NOVEL MECHANISMS UNDERLYING HYALURONAN-MEDIATED REMYELINATION FAILURE

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4-MU	4-methylumbelliferone
BBB	blood brain barrier
BMP	bone morphogenic factor
BTH	Bovine Testicular Hyaluronidase
CC	corpus callosum
CNS	central nervous system
CSPG	chondroitin sulfate proteoglycans
CS'ase	chondroitinase ABC
CFA	complete Freud's adjuvant
DG	dentate gyrus
EBV	Epstein-Barr virus
EAE	experimental autoimmune encephalomyelitis
ECM	extracellular matrix
ERK1/2	extracellular signal-regulated kinase 1 and 2
ERM	Ezrin/Radixin/Moezin
GalC	galactocerebrosidase
GE	ganglionic eminence
GSK3b	glycogen synthase kinase-3b
GAG	glycosaminoglycan
HA	Hyaluronan, Hyaluronate, or Hyaluronic acid
HARE	HA receptor for endocytosis
HSC	hemopoietic stem cell
HMW	high molecular weight
IL	interleukin

JME	Japanese macaque encephalomyelitis
LGE	lateral ganglionic eminence
LMW	low molecular weight
MGE	medial ganglionic eminence
MAPK	mitogen-activated protein kinase
pMN	motor neuron progenitor domain
MS	Multiple Sclerosis
MHV	murine hepatitis virus
MAG	myelin associated glycoprotein
MBP	myelin basic protein
NAC	N-acetyl-L-cysteine
NGF	nerve growth factor
NSPC	neural stem/progenitor cell
O-2A	oligodendrocyte – type2 astrocyte
OPC	oligodendrocyte progenitor cell
OL	oligodendrocyte
PNS	peripheral nervous system (PNS)
PDGFR-α	platelet-derived growth factor receptor subtype alpha
preOL	pre-myelinating oligodendrocytes
PG	proteoglycan
PLP	proteolipid protein
qRT-PCR	quantitative real time polymerase chain reaction
RTK	receptor tyrosine kinases
SCI	spinal cord injury
StrepH	*Streptomyces* hyaluronidase
SVZ	subventricular zone

RHAMM	receptor for HA-mediated motility (RHAMM)
SGZ	subgranular zone
TVME	Theiler's murine encephalomyelitis
TLR	Toll-Like Receptor
TNF	transforming necrosis factor
T3	triiodothyronine, thyroid hormone
VCPAL	6-O-Palmitoyl-L-ascorbic acid
WNT	*wingless*

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ABSTRACT

Large caliber axons are wrapped in a thick insulating membrane known as myelin, which vastly improves the speed and efficiency of electrical signaling in the nervous system. Central nervous system (CNS) myelin is synthesized by glia cells named oligodendrocytes (OLs), which arise from oligodendrocyte progenitor cells (OPCs). Both myelin and OLs are destroyed following a number of insults to the adult CNS, including traumatic injury, ischemia, and diseases like multiple sclerosis. Remyelination following each of these insults requires the recruitment of OPCs to demyelinated lesions and their subsequent maturation into myelin-producing OLs. Remyelination is typically an incomplete process in the CNS, likely due to inhibitory molecules found within demyelinated lesions. Hyaluronic acid (HA; also called hyaluronan) is a core component of the extracellular matrix (ECM) of all tissues and is secreted by glia in demyelinated lesions. HA accumulates in chronic demyelinated lesions and has been shown to inhibit OPC maturation and contribute to remyelination failure. The mechanism by which this accumulated HA influences OPC maturation was not previously known.

