and b), suggesting that the proliferation decrease observed could be due to G0/G1 arrest. I additionally found that gene transcripts of cell cycle regulators (cyclins D1, D3 and E) were downregulated upon inhibitor treatment, along with Immediate-Early-Response Genes c-jun, c-fos and Id2 (Table 3). Concomitantly, gene transcripts of cell cycle progression inhibitors p21 and p27 appeared to be mildly upregulated upon inhibitor treatment. At the protein level, cyclin D1 and cdk2, which form complexes with cdk4/6 and cyclin E, respectively, to promote entry into S phase after G1, displayed expression patterns supporting that of a G1 arrest where cyclin D1 showed a non-significant decreased trend (Fig. 3-11a and b: Fold change relative to DMSO control: 12 hr BiPS treatment: 0.8±0.1; 24 hr BiPS treatment: 0.6±0.1. No statistically significance resulted from a Student's t-test (paired, 2 –tailed) comparing the DMSO and BiPS treatment groups, n=7), while cdk2 showed a significant decrease (Fig. 3-11c and d: Fold change relative to DMSO control: 12 hr BiPS treatment: 0.8±0.2; 24 hr BiPS treatment: 0.6±0.1. No statistically significance resulted from a Student's t-

test (paired, 2 –tailed) comparing the DMSO and BiPS treatment groups at 12 hrs (n=5). However, at 24 hrs: p-value at 0.045, n=5). Inversely, p27 transiently increased (Fig. 3-11e and f: Fold change relative to DMSO control: 12 hr BiPS treatment: 1.2±0.4; 24 hr BiPS treatment: 0.8±0.2. No statistically significance resulted from a Student's t-test (paired, 2 –tailed) comparing the DMSO and BiPS treatment groups, n=5). While most of the results were trends in changes, they do suggest that metalloproteinase inhibition correlated with decreased β-DG cleavage are involved in G0/G1 cell cycle progression and regulation of OPC proliferation.

Given that BiPS as well as other MMP inhibitors can have effects on proliferation, I turned to a gain of function approach to determine the role of β-DG cleavage in OPCs. The β-DG intracellular domain (ICD) is a region on dystroglycan spanning various domains that bind intracellular signaling proteins such as dystrophin, F-actin, MAPK, Src tyrosine kinase and other important signaling components. To mimic overexpression of cleaved β-DG *in vitro,* I transfected OPCs with 2 constructs: 1) EGFP vector (CTRL-EGFP), and 2) EGFP tagged β-DG-intracellular domain (β-DG-ICD-EGFP) (Fig. 3-12a). Following transfection, OPCs were plated in proliferation media, and 12 hours later, switched to differentiation media in the presence of BrdU for 8 hours. The percent of BrdU+ cells out of EGFP+ cells was determined (Fig. 3-11 b). I found that OPCs appeared to proliferate more on laminin versus PDL (Fig. 3-12b: Fold change in percent of BrdU+ cells out of EGFP+ cells: PDL CTRL: 1.0±0.0; PDL β-DG-ICD: 1.2±0.0; LM CTRL: 1.2±0.2; LM β-DG-ICD: 1.1±0.1. No statistical significance resulted from a Student's t-test (paired, 2 –tailed) comparing the any of the groups, n=3). Interestingly, I also found that OPCs expressing β-DG-ICD-EGFP on PDL, displayed proliferation rates similar to those expressing CTRL-EGFP on laminin and higher than that of PDL CTRL, suggesting that β-DG-ICD-EGFP promotes OPC proliferation.

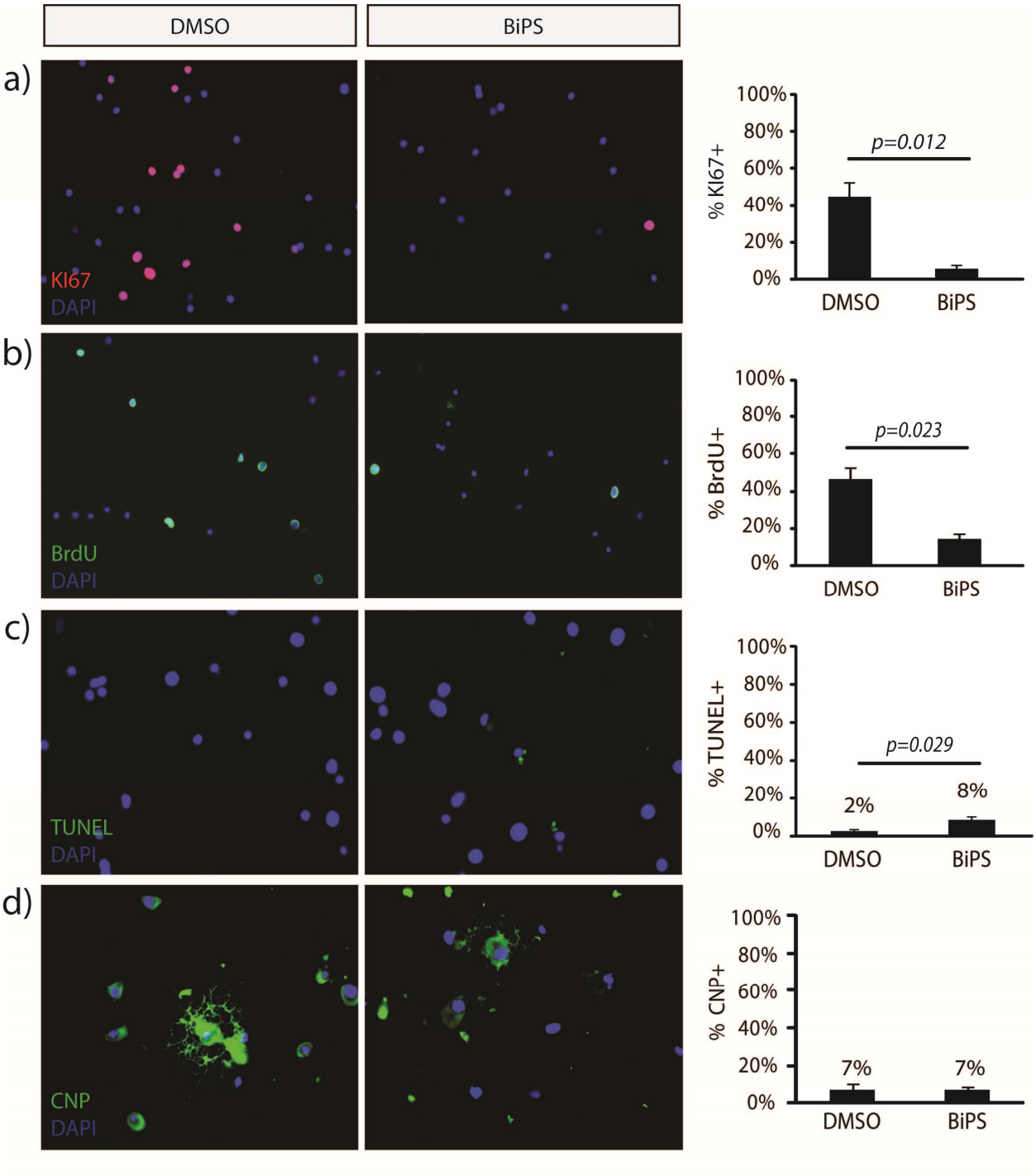
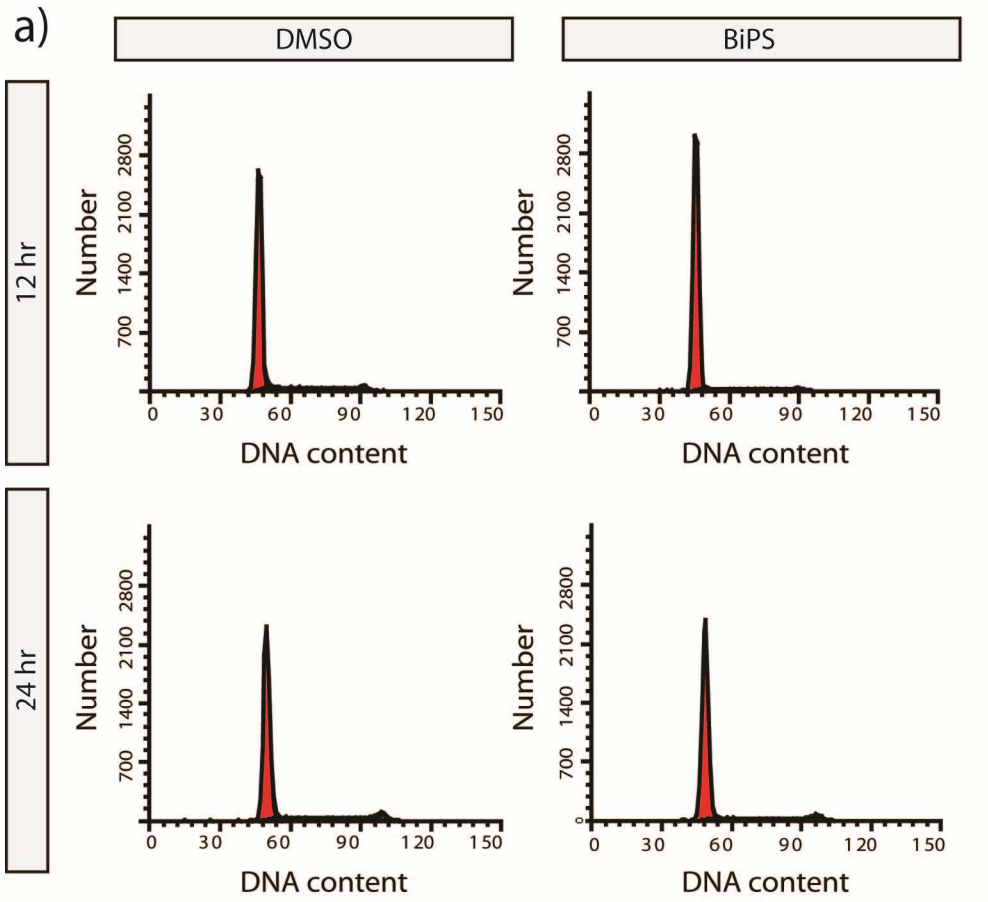


Figure 3-9: Metalloproteinase inhibitor treatment results in decreased OPC proliferation, not due to increased cell death or precocious differentiation. OPCs plated on laminin-2 and treated with BiPS for 24 hours under proliferating conditions were assessed for proliferative changes via (a) Ki67 and (b) BrdU. OPC apoptosis was evaluated via TUNEL labeling and immunodetection (c). OPC differentiation was assessed via CNP immunodetection (d).



b)

Average percent of cell cycle phases and ratios

	12h		24h	
	DMSO	BiPS	DMSO	BiPS
% G1	*81.9 ± 1.7	*85.1 ± 3.1	83.9 ± 3.5	84.6 ± 3.0
% S	**12.7 ± 1.3	**9.2 ± 1.3	11.1 ± 3.1	10.7 ± 2.6
% G2	5.4 ± 1.9	5.7 ± 2.2	*6.0 ± 1.4	*5.0 ± 1.0
Mean G1/S Ratio	*6.5 ± 0.8	*9.5 ± 1.7	8.2 ± 3.2	8.4 ± 2.7
Mean G2/S Ratio	*0.4 ± 0.2	*0.6 ± 0.2	0.6 ± 0.3	0.5 ± 0.2

Figure 3-10: Metalloproteinase inhibitor treatment results in increased OPC populations at the G0/G1 cell cycle phase. a) Flow cytometry curves depicting populations at each cell cycle phase at 12 and 24 hours after BiPS treatment. b) Average percent of cell cycle phases and ratios between DMSO and BiPS treatment percent at 12 and 24 hours. Student's t-test (paired, 2-tailed) significance is indicated by an asterisk where * = p-value < 0.05, and ** = p-value < 0.001. A sample size of 6 was analyzed for both groups and time points.