HA is degraded by a family of enzymes named hyaluronidases, and distinct sizes of HA has been shown to influence a diverse set of cellular behaviors via interaction with a variety of intra- and extracellular HA-binding receptors. The body of work presented in this dissertation shows that hyaluronidase activity generates HA degradation products that inhibit OL maturation and contribute to HA-mediated remyelination failure. Next, this work identifies that one specific hyaluronidase, PH20, is found in chronic

demyelinated lesions in MS patients and in mice with experimental autoimmune encephalomyelitis (EAE), an animal model of MS. These data indicate that PH20 is a potential pharmacological target for therapies aimed at promoting remyelination. Additionally, this work supports potential roles for controlled HA catabolism in driving a diverse collection of cellular behaviors needed for the repair of damaged myelin, such as neural stem cell quiescence, progenitor specification, OPC proliferation, and migration. Collectively, the studies described in this dissertation highlight the importance of the integration of extracellular cues in driving cellular processes critical for the generation and repair of white matter.

CHAPTER 1: BACKGROUND & INTRODUCTION

Structure & Function of Myelin

A major step in the evolution of the nervous system of higher vertebrates is the development of saltatory conduction, a process that allows for the rapid propagation of action potentials along large diameter axons. Saltatory conduction is possible because of the formation of myelin, a thick ensheathment of an axon by multiple layers of insulating glial membrane. Myelin functions to reduce the passive loss of current through the axonal membrane and increase the flow of current down the axon (i.e. increasing the space constant). Myelin also increases the capacitance of the axon, allowing for a more rapid depolarization of the membrane and generation of action potentials. The generation of action potentials is a metabolically costly process for neurons, requiring the continuous activity of ATP-driven sodium-potassium pumps to generate ion gradients across the axonal membrane. Following myelination, small areas of the axonal membrane that are left bare are known as the nodes of Ranvier, where ion gradients are established and voltage-gated ion channels are concentrated. Action potentials are regenerated at the nodes of Ranvier, and current flows down the axon by jumping from node to node, vastly increasing the conduction velocity and efficiency of electrical signaling. Disruption of myelin leads to conduction deficits that cause significant functional impairments. This dissertation is focused on signals that limit the replacement of damaged myelin with the goal of finding ways to promote myelin repair.

The German anatomist Rudolf Virchow coined the term myelin, from the Greek word myelos or “marrow”, in 1854. However, the first descriptions of myelin were likely made by Anthony Von Leeuwenhoek in 1717, who described “fatty parts” surrounding nerves in animal tissue (from Clarke & O’Malley, 1968). More than a hundred years later, Christian Ehrenberg observed that some nerve fibers contain inner (axon) and outer (myelin) boundaries (Ehrenberg, 1837). Robert Remak noted in 1838 that some nerve fibers have a “double border”, with the axon being surrounded by a “pellucid” material (reviewed in (Rosenbluth, 1999)). Characterization of myelin continued over the next 100 years, starting with the first description of myelinating cells in peripheral nerves by Theodor Schwann (Schwann, 1839), and the observation that there are regular gaps (or constrictions) in myelinated nerves by Louis-Antoine Ranvier in 1871 (Ranvier, 1871). Virchow suggested in 1858 that while myelin is not necessary for nerve function, the sheath serves to electrically insulate axons, and hypothesized that current ‘discharge’ likely occurs only at unmyelinated regions of the nerve membrane (cited in (Rosenbluth, 1999)). Consistent with this notion, Ichiji Tasaki in 1939, followed by Andrew Huxley and R. Stämpfli in 1949, demonstrated that myelinated nerve fibers display saltatory conduction (Tasaki, I. 1939, Huxley & Stämpfli, 1949). Over the next 30 years, better fixation techniques, electron microscopy and freeze fracture preparations began to reveal the intricate structure of myelin. Furthermore, electrophysiology studies and the discovery of naturally demyelinated or dysmyelinated animals established myelin’s functional importance in saltatory conduction and neurological function (see Rosenbluth, 1999 for a more detail description).

The thickness of myelin is tightly regulated, and multiple studies have shown that both overwrapping and underwrapping of axons can produce severe neurological deficits. It is still unclear how the relationship between axon diameter and myelin thickness, also known as the G-ratio, is established and maintained. While molecules such as nerve growth factor (NGF) (Urschel and Hulsebosch, 1990), Neuregulin, and ErbB2/3 (Michailov et al., 2004) have been shown to influence myelin thickness, the mechanisms controlling myelin structure are still being elucidated. Nonetheless, the G-ratio appears to be highly conserved between such diverse species as fish, rodents and primates (Hildebrand and Hahn, 1978). Taken together, these observations support a critical role for myelination in the function of vertebrate axons.

Experimental evidence also indicates that myelin may provide trophic and metabolic support to axons (Friedman et al., 1996; Riopelle et al., 1981), serving to maintain axonal architecture (Kirkpatrick et al., 2001; Wilkins et al., 2003), and to influence microtubule-driven transport of vesicles between the soma and synaptic boutons of neurons (Kirkpatrick and Brady, 1994; Kirkpatrick et al., 2001) . Multiple developmental diseases are linked to dysmyelination, including Marie-Charcot-tooth disease and a collection of devastating disorders known as leukodystrophies (see (Faust et al., 2010; Meyer zu Horste et al., 2006). Myelination disturbances that occur during postnatal development can also lead to a variety of conditions, including cerebral palsy (Azzarelli et al., 1980), and they have been implicated in schizophrenias (Walterfang et al., 2005). On the whole, dysmyelinating diseases tend to result in severe neurological decline and

premature death, highlighting the importance of myelin to healthy nervous system function.

Oligodendrogliogenesis

Ramón Y. Cajal began to question the source of myelin in 1909 and hypothesized that it was produced by axons; however, by 1932, the maintenance of myelin in the CNS was credited to "oligodendroglia" (reviewed in (Rosenbluth, 1999))**.** Seminal work by Betty Geren in 1954 reported that myelin arose from the spiral wrapping of axons by glial cells in chick nerves, and confirmed that myelin was a living cellular structure and not just an inert secreted substance (Geren and Raskind, 1953). Myelin is produced by specialized glial cells known as oligodendrocytes (OLs) in the central nervous system (CNS) and Schwann cells in the peripheral nervous system (PNS). During embryonic and postnatal development of the mammalian CNS, OLs arise from the differentiation of neural stem/progenitor cells (NSPCs) within neural stem cell niches into a specialized progenitor know as the oligodendrocyte progenitor cell (OPC). OPCs then proliferate and migrate throughout the expanding cortex, where they become dispersed along axonal tracks. Once sufficient numbers of OPCs are generated and distributed along axons, myelination begins with the differentiation of OPCs into pre-myelinating oligodendrocytes (preOL). Each preOL extends multiple processes that contact an axon and begins generating copious amounts of ensheathing membrane. Cytoplasm is then squeezed out as the spiraling membrane is compacted to create a segment or "internode" of myelin. Each OL can produce 40 or more individual myelin internodes

and myelinate multiple axons, thereby creating one of the most specialized structures in the body (for a more extensive description of myelin structure see Miller, 2002; Rogister et al., 1999).

The maturation of OPCs into myelinating OLs has been extensively characterized both *in vitro* and *in vivo* during normal development (Figure 1.1). In rodents, embryonic and adult OPCs are identified as small bipolar and multipolar cells expressing the platelet-derived growth factor receptor subtype alpha (PDGFR-α), the basic helix-loop-helix transcription factor OLIG2, and the proteoglycans NG2 and A2B5. OPCs next exit the cell cycle and become highly branched multipolar immature OLs (or preOLs), predominantly expressing a sulfatide identified by the O4 antigen (Mirsky et al., 1990) and galactocerebrosidase (GalC). Finally, mature myelinating OLs are post mitotic cells with a complex, extensively branched morphology that synthesize huge quantities of membrane and are identified by the expression of myelin components such as myelin basic protein (MBP), myelin associated glycoprotein (MAG) and proteolipid protein (PLP).