Table 3: Average Fold Ratio of BiPS/DMSO treament per time point

		BiPS/DMSO Ratio at:				
		1 hr	4 hr	8 hr	12 hr	24 hr
G1 to S progression	Cyclin D1	0.88±0.11	*0.64±0.05	*0.68±0.08	0.72±0.12	0.87±0.11
	Cyclin D3	**0.79±0.02	**0.73±0.02	0.67±0.14	0.63±0.09	0.61±0.11
	Cyclin E	1.04±0.12	*0.50±0.05	*0.67±0.04	*0.53±0.05	0.73±0.12
Immediate-Early-Genes	c-Fos	0.95±0.03	**0.48±0.02	**0.62±0.02	*0.67±0.04	0.67±0.11
	c-Jun	**0.71±0.02	0.98±0.04	0.81±0.15	0.77±0.14	1.00±0.05
	Id2	0.88±0.13	*0.62±0.04	0.90±0.11	0.80±0.10	0.86±0.12
Cell cycle inhibitors	p21	*0.83±0.02	0.83±0.05	1.13±0.12	1.41±.036	*1.31±0.08
	p27	0.76±0.06	0.86±0.05	1.06±0.14	1.33±0.38	0.99±0.02

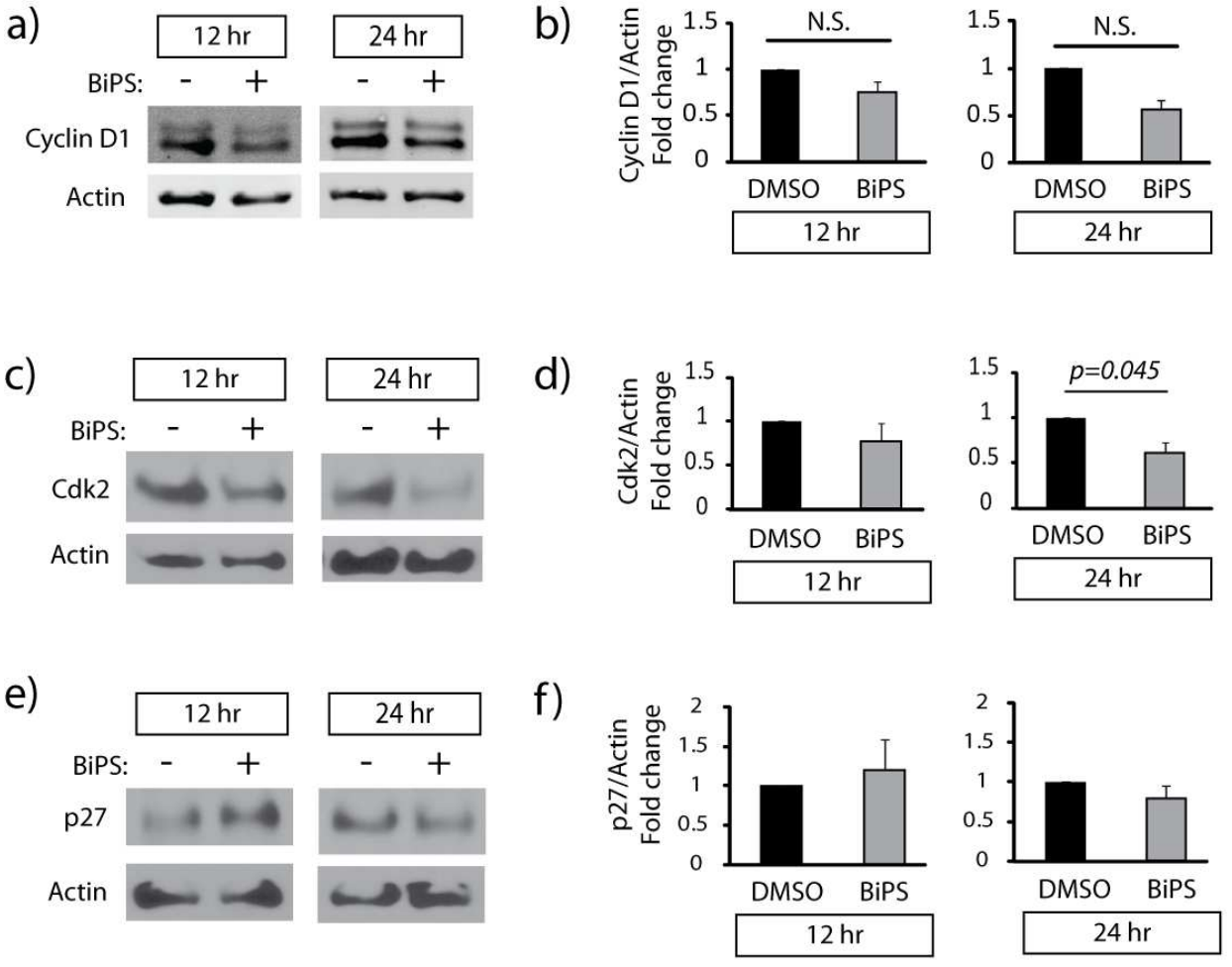


Figure 3-11: BiPS treatment results in changes in cell cycle regulating proteins in OPCS. a) and b) depict changes in cyclin D1, b) and c) in Cdk2, and e) and f) in p27.

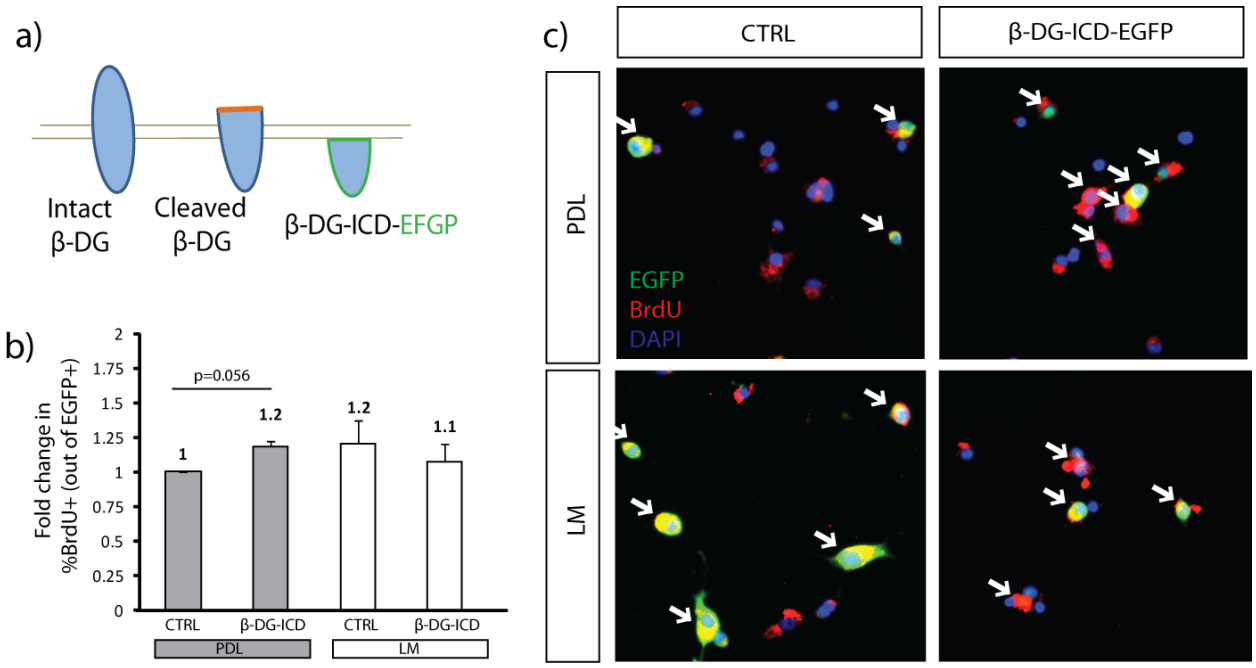


Figure 3-12: β-DG-ICD-EGFP in OPC proliferation. OPCs transfected with either CTRL-EGFP or β-DG-ICD-EGFP and pulsed with BrdU for 8 hours without mitogenic stimulation. a) Schematic depicting the expression of β-DG-ICD-EGFP which mimics the intracellular domain of β-DG, and differs from cleaved β-DG in that it does not contain a transmembrane or ectodomain. b) Fold change in the percent of BrdU+ cells out of EGFP+ expressing OPCs on PDL and laminin. c) Representative images: EGFP = green, BrdU = red, and DAPI = blue.

Discussion:

Here I described dystroglycan expression and cleavage during postnatal brain development. β-DG cleavage is highest at postnatal day 1 when neural stem cells are highly proliferative in the cerebral cortex. Furthermore, a decrease in β-DG cleavage is observed from P5 into adulthood, correlating with time points known for cellular differentiation in the brain. I took into consideration that cortical lysates do not specifically detect β-DG cleavage from oligodendroglia alone, particularly because it is known that other cell types (i.e. astrocytes and neurons) also express dystroglycan. I therefore isolated OPCs in culture, and found that β-DG cleavage is highest during OPC proliferation but decreases as OPCs undergo cell cycle exit and

proceed to differentiate. These observations suggest that β-DG cleavage has a function during brain development since it is regulated in a time-dependent manner, and that it may play a role in OPC development due to the similar trend that takes place *in vitro*. Additionally, while cleavage of β-DG has been associated with injury and disease in most published models, I show that this proteolytic event can also occur during development (in the brain), and that it can impact normal cellular behaviors (in primary OPCs).

One possibility that can explain why very little cleaved β-DG is identified in cells grown on PDL is that upon cleavage of β-DG, cleaved β-DG may be rapidly degraded unless a receptor substrate (like laminin-2) stabilizes dystroglycan and signals to prevent its degradation. In this way, cleaved β-DG becomes detectable only when OPCs are plated on laminin-2. To determine if this is the case, I plated OPCs on PDL in the presence or absence of MG132, a proteosomal inhibitor. While we observed a small increase in β-DG cleavage product upon MG132 treatment, this increase was not nearly as high as that of the level of β-DG cleavage that occurs on laminin-2 (data not shown). This suggests that β-DG cleavage is induced, not stabilized, by laminin-2, and I can rule out proteosomal degradation as a primary reason for why very little β-DG cleavage is detected in cells grown on PDL. However, treatment of OPCs on laminin-2 with MG132 results in an increase in β-DG cleavage almost twice that on laminin-2 without inhibitor, suggesting that when β-DG proteolysis takes place, a pool of the cleaved product is usually targeted for proteosomal degradation. This raises interesting questions about how different dystroglycan pools are differentially segregated, and if it occurs, how this process is regulated. Also in question is whether the pool *not* selected for degradation is targeted for a different compartment, or function, in the cell.

Dystroglycan is an ECM receptor that binds various extracellular ligands, including several members of the laminin family [148]. To my surprise, I found that laminin-2, but not laminin-1, promoted β-DG cleavage in OPCs. It is known that laminin-2 has a higher affinity for dystroglycan than does laminin-1, and this could be a reason for why cleavage is preferentially promoted on laminin-2. Other dystroglycan ligands such as perlecan and agrin are expressed in the developing and adult brain [149, 150]. It will be important to determine if these ligands are also involved in promoting β-DG cleavage and thereby may regulate OPC proliferation. Nevertheless, to our knowledge, this study is the first report of a specific ligand type that modulates dystroglycan processing.

One of our preliminary strategies was to block the known mediators of β-DG cleavage as a loss-of-function approach to determine what the role of cleaved dystroglycan is in developing OPCs. Using a broad spectrum metalloproteinase inhibitor as well as an inhibitor that is more selective to MMP-2/9 (BiPS), I observed a decrease in β-DG cleavage. However, at concentrations where BiPS has been characterized as specific for MMP-2 and 9, there was no change in β-DG cleavage. Through loss- and gain-of-function approaches, I determined that MMP-2 and 9 are neither necessary nor sufficient for β-DG cleavage in OPCs. At least 2 other studies have characterized a similar result in which they could not identify the proteinases cleaving β-DG, but in which MMP-2 and MMP-9 were ruled out [151, 152]. In one study, the authors identified a pharmacologic inducer of β-DG cleavage (chloranil) that appears to mediate the activation of a metalloproteinase through generation of reactive oxygen species. Using broad spectrum MMP inhibitors, the authors of this study concluded that a metalloproteinase is activated by chloranil, and through the use of recombinant TIMPs, they concluded that a metalloproteinase that is inhibited by both TIMP-2 and 3 could be involved. However, they did

not identify which specific proteinases are involved in cleaving β-DG. I reproduced similar results in that oligodendrocytes treated with chloranil also resulted in β-DG cleavage that could be inhibited by broad spectrum MMP inhibitors (data not shown), however, I did not test whether TIMP proteins could inhibit chloranil-induced β-DG cleavage. Given that it is known that TIMP-3 can block ADAM proteinases, I became interested in whether ADAM-10 or 17 could be involved in cleaving β-DG in OPCs, however, I did not find that ADAM-10 or 17 were involved. This is supported by other experiments performed in the lab where an ADAM-10 inhibitor (DAPT) was used to block ADAM-10 activity in OPCs, but no changes in β-DG cleavage were observed with treatment. Furthermore, rTIMP treatment of proliferating OPCs did not result in any changes in β-DG cleavage, suggesting that a metalloproteinase that is not targeted by the TIMPs may be involved. This result raised the possibility of the involvement of a metalloproteinase that fits a profile of evading TIMP inhibition. Through our search for candidate metalloproteinases, I identified the meprin proteases which are involved in ECM shedding and have developmental roles as characterized in urothelial function [153]. However, using a meprin inhibitor (actinonin) at various concentrations known to block meprin function, we did not identify any changes in β-DG cleavage in OPCs. Therefore, the enzyme involved in OPC β-DG cleavage remains unknown.

A completely alternative (and intriguing) hypothesis is that perhaps β-DG cleavage does not occur at the plasma membrane, and perhaps laminin-2 promotes the expression of a metalloproteinase that cleaved β-DG intracellularly. This model would explain why the addition of recombinant MMPs and TIMPs do not have an effect on cleavage since they do not enter the cell, and since MMP inhibitors are relatively soluble, they inhibit an intracellularly situated metalloproteinase that normally cleaves β-DG. In either case, it appears that MMP-2 and 9 are

not involved because knock down of either or both enzymes does not affect β-DG cleavage. In addition, if β-DG is cleaved intracellularly, it would be interesting to understand what functions it modulates in OPCs. Further studies will be required to determine where β-DG is cleaved.

Since BiPS treatment in OPCs resulted in ablation of β-DG cleavage, I continued with this strategy and used it as a loss of function approach. Using this method, I found that in the absence of cleaved β-DG, i.e. in the presence of BiPS, OPC proliferation decreases. After finding that the observed decrease in proliferation was not due to cell death or differentiation, a closer look at the cell cycle revealed a G0/G1 arrest phenotype. This finding was interesting to us because in a study in which dystroglycan is knocked down, a G2/M arrest is observed, suggesting that β-DG regulates cell cycle progression [141]. Furthermore, it was found that β-DG can be found at the cleavage furrow during cytokinesis, implicating dystroglycan in other aspects of cell division [141]. However, more studies will be required to determine what the role of dystroglycan is in terms of the "OPC timer" that regulates cell cycle exit and differentiation. While it is known that the timer is dependent on PDGF to induce the number of cell division counts, and on thyroid hormone to control the timing of differentiation, it is interesting that β-DG cleavage correlates with proliferation and sharply decreases with OPC differentiation. Whether β-DG cleavage is a cause or effect of OPC proliferation is unknown but is an interesting avenue to pursue since it would explain how extracellular signals like laminin may influence the OPC timer mechanism through a ligand-binding event.

To confirm that cleaved β-DG plays a role during OPC proliferation, I expressed a construct in which the cytoplasmic fraction of β-DG was EGFP tagged, allowing us to track transfected OPCs in combination with BrdU labeling to detect proliferating cells. I found that EGFP+ OPCs expressing the β-DG-ICD-EGFP construct were more likely to be BrdU labeled,

suggesting that β-DG cleavage modulates OPC proliferation. Current studies in our lab are focused on understanding if gene expression changes mediate this increase in proliferation, and where this protein is targeted to in the cell to result in proliferative changes.

In future studies it will also be important to identify the intracellular signaling modulators through which dystroglycan directs its effects (i.e. dystrophin, utrophin, focal adhesion kinase, etc) to better understand how β-DG affects OPC proliferation. In Schwann cells, the only other cell type where β-DG cleavage has been characterized during development, it was found that different pools of β-DG are associated with different intracellular adaptor proteins and play different functions, all vital to the proper function of the myelinated sheath in the peripheral nervous system. This strongly suggests that understanding the signaling partners of β-DG may reveal information about the functions it plays, when and where in OPCs.

Following BiPS treatment of OPCs, I identified decreased Erk phosphorylation in acute time points post-treatment (30 minutes – 1 hour) (data not shown). Dystroglycan has predicted MAPK binding sequences in its cytoplasmic region, and is furthermore known to bind MEK and ERK in different cellular locations to influence cellular behaviors [154]. The changes in Erk phosphorylation at short time points post-treatment could reflect the effect of the inhibitor blocking β-DG cleavage and affecting Erk signaling. Furthermore, Immediate-Early-Response gene transcription was decreased at slightly later time points (1 hr – 12 hr), which could suggest a transcriptional response to blocking β-DG cleavage. In addition, some of the cyclin genes transcripts analyzed suggested a direct effect on cell proliferation due to inhibitor treatment. Because MMP inhibitors could interrupt cell proliferation by a different mechanism that does not relate to that of decreased β-DG cleavage, I tested BrdU incorporation under increasing concentrations (17nM – 12.5 μM) of BiPS to determine if concentrations less than the one that

blocks β-DG cleavage also result in decreased proliferation. However, I observed no changes in BrdU incorporation in concentrations prior to reaching the one at which ablation of β-DG cleavage occurred, thereby at least correlating decreased β-DG cleavage and decreased proliferation (data not shown).

In conclusion, I have described protease-mediated alterations in OPC biology, likely via alterations in dystroglycan processing that regulate OPC proliferation and cell cycle progression. I also found that while canonical MMP-2 and 9 mediated dystroglycan cleavage does not mediate dystroglycan processing, other metalloproteinase(s) are likely involved, and future work will be required to understand more in detail how cleaved dystroglycan functions in OPCs to regulate proliferation and perhaps other aspects of OPC development.

Chapter 4:

MMP-7 delays oligodendrocyte differentiation during early stages of oligodendroglial development

Introduction:

Oligodendrocyte differentiation is a finely tuned process whose key molecular regulators are not completely understood. However, interactions of developing oligodendrocytes with ECM proteins such as laminin have profound consequences in proper timing of oligodendrocyte development and myelination [57, 58]. In addition to laminin, various other ECM ligands (i.e. tenascin, vitronectin and fibronectin) have been also been described to regulate various aspects of oligodendrocyte development including proliferation, survival, and process extension to name a few [48, 52, 56]. In the brain, it is known that ECM availability is more abundant during early postnatal development, and later in adulthood, confined to specific ECM structures in the adult brain [155]. How this drastic change in expression and bioavailability of ECM proteins occurs is not understood.

Matrix metalloproteinases are zinc-dependent enzymes whose primary function (among many others ascribed) include the proteolysis of extracellular matrix proteins that can result in the release of ECM protein fragments to stimulate cell behavior and liberation of growth factors and cytokines to activate other signaling cascades [156]. More recently, a small set of MMPs have been discovered to play important roles in the developing brain, but the role of the rest of the MMP family members remain elusive [79].

Through a gene expression screen I identified the expression of MMP-7 in differentiating oligodendrocytes. MMP-7 is a minimal domain MMP whose expression has been identified in the brain, and whose activity is implicated in cancer, immunity, epithelial repair and exacerbation of disease onset in mouse models of multiple sclerosis [157, 158]. However, MMP-7 has not previously been identified in glial cells, and therefore, its role has not been explored in this context.

Here I describe a preliminary set of data that implicate MMP-7 as a myelination inhibitor. First, I found that differentiating oligodendrocytes upregulate MMP-7 gene expression an average of 23 fold from the OPC to mature oligodendrocyte stage. This increase is also observed at the protein level where western blot analysis of OPC, pre-mature and mature oligodendrocytes *in vitro* show an increased trend in MMP-7 expression. To test MMP-7 function, recombinant active MMP was added to OPCs for 1 day in culture where I observed decreased numbers of NG2+ cells, suggesting that MMP-7 promotes differentiation. However, OPCs treated daily with rMMP-7 for 3 and 5 days led to decreases in CNP+ and MBP+ cells, suggesting that MMP-7 delays oligodendrocyte differentiation. MMP-7 pharmacological inhibition for the same time schemes resulted in mild increases in CNP+ and MBP+ oligodendrocytes, further supporting that MMP-7 is an oligodendrocyte differentiation inhibitor. To get a better understanding of when oligodendrocytes are affected by MMP-7 treatment that leads to decreased differentiation, pre-myelinating oligodendrocytes at day 3 were treated with rMMP-7 until day 5 of differentiation where no changes in MBP+ cells were observed. In addition, day 3 cells were also treated with MMP-7 inhibitor and a small but significant decrease in MBP+ cells was observed, suggesting that MMP-7 inhibition in the late stages of differentiation is unfavorable to differentiating oligodendrocytes. Together, these results suggest that MMP-7 is inhibitory in early stages of

oligodendrocyte differentiation, but supporting, in the later stages of differentiation. Finally, I show that expression of MMP-7 occurs in the early postnatal brain as is associated with the developing striatum, cortex, corpus callosum and neuroepithelium, suggesting that MMP-7 may play a role in the development or function of cells in these regions.