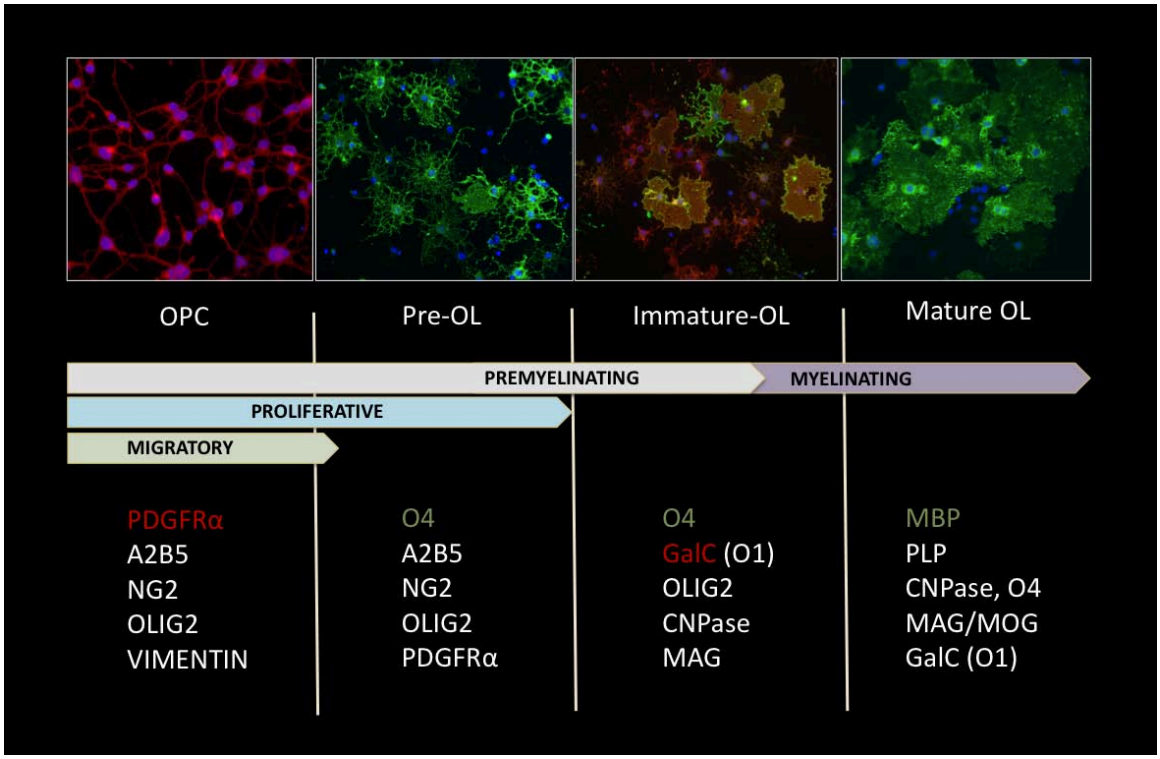

Figure 1.1 Oligodendrogliogenesis is recapitulated *in vitro*. Distinct stages of oligodendrogliogenesis are characterized by cell morphology, expression of histological markers and cellular behavior (such as proliferation or migration). *In vitro* maturation is thought to faithfully recapitulate normal developmental OL-genesis *in vivo (for further information see Asou et al., 1995)*.

Although myelination of the mammalian brain occurs during the first weeks of postnatal development in rodents and continues into the first decades of life in humans, OPCs are born much earlier. In the mouse, a wave of OPCs arises from the motor neuron progenitor (pMN) domain of the ventral neuroepithelium in the spinal cord, starting at embryonic day 12.5 (Noll and Miller, 1993; Timsit et al., 1995; Warf et al., 1991). In the cortex, overlapping waves of OPCs arise first from the medial ganglionic eminences (MGE) then from the lateral ganglionic eminences (LGE) between embryonic days 12.5 and 15.5, respectively (Kessaris et al., 2006). In both the spinal cord and cortex these early OPCs arise, then proliferate and migrate laterally and dorsally throughout the