Results:

I explored the possible changes taking place in the ECM during oligodendrocyte differentiation through analysis of gene expression between OPCs and mature oligodendrocytes. I employed the use of a qRT-PCR array designed to screen gene expression changes of ECM proteins, receptors and remodeling enzymes. Among the group of proteins whose expression was upregulated from early to late differentiation, MMP-7 expression was detected to increase an average of 21 fold (Fig. 4-1a: Fold change in MMP-7 transcript expression at day 5 of differentiation relative to day 1 of differentiation: 21.3±5.1, Student's t-test (paired, 2-tailed) p-value: 0.001, n=4). I confirmed the expression of MMP-7 protein through western blot analysis of differentiating oligodendrocyte lysates (Fig. 4-1b). To determine where MMP-7 is expressed in oligodendrocytes, I performed immunocytochemistry to detect NG2+ OPCs after 1 day in differentiation media and found that many of them were MMP-7 positive, and that MMP-7 appeared to be localized to areas of the cell soma that were associated with growing processes, as well as in the extending processes themselves (Fig. 4-1 c). This confirmed the expression of MMP-7 in oligodendrocytes and provided us initial clues about where MMP-7 is localized in OPCs. To determine if other glial cells express MMP-7, I performed immunocytochemistry for GFAP to identify astrocytes and found that MMP-7 expression was associated with GFAP+ cells (Fig. 4-1d). However, immunocytochemistry for Iba1, a marker for microglia, did not appear to

be associated with MMP-7, suggesting that these cells do not express MMP-7 under these conditions (Fig. 4-1 e).

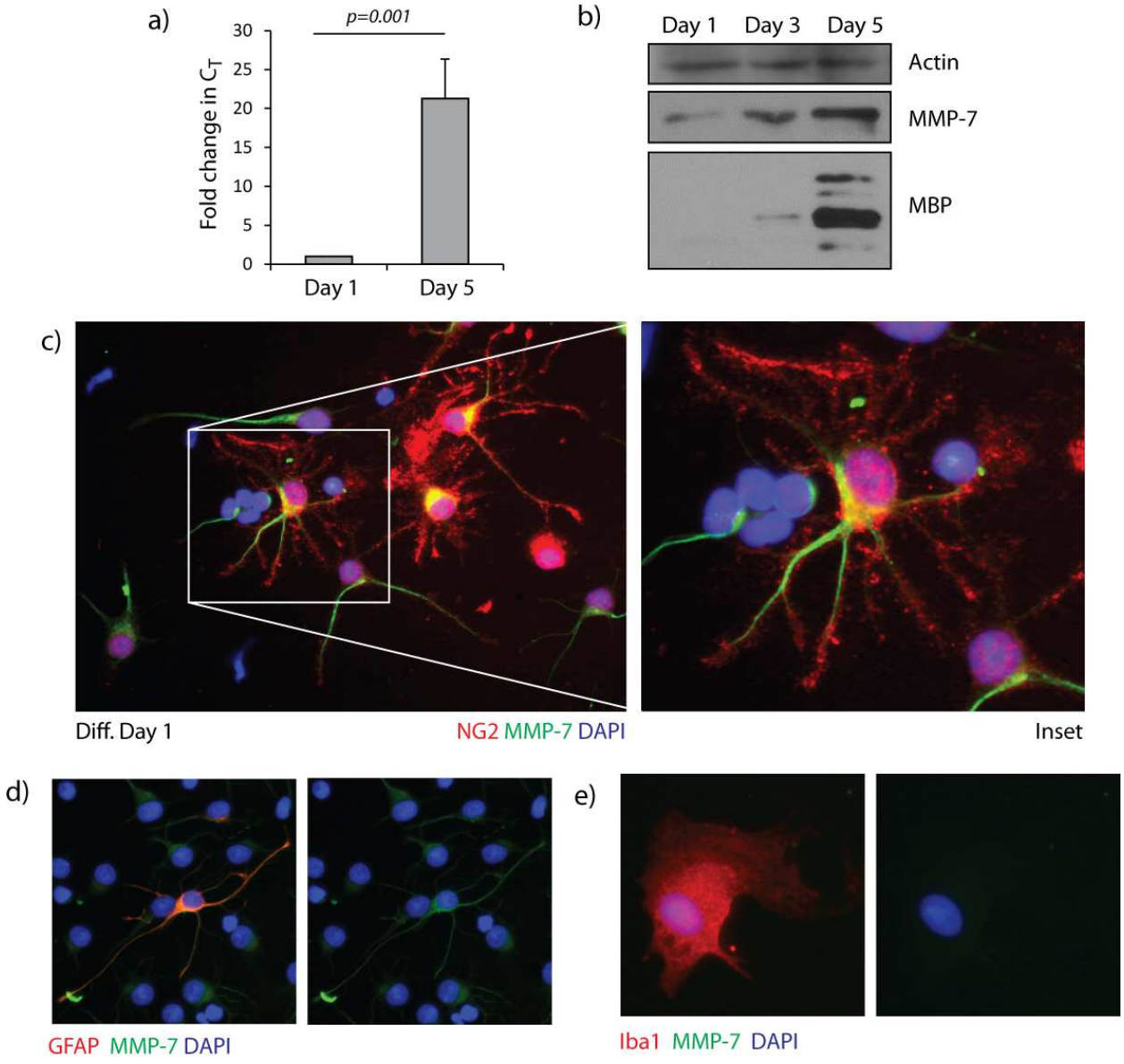


Figure 4-1: MMP-7 expression in oligodendrocyte development. a) OPCs were plated on PDL and differentiation media for 1 and 5 days. At these time points, RNAs were extracted and analyzed via qRT-PCR. MMP-7 Fold change in C_T values averaged from 4n yielded a 23 fold increase in MMP-7 expression. b) Differentiating oligodendroglia were lysed for protein and analyzed via western blot for MMP-7 protein expression. c) Immunocytochemistry for MMP-7 in NG2+ oligodendrocyte progenitor cells. d) GFAP+ cells express MMP-7, while e) Iba1+ cells do not.

While I found MMP-7 expression in the processes of NG2+ OPCs, not all regions of the processes appear to contain MMP-7. A closer look at the tips, or growth cones, of OPC processes that are marked by NG2, are areas where MMP-7 is absent (Fig. 4-2, white arrows), suggesting that the growing tips of processes specifically restrict MMP-7. Interestingly, MMP-7 expression was also found to correlate with areas of the cell body where the cell surface is in close apposition to the nuclear membrane, as well as slightly in the nucleus itself. Further studies are required to assess the potential complexity of MMP-7 expression in developing oligodendroglial nuclear areas (yellow arrows).

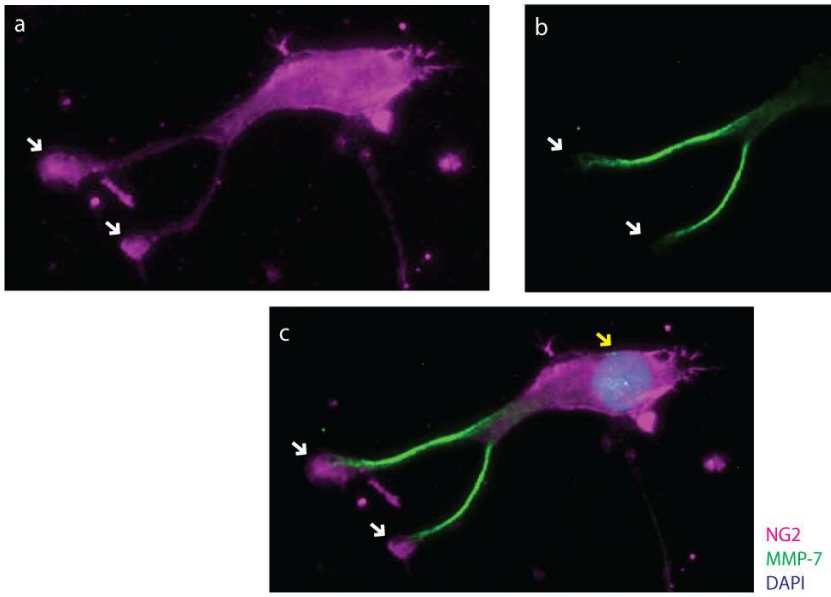


Figure 4-2: MMP-7 localization in oligodendrocyte progenitor cells. NG2+ oligodendrocyte progenitor cells (a) were co-labelled for MMP-7 (b). Merge in (c). White arrows point to tips of processes where MMP-7 is absent, and yellow arrows points to areas consistent with cell nucleus where MMP-7 is also observed.

Given that MMP-7 is expressed in growing OPC processes, I performed gain and loss of function experiments aimed at increasing or ablating MMP-7 activity during early OPC differentiation. Because laminins have been characterized as MMP-7 substrates, and OPC differentiation is increased on laminin substrate, I plated cells on either non-physiological

adherent substrate (Poly-D-Lysine, PDL) or on laminin-2 (LM). First, I treated OPCs with either DMSO vehicle or MMP-7 inhibitor for 1 day of differentiation and then assessed the NG2+ cell pools in each condition. NG2 expression in OPCs is associated with a progenitor phenotype, whereas NG2 shedding, and therefore loss of NG2 expression, is associated with an oligodendrocyte reaching a pre-myelinating stage. After MMP-7 inhibitor treatment, I did not identify any differences in NG2+ cell populations (Fig. 4-3e: Percent of NG2+ cells in: PDL DMSO: 29.5%±6.1%; PDL MMP-7 inhibitor: 27.1%±1.5%; LM DMSO: 27.2%±0.4%; 25.0%±1.6%. n=2), suggesting that MMP-7 is not required for early phases of oligodendrocyte differentiation.

Inversely, treatment of OPCs with recombinant MMP-7 resulted in decreased numbers of NG2+ cells (Fig. 4-3c: Percent of NG2+ cells in: PDL CTRL: 57.7%±6.5%; PDL rMMP-7: 18.6%±0.7%; LM CTRL: 60.5%±2.3%; LM rMMP-7: 10.7%±5.1%. n=2) suggesting that MMP-7 may promote oligodendrocyte differentiation. However, looking at the phase micrographs of corresponding NG2 immunocytochemically labeled images, it did not appear that oligodendrocytes were more differentiated, given that a characteristic of process complexity and morphology that is associated with differentiation ("branchiness") appeared less complex in the rMMP-7 treated cells (Fig. 4-3a and b). Together, these results suggest that MMP-7 is not necessary for normal oligodendrocyte differentiation in the early stages, but if present in levels above that of endogenous, may delay or stall oligodendrocyte differentiation. This is supported by a decreased trend in the percentage of CNP+ cells upon rMMP-7 treatment at day 1 of differentiation (Fig. 4-3d: Percent of CNP+ cells at day 1 of differentiation: PDL CTRL: 41.5%±9.1%; PDL rMMP-7: 23.7%±0.5%; LM CTRL: 60.5%±2.3%; LM rMMP-7: 10.7%±5.1%. n=2).

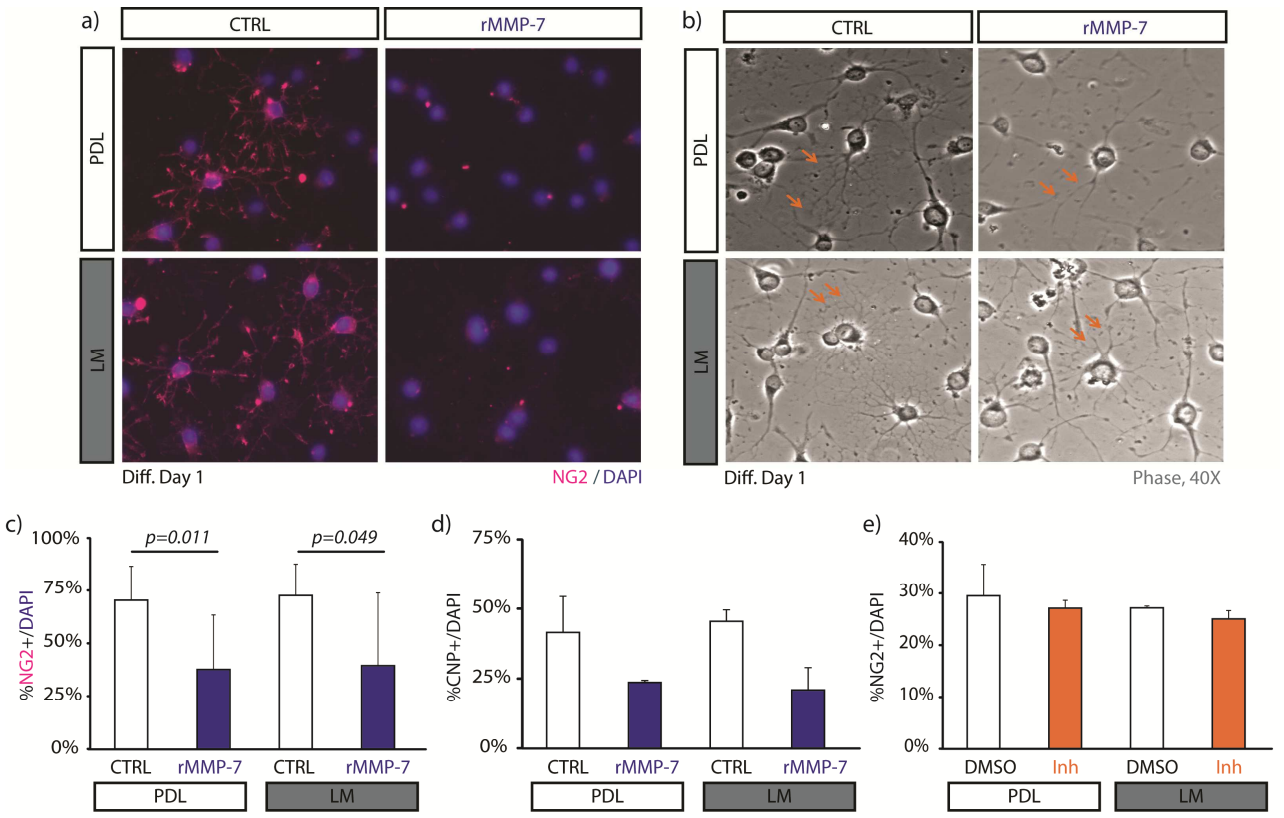


Figure 4-3: Exogenous MMP-7 results in decreased NG2 expression in differentiating oligodendrocyte progenitor cells. a) OPCs treated with recombinant active MMP-7 (rMMP-7) for 1 day in differentiating conditions. b) Percent of NG2+ cells in control (CTRL) and rMMP-7 treated OPCs (n=3). c) Percent of CNP+ cells in control and rMMP-7 treatment conditions (n=2). d) Percent of NG2+ cells after 1 day of either vehicle (DMSO) or MMP-7 inhibitor (Inh) (n=2).

To determine the function of MMP-7 during later stages of oligodendrocyte differentiation, I first added recombinant-active MMP-7 daily to differentiating OPCs for either 3 or 5 days at which point the cell populations of CNP+ (at day 3) or MBP+ (at day 5) were counted in control or treatment conditions. In both PDL and LM conditions, I found that exogenous MMP-7 treatment led to decreased percentages of CNP+ and MBP+ cells at day 3 and day 5, respectively (Fig. 4-4 b: Percent of CNP+ cells at day 3 of differentiation upon rMMP-7 treatment: PDL CTRL: 35.2%±1.2%; PDL rMMP-7: 21.1%±7.5%; LM CTRL: 36.4%±2.4%; LM rMMP-7: 20.6%±9.6%. Student's t-tests (paired, 2-tailed) comparing any of

the groups were not significant, n=3. Fig. 4-4d: Percent of MBP+ cells at day 5 of differentiation upon rMMP-7 treatment: PDL CTRL: 58.9%±17.8%; PDL rMMP-7: 24.4%±13.0%; LM CTRL: 74.9%±10.2%; LM rMMP-7: 24.3%±3.4%. PDL CTRL vs. PDL rMMP-7: Student's t-test (paired, 2-tailed) p-value: not significant, n=3; LM CTRL vs. LM rMMP-7: Student's t-test (paired, 2-tailed) p-value: 0.02, n=3). Furthermore, MBP+ area quantified at day 5 of differentiation was also decreased upon rMMP-7 treatment (Fig. 4-4e: Fold change in MBP+ area relative to substrate control (PDL or LM): PDL rMMP-7: 0.5±0.05; LM rMMP-7: 0.2±0.06; Student's t-test (paired, 2-tailed) p-value: comparing either of the groups was not significant; n=3). MBP+ cell area reflects the growth of myelin membrane sheets, a hallmark of the most mature, myelination-competent cells. These results suggest that MMP-7 represses oligodendrocyte differentiation.

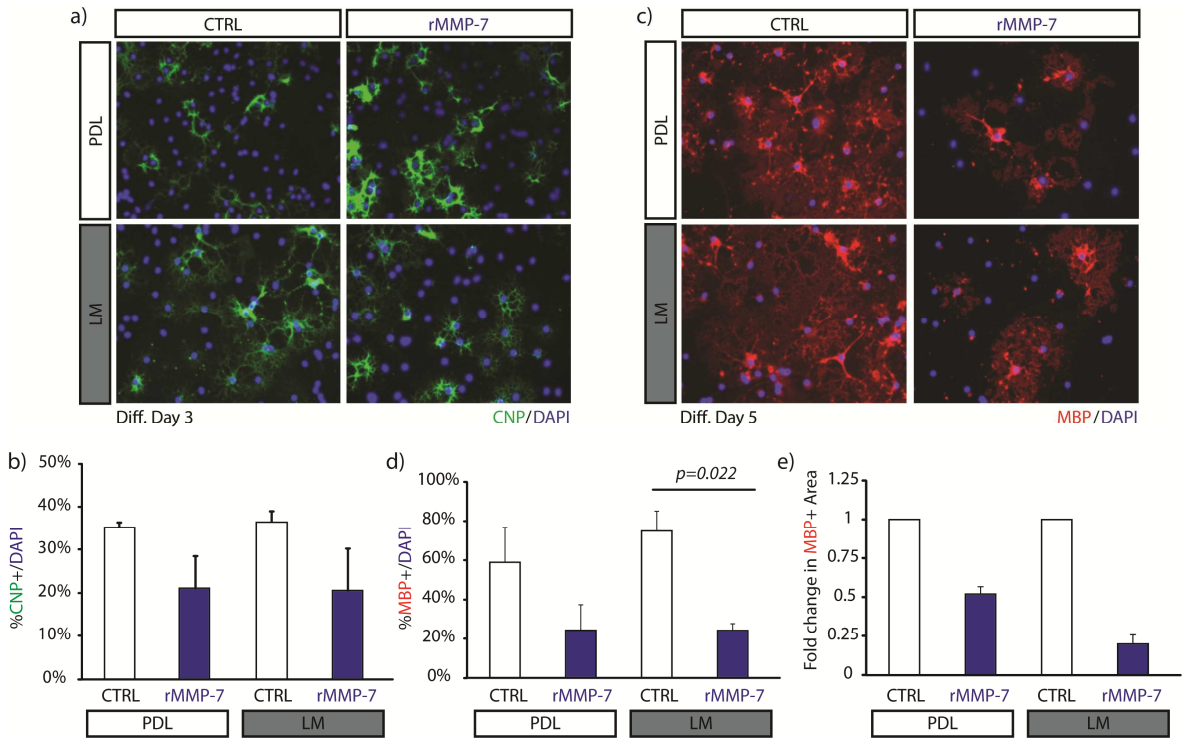


Figure 4-4: Exogenous MMP-7 inhibits oligodendrocyte differentiation. Differentiating oligodendrocytes in the presence of recombinant active MMP-7 (rMMP-7) at differentiation day 3 (a) or 5 (c). Percent of CNP+ cells at day 3 of differentiation (b). Percent of MBP+ cells (d) and MBP+ area (e) at day 5 of differentiation.

To determine the effects of MMP-7 inhibition during oligodendrocyte differentiation, I blocked MMP-7 activity via pharmacological inhibition following the same treatment schemes described above. Analysis of the CNP+ and MBP+ cell populations at day 3 and 5 revealed that MMP-7 inhibition resulted in mild, but significant increases in the percentages of CNP+ cells (on PDL and LM) and MBP+ (LM only) (Fig. 4-5b: Percent of CNP+ cells treated with MMP-7 inhibitor at differentiation day 3: PDL DMSO: 56.6%±9.5%; PDL Inh: 65.7±7.3%; LM DMSO: 62.5%±1.9%; LM Inh: 72.7%±3.8%. PDL DMSO vs. PDL Inh: Student's t-test (paired, 2-tailed) p-value: 0.026, n=4. LM DMSO vs. LM Inh: Student's t-test (paired, 2-tailed) p-value: 0.016, n=4. Fig. 4-5d: Percent of MBP+ cells treated with MMP-7 inhibitor at differentiation day 5: PDL DMSO: 39.9%±10.4%; PDL Inh: 56.8±2.6%; LM DMSO: 44.5%±5.3%; LM Inh: 53.0%±5.0%. PDL DMSO vs. PDL Inh: Student's t-test (paired, 2-tailed) p-value: not significant, n=3. LM DMSO vs. LM Inh: Student's t-test (paired, 2-tailed) p-value: 0.02, n=3) in addition to an increased trend in MBP+ areas on PDL, but not on LM (Fig. 4-5e: Fold change in MBP+ area relative to substrate DMSO control (PDL or LM): PDL Inh: 1.8±0.9; LM rMMP-7: 1.2±0.1; Student's t-test (paired, 2-tailed) p-value: comparing either of the groups was not significant; n=3). These results suggest that MMP-7 inhibition results in a mild promotion of oligodendrocyte differentiation, and that MMP-7 is unfavorable to oligodendrocyte differentiation.

These results suggest a paradoxical situation that occurs during oligodendrocyte development and raise questions about the expression and function of MMP-7 during this process. If oligodendrocytes upregulate MMP-7 expression endogenously during their differentiation, why would increasing MMP-7 prevent their differentiation? In addition, why would blocking MMP-7 activity result in increased differentiation response at later stages? To

answer these questions, I turned to experiments that would allow us to identify the time window during which MMP-7 exerts its effects.