developing spinal cord and cortex. A final wave of OPCs arises from the periventricular zones of the dorsal spinal cord beginning day e15.5 or from the ventricular zone of the expanding cortex around the time of birth (postnatal day 0, P0) (Fogarty et al., 2005; Strathmann et al., 2007; Vallstedt et al., 2005). Both ventral and dorsal OPC populations are capable of producing myelinating OLs and appear to be histologically undistinguishable. However, experimental evidence indicates that competition between the so-called 'ventral OPCs' and 'dorsal OPCs' ensues, with the dorsally derived population eventually making up the bulk of myelinating OLs in the early postnatal and adult nervous system (Aszmann, 2000; Kessaris et al., 2006). The functional significance and fate of the majority of early ventrally derived OPCs following myelination remains unknown. Furthermore, despite being morphologically identical, two distinct classes of OPCs are found in the postnatal rat, spiking OPCs that express voltage gated ion channels and fire action potentials, and non-spiking OPCs that do not (De Biase et al., 2010; Karadottir et al., 2008; Paez et al., 2009). It is also reported that migrating OPCs and NG2 positive cells (see below) receive synaptic connections and electrical input from neurons (Etxeberria et al., 2010; Kukley et al., 2010; Muller et al., 2009), but lose these connections as they mature into OLs (Kukley et al., 2010; Velez-Fort et al., 2010). It remains to be seen whether the spatial (i.e dorsal versus ventral) or temporal (i.e embryonic versus postnatal) developmental origins of OPCs contributes to the functional heterogeneity of OPCs in the adult brain; or if distinct OPC populations supply progenitors for the repair of myelin during normal tissue homeostasis and following demyelinating injury or disease.

During development, OPCs spread evenly throughout the entire adult spinal cord and brain. Not all OPCs will become mature OLs and produce myelin. It appears that more OLs than are needed are produced, and a large fraction of these surplus OLs subsequently undergo apoptosis during the process of myelination (Muir and Compston, 1996; Yasuda et al., 1995), likely due to competition for growth factor and trophic support from axons (Barres et al., 1993; Hashimoto et al., 2011; Ma et al., 2011; Ye et al., 1995). Recent studies also suggest that intercellular communication between OPCs, preOLs and mature OLs might be critical for the initiation of myelination and the repair or replacement of damaged myelin (Yang et al., 2011). After myelination, a fraction of embryonic OPCs remain distributed evenly throughout both the white and gray mater and become a population of cells known as adult OPCs (Hart et al., 1989). It is unclear how much similarity an adult OPC shares with its embryonic counterpart or their role in the adult nervous system (i.e. ongoing myelination or remyelination, stem-like progenitors, or synaptic modulation; see below) (Windrem et al., 2004). Additionally, two distinct populations of OPCs expressing either PDGFRα or PLP/DM20 have been reported (Fruttiger et al., 1999; Perez Villegas et al., 1999; Spassky et al., 1998). It is not known how these heterogeneous OPC populations contribute to myelination or the generation and maintenance of adult OPCs.

Finally, controversy continues over which cells can be considered true adult OPCs. A large amount of attention has been focused on parenchymal cells expressing the NG2 proteoglycan, called NG2 cells or NG2 glia (Raff et al., 1983). The term NG2 cell is often used synonymously with adult OPC, due to their ability to produce mature OLs both *in*