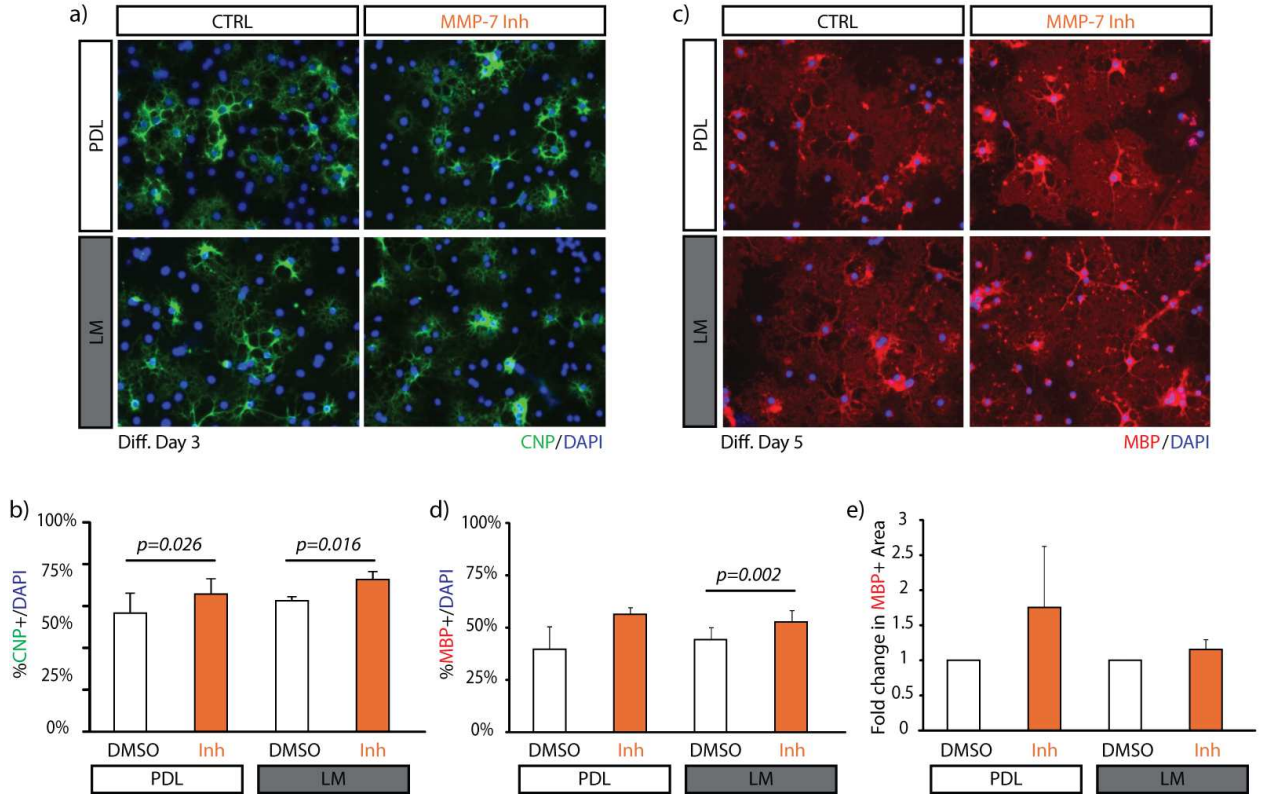


Figure 4-5: MMP-7 inhibition promotes oligodendrocyte differentiation. CNP+ and MBP+ cells at day 3 and day 5 of differentiation after MMP-7 inhibitor treatment (MMP-7 Inh) (a) and (c), respectively. (b) Percent of CNP+ cells after MMP-7 inhibitor treatment (Inh). (d) MBP+ cell percentages and MBP+ area (e) after MMP-7 inhibitor treatment (Inh) at day 5 of differentiation.

One of the strategies that I employed was to limit the window of treatment to identify stage-specific MMP- function. To determine the function of MMP-7 in late stages of differentiation, I allowed OPCs to form processes normally and commit to differentiation. At this point (day 3 of differentiation), I then modulated MMP-7 activity via rMMP-7 or MMP-7 inhibitor until day 5 of differentiation and then assessed the percentage of MBP+ cells (Fig. 4-6a, schematic). Interestingly, I do not see changes in the percentages of MBP+ cells (Fig. 4-6b: Percent of MBP+ cells upon rMMP-7 treatment from day 3 to day 5 of differentiation: PDL

CTRL: 55.6%±7.1%; PDL rMMP-7: 54.0%±6.6%; LM CTRL: 59.7%±4.3%; LM rMMP-7: 53.1%±2.1%), except slightly in the PDL MMP-7 inhibition condition (Fig. 4-6c: Percent of MBP+ cells upon MMP-7 inhibitor treatment from day 3 to day 5 of differentiation: PDL DMSO: 61.0%±9.7%; PDL Inh: 50.2%±10.3%; LM DMSO: 60.3%±6.3%; LM Inh: 60.2%±9.1%. PDL DMSO vs. PDL Inh: Student's t-test (paired, 2-tailed) p-value = 0.006, n=4), which suggests that MMP-7 is promoting oligodendrocyte differentiation after day 3. However, rMMP-7 treatment did not prevent oligodendrocyte differentiation after differentiation day 3, suggesting that the inhibitory effects that rMMP-7 has on differentiation are established during the day 1-3 differentiation window, and reflect a reason why MMP-7 may be expressed at low levels in OPCs, but upregulated later. However, MMP-7 expression patterns may not reflect MMP-7 activity patterns, and therefore, further studies are required to separate out these possibilities.

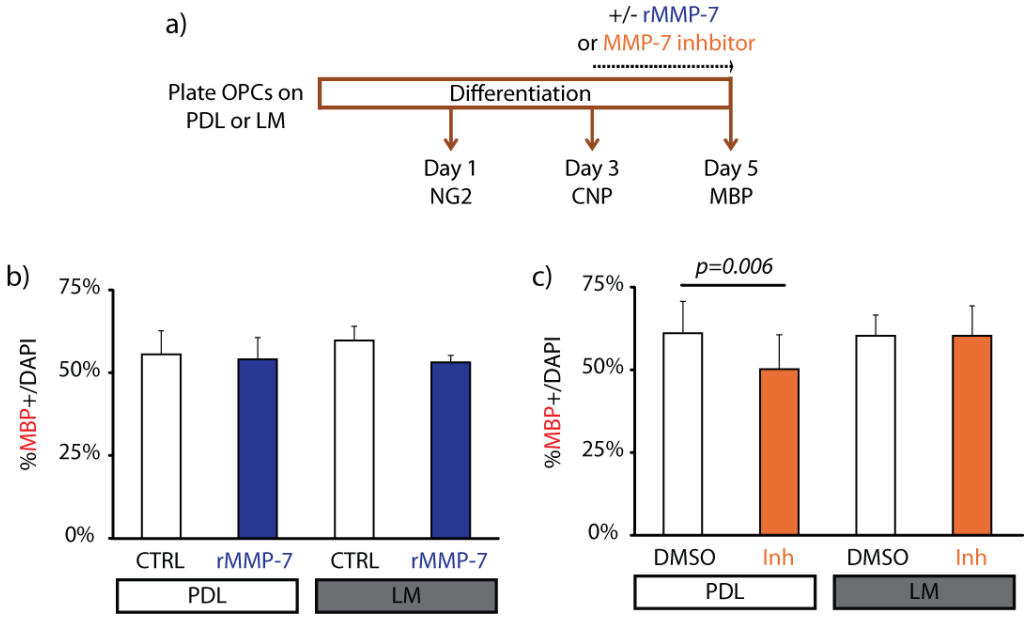


Figure 4-6: MMP-7 alters oligodendrocyte differentiation by acting early in differentiation. a) Treatment strategy employed in experiments (b) and (c) where OPCs are differentiated for 3 days are then treated with recombinant active MMP-7 (rMMP-7) or MMP-7 inhibitor (Inh) until day 5. (b) and (c) show MBP+ cell results upon treatment with rMMP-7 and MMP-7 inhibitor, respectively.

To determine when MMP-7 is expressed during cortical development relative to myelination, I dissected mouse cerebral cortices at time points leading up to postnatal day 21, a time point at which myelination is mostly complete in the murine cortex. I found that MMP-7 is expressed at higher levels in the early postnatal time points (P1-P5) and decreases sharply thereafter, resulting in little to no expression by postnatal day 21 (Fig. 4-7, n=3). This pattern of expression inversely correlates with myelination, supporting our earlier findings in that MMP-7 is a myelination inhibitor. However, given that oligodendrocytes appear to increase MMP-7 expression during differentiation, it is unclear why during time points that correlate with oligodendrocyte differentiation in the cortex (P8-P21), there is a decrease in MMP-7. Further studies will be required to determine what the expression of MMP-7 is *in vivo* in oligodendrocytes.

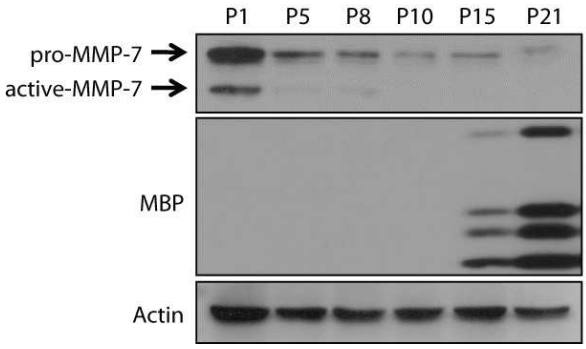


Figure 4-7: MMP-7 expression in the developing brain. Cerebral cortical lysates at postnatal days 1,5,8,10,15, and 21 (P1-P21) were analyzed for MMP-7 and Myelin Basic Protein (MBP) expression.

After observing that MMP-7 expression was highest at postnatal day 1 in the cerebral cortex, coronal sections of P1 brains brain were immunohistochemically processed for MMP-7 and nestin to identify gross, regional MMP-7 expression patterns. Nestin was used to visualize radial glia, neural stem cells and some early progenitors of the developing cerebral cortex. Fig. 4-8a shows that MMP-7 (red) is expressed in the developing striatum in areas that, by DAPI (blue), characteristically appear to be cross-sections of axon bundles. In cortical areas of the brain above the corpus callosum (Fig. 4-8b, nestin = green, MMP-7 = red), high MMP-7

immunoreactivity is found associated with what is likely to be neurons in the cerebral cortex. This suggests that MMP-7 could play a role in neuronal migration and/or establishment of neurons as they reach their target sites. In addition, MMP-7 appeared to correlate with nestin expression in the regions below the cingulum in the corpus callosum and also at the edges of the subventricular zone where radial glia line the lateral ventricular wall (Fig. 4-8c, white arrows). I also identified expression of MMP-7 in areas of the corpus callosum following a pattern to that of the axonal tracts (Fig. 4-8d-f, white arrows).