vitro and *in vivo*. NG2 cells also express PDGFRα and/or the A2B5 antigen, both markers of proposed adult OPC populations (Abney et al., 1983; Nishiyama et al., 1996; Scolding et al., 1999) (Hart et al., 1989). Currently it is unclear whether the differences in marker expression between NG2 cells or adult OPC populations represent distinct lineages or simply differences in the maturation state of the cell (Greenwood and Butt, 2003; Mallon et al., 2002). NG2 positive cells comprise 5-10% of the total cells in the adult CNS, are distributed ubiquitously throughout the white and gray matter and appear to be the primary proliferative population of cells in adult CNS (Geha et al., 2010; Horner et al., 2000). Intriguingly, in the adult brain, the processes of NG2 positive cells are found in close association with nodes of Ranvier, where they may receive and transmit signals in response to axonal activity (Hamilton, et al 2010). Experimental evidence also suggests that they are multipotent cells that can produce neurons, astrocytes and myelinating OLs, *in vitro* and *in vivo,* and they are rapidly activated and proliferate in response to CNS injury (Chang et al., 2000; Guo et al., 2009; Rivers et al., 2008; Tripathi and McTigue, 2007; Zhu et al., 2011). These observations have led to the speculation that the NG2/adult OPC may be another *de facto* population of neural stem cells in the CNS (Richardson et al., 2011).

Unsurprisingly, due to their exquisite architecture, myelinating OLs are extremely susceptible to neurological insults such as stroke and spinal cord injury. It has been proposed that during peak periods of myelination, an individual OL produces up to 300 times the weight of its cell body in membrane a day (see Miller, 2002), putting tremendous load on the lipid and protein synthesis machinery of the cell (Leblanc et al.,

2005; Narayanan et al., 2009). Following myelination, a mature OL must maintain 40 or more nodes of myelin and the soma must support 100 times its weight in myelin, making the mature OL extremely sensitive to metabolic stress (Friede and Bischhausen, 1980). Extensive experimental evidence has shown that OL death can be rapidly induced under conditions of oxidative stress (Back et al., 2005a; Gerstner et al., 2008), excessive intracellular calcium (Benjamins and Nedelkoska, 1996; Mato et al., 2010; Ruiz et al., 2010; Tzeng et al., 1995), induction of ER stress cascades (Bauer et al., 2002; Ohri et al., 2011), and excessive glutamate release (Chen et al., 2007; Liu et al., 2002; Sanchez-Gomez et al., 2011). Even processes associated with aging have been correlated to decreases in white matter volume due to OL loss (Andersen et al., 2003; Sala et al., 2010; Zhang et al., 2009). Because of the extensive communication and interactions between axons and the myelin membrane, OL death and demyelination in general is strongly correlated to declining axonal health and function (Rasminsky and Sears, 1972). As such, following insult or injury, extensive OL death may also trigger damage to axons and/or nearby OLs, inducing a secondary wave of escalating damage much greater in scale and scope than the initial insult.

Multiple Sclerosis: modeling demyelination & remyelination.

An example of a disease in which adult myelin is specifically targeted for degradation is the autoimmune-mediated disease Multiple Sclerosis (MS). First described by Jean-Martin Charcot (Charcot, 1868), MS is currently estimated to affect roughly 400,000 Americans and 2.5 million people worldwide (Freedman, 2006). In response to an

unknown etiological trigger, the immune system becomes sensitized to myelin proteins and invading immune cells such as T-cells, neutrophils, and macrophages attack and destroy the myelin sheath, axons and OLs. Furthermore, invading immune cells can induce cytokine and chemokine release from resident glia, such as astrocytes and microglia, further exacerbating OL death and axonapathy (for an extensive review see Szczucinski and Losy, 2007). Once the inflammatory phase of an MS attack subsides, damaged cells and myelin debris are cleared, and the repair and replacement of myelin may occur. Substantial repair and recovery occurs early in MS pathology (i.e. relapsing-remitting MS) (Patani et al., 2007), but as frequency and severity of attacks increase, the efficiency of repair declines, and large demyelinated lesions begin to form. Chronically demyelinated axons display conduction deficits and are more susceptible to atrophy and death, resulting in rapid deterioration of neurological function (i.e. primary and secondary progressive MS) (for more information see Miller and Leary, 2007; Trojano et al., 2003).

MS is a complicated disease, with affected individuals presenting a spectrum of disabilities and varying patterns of disease progression. No one animal model is able to completely recapitulate MS; therefore multiple models are used to investigate different aspects of disease progression and the repair process. The most widely used animal model of MS is Experimental Autoimmune Enchapholomyelitis (EAE), an extensively characterized model of inflammatory-induced demyelination similar to that seen in MS. In EAE, rodents are injected subcutaneously with synthetic myelin peptides such as myelin basic protein (MBP), myelin associated glycoprotein (MAG) or proteolipid protein

(PLP), along with Complete Freud's Adjuvant (CFA) and/or pertussus toxin to increase blood brain barrier penetration (BBB). Different peptides in different strains of mice can result in either a progressive or relapsing-remitting disease course. Peripheral activation of the immune system generates myelin-sensitive monocytes and T-cells that attack and destroy myelin. Disease progression can be tracked through symptomatic deficits, such as progressively ascending paralysis and weight loss, and through histological analysis of immune cell invasion and the formation of inflammatory demyelinated lesions (see Pachner, 2011). However, it is becoming apparent that despite recovery of function in EAE animals, remyelination is limited, perhaps due to axonal damage (Soulika et al., 2009; Vogt et al., 2009; Ziehn et al., 2010). The relapsing-remitting pathology of MS is poorly reproduced in the majority of EAE models. Furthermore, EAE in other species, including non-human primates, can be very severe and is therefore used infrequently as to model MS in larger animals.

Another animal model commonly used to study MS is viral-induced demyelination. While it is still unclear what factor(s) sensitize the immune system to myelin components in MS, a variety of viral infections have been shown to cause demyelination in animals, including canine distemper in dogs, visna virus in sheep, and Semliki Forest virus in mice (reviewed in Pachner, 2011). In humans, the Epstein-Barr virus (EBV) proteins have been shown to share consensus sequences with multiple autoantigens present in MS patients (Carter 2011), though a definitive link between EBV and MS has yet to be established (Otto, et al. 2011). Theiler's murine encephalomyelitis (TVME) or murine hepatitis virus (MHV) are the most popular rodent models of viral-induced

demyelination (see Tsunoda and Fujinami, 2010). Injection of either virus produces CNS inflammation, axonal damage, and demyelination/remyelination. Viral particles can persist in neural cells, particularly neurons, microglia, and astrocytes, inducing chronic demyelination such as seen in MS patients (reviewed in Pachner, 2011). However, finding the proper titer of virus to faithfully replicate demyelination can be difficult and infection of immune-compromised animals is often fatal. Recently, a herpes virus has been isolated from non-human primates that display spontaneous demyelination and a MS-like disease (Axthelm et al., 2011). This new model, named Japanese macaque encephalomyelitis (JME), may provide an alternative model for studying MS and demyelinating diseases in primates.

Alternatively, chemical demyelination is used to investigate mechanisms underlying spontaneous remyelination. In these models, chemicals such as the detergent lysolecithin (Hall, 1972) or ethidium bromide (Yajima and Suzuki, 1979) are injected directly into white matter and induce myelin destruction and OL death, while leaving axons largely intact. Rodents may also be fed a diet containing the copper-chelating agent cuprizone (Blakemore, 1973), which is selectively taken up by OLs, robustly inducing demyelination, particularly in the corpus callosum. Advantages of these models are that the generation, location, and severity of demyelinated lesions are well controlled and reproducible. Furthermore, robust spontaneous remyelination occurs within days of toxin injection (i.e. lysolecithin) or within weeks of removal of cuprizone from the diet, allowing for detailed studies of distinct phases of repair and remyelination, such as progenitor activation, migration and maturation.