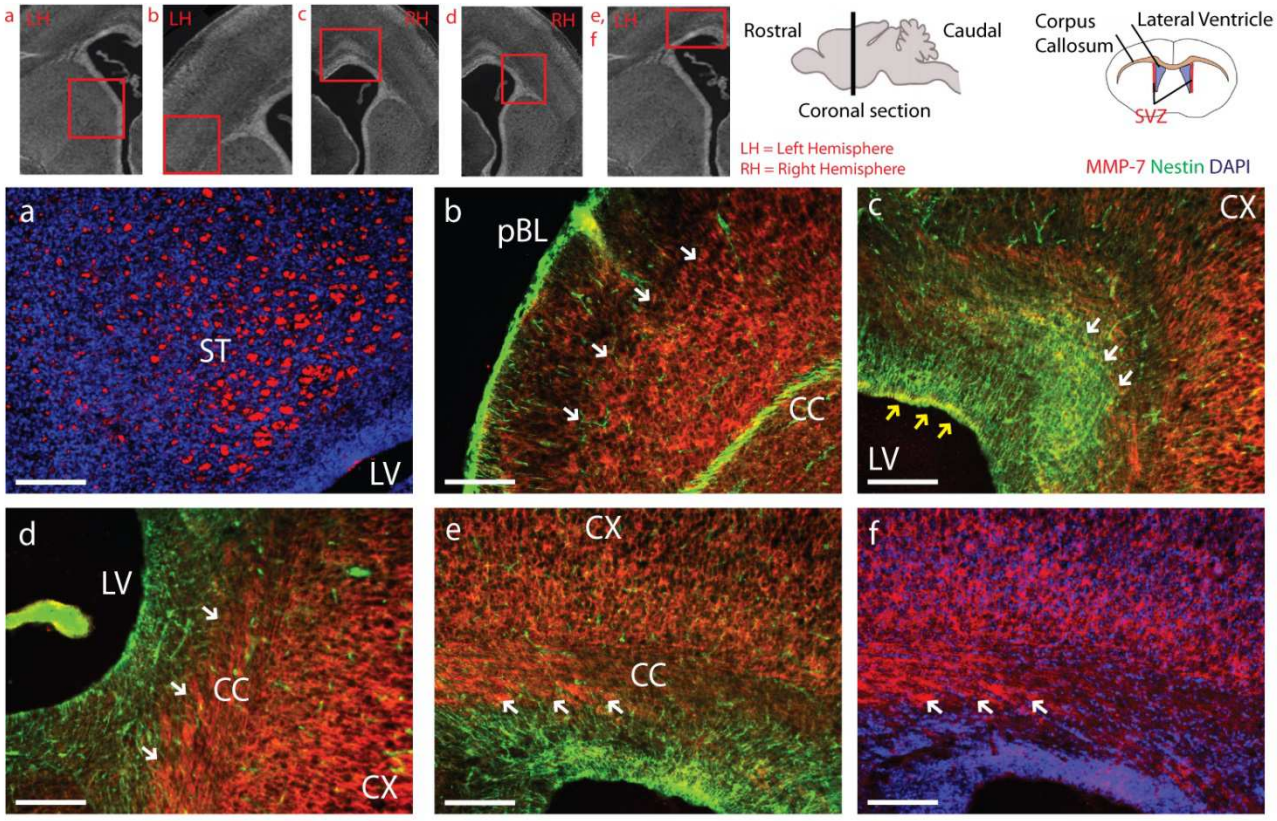


Figure 4-8: MMP-7 expression in the early postnatal brain. Coronal sections of p1 brains were analyzed for MMP-7 (red) and DAPI (blue) expression by immunohistochemical detection. Nestin (green) was used to demarcate the areas of neural stem cells, radial glial and some neural progenitors. MMP-7 expression in (a) the developing striatum, (b) cerebral cortical layers above the corpus callosum (white arrows), (c) Regions below the cingulum (white arrows) and cells bordering the ventricle (yellow arrows), (d, e) axonal tracts in the corpus callosum (white arrows) and the cortex. Regional locations for a-f are indicated in 10X images (a-f) in red boxes. Scale bars = 200 µm. ST = striatum; pBL = pial basal lamina; CC= corpus callosum; LV = lateral ventricle; CX = cortex.

Discussion:

Here I described a new role for MMP-7 in the myelin forming cells in the CNS, in addition to describing its expression pattern in the early postnatal brain. I identified this metalloproteinase from a screen of ECM protein, receptor and proteinase gene expression, during which MMP-7 exhibited a relatively high upregulation from the OPC to mature oligodendrocyte stages. I confirmed MMP-7 expression at the protein level in these cells, and found a specific pattern of localization in the cell processes where it is absent from the growing tips. Furthermore, MMP-7 appears to be localized to areas correlating with the nucleus, suggesting that it may play a role in this cellular compartment. Interestingly, other studies have found MMPs in the nucleus of other cell types, where they mediate cell behaviors independent of their more widely known roles in the extracellular milieu [159]. Further studies will be required to determine if MMP-7 is indeed localizing to the OPC nucleus and whether it facilitates functions in this cellular compartment.

Through gain and loss of function studies I found that MMP-7 can alter oligodendrocyte differentiation. The functional consequences of adding exogenous-active MMP-7 to differentiating cells are decreased numbers of NG2+ cells and less complex branching, phenotypes that could be explained through a variety of mechanisms. First, MMP-7 has been shown to activate MMP-9, a metalloproteinase that has been implicated in the shedding of NG2+ in the spinal cord and the brain [89, 94]. Some reports have shown that MMP-7 can activate MMP-9 [119]. Given that MMP-7 treatment results in decreased expression of NG2, it is feasible that either MMP-7 could be shedding NG2 directly, or, that MMP-7 is activating MMP-9 and thereby inducing NG2 shedding. Furthermore, a reason why process complexity is observed could be explained by the evidence that MMP-9 is required for normal oligodendrocyte process

outgrowth and differentiation [87]. In addition, decreased NG2+ cell number was not accompanied by decreased cell densities in the OPC cultures upon rMMP-7 treatment (total number of cells were not different between conditions; data not shown), suggesting that the decrease in NG2+ cells is also most likely not due to cell death.

MMP-7 treatment correlated with altered OPC process complexity, suggesting that MMP-7 affects cell morphology. In cultured mature hippocampal neurons, Bilusova et al., showed that MMP-7 treatment results in loss of dendritic spines where the mature dendritic spines transform into long, thin filopodia that are characteristic of immature dendrites [108]. Furthermore, the actin cytoskeleton moved away from the dendritic shaft into the axon, suggesting that MMP-7 treatment leads to changes in actin cytoskeleton. Although the exact mechanism through which MMP-7 mediates this effect on dendritic morphology is unknown, this study serves as a template to view the change in process dynamics that I observe upon OPC MMP-7 treatment. In OPCs, actin cytoskeleton remodeling is crucial for proper process formation and branching [160, 161]. MMP-7 treatment in OPCs may similarly stall or delay the normal actin cytoskeleton arrangement, and thereby, process branching in OPCs.

Interestingly, MMP-7 inhibition for 1 day of OPC differentiation did not result in changes in NG2+ cells, suggesting that OPCs may normally keep MMP-7 activity low during their earlier development to prevent MMP-7-mediated effects on cell morphology. However, this result is in itself surprising given that OPCs appear to express a very tightly localized pool of MMP-7 at the cell processes. Although MMP inhibition can in some circumstances result in various forms of compensation (i.e., upregulation of other MMPs) and may be a reason to explain why no changes in NG2+ numbers were changed, perhaps MMP-7 is not necessary for process formation. Other,

more direct studies assessing cell morphology may be required to understand what the role of MMP-7 is during the early stages of OPC differentiation in the processes.

To determine the long-term effects of MMP-7 treatment on oligodendrocyte formation, I performed rMMP-7 or MMP-7 inhibitor treatments daily starting at the OPC stage and into the maturation stage. OPCs treated with MMP-7 for several days (3 and 5) resulted in decreases in the percentages of CNP+ and MBP+ cells, respectively, further supporting the initial results that MMP-7 may repress differentiation. One way to explain this delay is by taking a closer look at Notch, another important signaling molecule involved in OPC differentiation [116]. Normally, axons display Notch ligands (Jagged and Delta) that come in contact with Notch-expressing OPCs and results in the release of the intracellular domain that function to repress oligodendrocyte differentiation. Because OPCs in my experiments were not co-cultured with neurons, Notch ligands are presumably not available, and therefore OPC differentiation can proceed normally as long as differentiating conditions are provided. MMP-7 has been shown to cleave Notch and release its intracellular domain in other cell types. rMMP-7 treatment in OPCs may therefore result in notch cleavage that may prevent OPC differentiation, and could be a contributing factor for why oligodendrocyte differentiation is delayed.

Treatment of differentiating OPCs with MMP-7 inhibitor resulted in mildly increasing percentages of CNP+ and MBP+ cells at day 3 and 5 of differentiation, which suggests that endogenous MMP-7 is also inhibitory to oligodendrocyte differentiation. These results unearth a paradox in OPC MMP-7 expression observed in the first part of the study: why do oligodendrocytes upregulate MMP-7 during their differentiation process if MMP-7 results in decreased mature CNP+ and MBP+ cells? One way to explain this is to define the window during which MMP-7 results in decreased OPC differentiation. When I treated oligodendrocytes

that have already partly differentiated and are committed to myelination (day 3 of differentiation) with rMMP-7 until day 5, I did not see any changes in MBP+ cells, suggesting that the window of MMP-7 action is during days 1-3. While I did not expect any changes in MBP+ cells due to inhibitor treatment, I found that a small but significant *decrease* in MBP+ cells in the PDL plated oligodendrocytes, but not on LM, suggesting that MMP-7 is important for oligodendrocytes not exposed to laminins. This result also suggests that the upregulation of endogenous MMP-7 begins to take effect at around day 3 of differentiation. Together, these results suggest that MMP-7 is an inhibitor of myelination (at early, immature time points) and in part explains why OPCs express low levels of MMP-7 early on in differentiation. Further work needs to address what the role of MMP-7 is in later stages of differentiation, although the data so far suggest that MMP-7 may be beneficial in the later stages of oligodendrocyte differentiation.

One possibility that cannot be precisely tested *in vitro*, but could be done *in vivo* is to determine whether MMP-7 acts as a myelin "break" at later stages of oligodendrocyte development. As oligodendrocytes form myelin sheaths around axons, it is known that somehow, these cells are able to determine how much to myelinate in order to reach the "best fit" for electrical conductivity depending on the axon size and caliber. The known molecular regulators that communicate a "stop" signal to oligodendrocytes are few, and for this reason, the discovery of others may be aid our understanding of the overall myelination process. To answer the question of whether MMP-7 acts as a myelination break, MMP-7 knock out and MMP-7 overexpressing transgenic mice may be used to study myelin thickness and compactness using electron microscopy on cross-sectioned axons in different regions of the CNS.

Clues about other MMP functions in myelination and in the brain can be obtained from staining brain tissues at different developmental time points and observing where MMP-7 is

located within specific regions. Using postnatal day 1 coronal brain sections, I identified 4 primary areas where MMP-7 protein is found. First, MMP-7 appears in the striatum, particularly in the DAPI-clear lumens characteristic of striatal neuron axonal cross-sections. Second, MMP-7 is expressed in areas of the cerebral cortex above the corpus callosum. Third, some MMP-7 appears to be associated with axon tracts in the corpus callosum, and fourth, MMP-7 appears to co-localize with nestin positive cells, for instance in radial glia lining the wall of subventricular zone. These observations suggest that MMP-7 may regulate some neuronal and glial functions due to its high expression in the striatum and cortical layers. Given the time point of development postnatally, it might be play a role in neuronal migration and specification during cortical lamination. Interestingly, notch is also expressed in cortical layer neurons and it is a critical regulator of cortical lamination [162]. It would be interesting to assess notch activity in MMP-7 null or over-expressing mice, to determine if MMP-7 is involved in this signaling cascade and thereby modulating neuronal phenotypes.

Laminin has been found on the surface of axons prior to myelination, and there, it is thought to provide trophic signals to migrating OPCs in a PDGF dependent manner [60]. Some reports have implicated MMP-7 in cleaving laminin, and others have implicated laminin fragments known are “cryptic sites” which can have effects on cellular behaviors [122]. I found that recombinant laminins 1 and 2 were cleaved by MMP-7 in a dose-dependent manner, suggesting that these fragments can be released *in vivo* (data not shown). However, I have not yet identified the amino acid composition of these products to determine if MMP-7 can release previously characterized cryptic domains released upon laminin proteolysis. With these preliminary findings it is tempting to predict that that perhaps MMP-7 is associated with axons in

order to release these cryptic peptides that provide OPCs with survival signals or other types of signal activation that can promote their differentiation once OPCs reach the axonal tract.

In conclusion, I identified a new member of the matrix metalloproteinase family involved in regulating oligodendrocyte differentiation and is expressed in a pattern inverse to myelination *in vivo*.

Chapter 5:

Conclusions & Future Directions

The molecular mechanisms that regulate OPC proliferation and differentiation are not completely understood. In this thesis project, I have identified 2 mechanisms that require further exploration to fully understand what their roles are in regulating oligodendrocyte development. In the first, I found that the laminin receptor dystroglycan is cleaved during OPC proliferation and differentiation, albeit at much higher levels during OPC proliferation. This suggested that dystroglycan cleavage is associated with the OPC stage of oligodendrocyte development, and upon further exploration, I found that cleaved dystroglycan appears to function in OPC cell cycle progression.

A closer look at OPC populations in different stages of interphase revealed a small but significant accumulation of OPCs at the G0/G1 phase. Support for a G1 arrest phenotype came from assessment of the cell cycle regulator and inhibitor protein expression in which the G1 phase progression proteins (i.e., cyclin D1 and cdk2) were decreased, while the cell cycle inhibitor, p27, was increased. Interestingly, a known mediator of the OPC timer mechanism is p27, which is slowly upregulated as OPCs cycle until they become "competent" to respond to a signal that promotes differentiation (like TH3). It is also known that during interphase, cdk2 expression is relatively unchanged, suggesting that as cells reach an increased ratio of p27/cdk2, they become increasingly competent to respond to a differentiation signal. This suggests that in the presence of metalloproteinase inhibitors, where G1 is arrested and there is increased p27 levels and decreased cdk2 levels, the p27/cdk2 ratio may be closer to that of a "competent" cell, and therefore, these cells could be more responsive to TH3. However, I did not directly assess

whether the G1 arrest occurs before or after the G1 restriction point. This demarcation is important in understanding the fate of these G1 arrested cells, in which G1 arrested OPCs would be able to drop out of the cell cycle and differentiate (if in the presence of TH3) if they are arrested before the restriction point, or, alternatively, if stalled after the restriction point for too long may result in cell death. If it were the case that BiPS treated OPCs were stalled before the G1 restriction point, I would have identified an increase in CNP+ cells upon BiPS treatment because TH3 was present in the media. However, I did not identify any changes in differentiating cells after BiPS treatment, and therefore this evidence supports that the BiPS-treated cells at G1 arrest are past the restriction point. Additionally, I identified a small but significant increase in cell death after BiPS treatment, further supporting that death is the fate of some of these G1 arrested cells. Further studies are needed to dissect the roles of these cell cycle proteins in relationship to dystroglycan to understand if OPC differentiation can be artificially advanced. Furthermore, a complete study would address the changes of the cell cycle and its molecular regulators in an inverse experiment in which the β-DG-ICD-EGFP (dystroglycan cleavage mimic) construct is expressed.

Further studies are also required to empirically determine if gain or loss of dystroglycan cleavage affects OPC proliferation. One way to address the loss of function aspect is to over-express an "uncleavable" form of dystroglycan in proliferating OPCs to determine if proliferation is again decreased. Inversely, overexpression of a β-DG construct that expresses the exact form of 31 kDa cleaved dystroglycan could be used to more precisely address dystroglycan cleavage gain of function. In both conditions, cell cycle stages, and their molecular regulators and inhibitors, will need to be analyzed to specifically determine how dystroglycan processing regulates the cell cycle.

Intriguingly, several recent studies have identified β-DG in the nuclear compartment where it appears to bind lamin proteins and localize to various sites of nuclear architecture. This implies that β-DG may play a key role in the nucleus, although not many aspects of this role have been explored. Preliminary studies from our lab have identified the β-DG 30 kDa fragment in the nucleus of both OPCs and oligodendrocytes, suggesting that the β-DG cleaved product may play a role in the oligodendroglial nucleus. Current work in our lab is focusing on expressing β-DG constructs that lack the nuclear localization signal at the c-terminus of β-DG and determine whether nuclear localization is involved in OPC proliferation or other cellular phenotypes. This work will be important to enable us to understand what happens to β-DG once it is processed at the membrane. With the knowledge that β-DG carries an NLS and that it is translocated to the nucleus, several questions arise: 1) If dystroglycan is cleaved at the cell membrane, how is it translocated to the nucleus? 2) If dystroglycan is not cleaved at the membrane, does this event occur intracellularly?

A couple of different scenarios remain a possibility: a) After β-DG is initially cleaved extracellularly, dystroglycan could undergo a secondary cleavage event by gamma secretase, resulting in release of the intracellular domain that can then be chaperoned to the nucleus. Gamma secretase has been shown to cleave β-DG in an in vitro system where a 26 kDa protein product is formed [163]. Whether gamma secretase is involved in β-DG cleavage in OPCs remains an open question. b) Dystroglycan could be endocytosed, sorted and transported to the nucleus in a mechanism similar to that of the epidermal growth factor receptor (EGFR) [164]. c) Following translation, dystroglycan could be proteolytically processed and then translocated to the nucleus (and thereby never see the plasma membrane at all). Given that MMPs have been shown to be have intracellular proteolytic effects in other cell types, this is a possible scenario

[80]. d) Because MMPs have been shown in other cell types to localize to the nucleus [159], dystroglycan could be synthesized and immediately translocated to the nucleus where it is then cleaved by nuclear MMPs. These possibilities open other novel and exciting questions about dystroglycan biology.

I determined that MMP-2 and 9 are not necessary or sufficient regulators of β-DG cleavage in OPCs, establishing a non-canonical mechanism that differs from various studies in which MMP-2 and 9 are clearly involved in β-DG cleavage. Because I did observe a decrease in dystroglycan cleavage upon broad spectrum MMP inhibitor treatment, however, it's probable that a metalloproteinase must be involved. However, metalloproteinases include MMPs in addition to ADAMs, ADAM-TS, and many other proteinase families. Given that ADAM-10 and 17 have been implicated in various membrane proteolysis events, I sought to determine if these enzymes were involved in dystroglycan cleavage in OPCs. However, ADAM-10 and 17, in addition to another class of metalloproteinases, the meprins, do not appear to be required for β-DG cleavage. One approach that can be used to determine candidate proteases that act on dystroglycan is based on protease expression during OPC proliferation on LM and not on PDL, by isolating gene transcripts from OPCs in these two conditions and performing a screen via qRT-PCR using MMP primers. If there are one or more proteases upregulated in the LM but not PDL condition, I can then confirm the change in expression via western blot analysis of lysates, as well as perform functional assays (i.e., recombinant protein treatment and siRNA knock down). An alternative way to find the β-DG cleaving enzyme(s) may be to immunoprecipitate β-DG from the PDL and LM proliferation conditions and use a mass spectrometry approach to determine what proteinases associate with β-DG that may act to process it. Perhaps both techniques may need to be utilized to determine which enzymes are involved.

One unanswered question in our studies is whether the GM6001 inhibitor has the same effects on OPC proliferation and cell cycle arrest as that of the BiPS inhibitor. If so, it confirms that a metalloproteinase is involved, but if not, I can expect that BiPS may simply target a smaller or different set of metalloproteinases which could help in identification of the metalloproteinase that cleaves dystroglycan.

Several questions remain in regards to the role of MMP-7 in oligodendrocyte development. Among them are whether MMP-7 can cleave NG2, and if so, whether it is direct or indirect, and furthermore, what the consequences of premature loss of NG2 is for MMP-7 treated OPCs. Some clues from the data suggest that NG2 loss may alter oligodendrocyte morphology. However, I do not know if NG2 is indeed shed from OPCs, or whether there is another mechanism involved in the loss of NG2, e.g. downregulation. Furthermore, the precise effects that exogenous MMP-7 has on oligodendrocyte cell morphology during differentiation are also unclear. I have observed that oligodendrocytes have altered morphology in the presence of exogenous MMP-7, which is confirmed by the decreased trend in MBP+ area in oligodendrocytes treated with MMP-7. However, I have not assessed whether decreased MBP+ area coverage is a direct result of MMP-7 effects on OPCs at the earlier stages of differentiation, or if there is a separate effect that also occurs later after those initial morphological changes. Our data suggests that MMP-7 acts early in OPC differentiation to delay it, suggesting that the modulating effects that MMP-7 has on OPCs in early differentiation are potentially long-lasting. From our data looking at MMP-7 *in vivo* at postnatal day 1 brain coronal sections, it appears that MMP-7 is found in many areas where migrating and proliferating OPCs are typically present, e.g. the corpus callosum. If this is the case, how do OPCs in vivo respond to these local MMP-7 signals? Importantly it will be critical to determine if MMP-7 levels are locally altered at the

onset of myelination. Perhaps OPCs express local pools of TIMPs to inhibit MMP-7 activity in the immediate area, or express other proteinases to degrade local MMP-7 as protective measures. These questions need exploration *in vivo,* and they hold important significance in understanding whether MMP-7 secreted at the sites of MS lesions act to repress OPC differentiation or simply modulate it in some way, and furthermore whether it is a main player in the stall of OPC differentiation at the sites of repair.

While I found that MMP-7 can cleave laminin-1 and 2 *in vitro,* I did not explore what the cleavage products are, whether they can modulate any aspect of OPC development, and whether they occur *in vivo*. These remain as interesting aspects of laminin biology in OPC development because laminins have been found on axonal tracts and have been thought to provide signaling cues to OPCs that support their health and survival once OPCs have reached the axonal tract. I found MMP-7 present in areas that correspond to axon tracts in the developing striatum as well as the corpus callosum, suggesting that MMP-7 at these sites could play a role in releasing and activating important signaling modulators that aid in neuronal and glial functions.

Given that MMP-7 appears to play a major role in repair and recovery from injury, it suggests that adult brains following injury may reveal a different role for MMP-7. Because many mouse models of MS have focused on the role of MMP-7 in the inflammatory response, it would be interesting to determine how MMP-7 null mice fare when repairing a demyelination lesion that is free of external inflammation factors such as recruited leukocytes and their secondary effects. One mouse model that can be used to answer this question is the cuprizone-induced demyelination model where mice are fed a diet of cuprizone-fixed chow for 4-6 weeks to induce the demyelination of the corpus callosum. After the 4-6 weeks, normal chow replaces the cuprizone-chow and remyelination is assessed. Although a local inflammatory response by the

brain's resident immune cells, the microglia, may also be observed, it is expected that perhaps microglia may not contribute dramatically to MMP-7-mediated alterations since I found that at least *in vitro*, microglia don't appear to produce MMP-7. It would be interesting to determine if laminin is re-expressed at the sites of demyelination in axons and whether MMP-7 plays a role in the remyelination process at these sites.

Finally, as oligodendrocytes appear to increase expression of MMP-7 during differentiation, and because MMP-7 appears to inhibit oligodendrocyte morphological changes, it would be interesting to determine if MMP-7 is secreted by mature oligodendrocytes as a "stop" signal that mediates the completion of a myelin sheath. Using co-cultures of neurons and oligodendrocytes some initial findings may be gleaned from *in vitro* studies. For instance, whether exogenous-active MMP-7 added to early myelinating cultures myelinate differently than non-MMP-7 treated ones. However, MMP-7 null mice should ultimately be used to determine if MMP-7 loss leads to thicker myelin sheaths.

In conclusion, further work will be required to determine 1) what protease(s) cleaves OPC dystroglycan, 2) whether dystroglycan cleavage affects or is affected by the OPC timer mechanism, and 3) whether MMP-7 is involved in myelination and remyelination.

Chapter 6:

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