Remyelination is generally considered to consist of two distinct phases: The recruitment of OPCs to areas of demyelination and the maturation OPCs and preOLs into myelinating OLs (for excellent reviews see Franklin, 2002; Keirstead and Blakemore, 1999). Remyelination recapitulates aspects of developmental myelination, in that OPCs must proliferate, migrate into lesions and associate with demyelinated axons. OPCs then appear to undergo the same morphological and histological changes seen during developmental myelination (see Figure 1.1). New myelin is markedly thinner than myelin produced during development, and a significant decrease in individual myelin node length has been reported (Clifford-Jones et al., 1980; Ludwin and Maitland, 1984; Weiner et al., 1980). However despite these structural differences, it has been shown that conduction velocity is restored to near pre-injury speeds (Felts and Smith, 1992; Honmou et al., 1996), and that significant functional recovery can be achieved with only a fraction of damaged fibers being remyelinated (Cao et al., 2005; Imaizumi et al., 1998; Papastefanaki et al., 2007).

There is currently no evidence that mature myelinating OLs extend new processes to generate new internodes of myelin. Furthermore, it is also unlikely that adult OLs are capable of dedifferentiating and re-entering the cell cycle to generate new OPCs, despite experimental evidence showing that Schwann cells in the PNS do so following demyelinating insults or traumatic nerve injury (Harrisingh et al., 2004; Nagoshi et al., 2011). Remyelination is generally considered to consist of two distinct phases: The recruitment of OPCs to areas of demyelination, and the maturation OPCs and preOLs into myelinating OLs (see Franklin, 2002). New OPCs are likely generated from neural

stem/progenitor cells (NSPCs) found in nearby neural stem cells niches (Nait-Oumesmar et al., 2007) or generated by the activation of adult OPCs or NG2 cells dispersed through out the nervous system (Tripathi et al., 2010). Multiple explanations have been suggested for why remyelination fails in large chronic lesions, including a failure in recruitment of progenitors to sites of demyelination or an exhaustion of progenitors following multiple demyelinating events (Fressinaud, 2005).

Experimental evidence has shown that the subventricular zone (SVZ) and dentate gyrus (DG), known neural stem cell niches in the adult brain, are activated near MS and EAE lesions in both humans and rodents (Nait-Oumesmar et al., 2008; Nait-Oumesmar et al., 2007; Yan et al., 2009). Demyelinating events such as spinal cord injury (Labombarda et al., 2011) or ischemia (Sizonenko et al., 2008) have also been shown to induce a robust response in parenchymal NG2 cells. Furthermore, rapid migration of transplanted OPCs to sites of injury has been observed (Baron-Van Evercooren et al., 1996; Vignais et al., 1993). Collectively, these data indicate that progenitor niches are being activated to supply OPCs to demyelinated lesions.

Furthermore, the exhaustion of OPC populations is not supported by data showing that PDGFR-α and NG2 positive OPCs can be found in perilesional tissue and to a lesser extent within the lesion itself (Picard-Riera et al., 2002; Scolding et al., 1998b; Tripathi and McTigue, 2007; Wilson et al., 2006; Wolswijk, 1998a). The failure of recruited OPCs to replace damaged and dying oligodendrocytes within large chronic lesions (Penderis et al., 2003) supports the hypothesis that *active inhibition of progenitor maturation within*

the glial scar accounts for some of the remyelination failure seen in MS and other chronic CNS lesions. Understanding how the microenvironment of the MS lesion or injured CNS influences OPC maturation is therefore critical for developing therapeutics aimed at promoting endogenous repair mechanisms.

Hyaluronan is a major component of the ECM in normal and injured CNS[1].

Local release of inflammatory cytokines from damaged tissue induces morphological and metabolic changes in glia. This leads to the formation of a glial scar, a common feature in numerous types of injury to the CNS, including MS, spinal cord damage, ischemia, and hypoxia. The glial scar is composed of a tight mesh of reactive astrocytic processes and secreted extracellular matrix (ECM) molecules, including proteoglycans (PGs) and glycosaminoglycans (GAGs). The glial scar is highly protective following neural insult, as it rapidly isolates and restricts inflammation and prevents the spread of damage to surrounding unaffected tissue. While the glial scar is initially beneficial, it impedes axonal sprouting, regeneration and remyelination. Molecules such as Lingo-1 (Zhang, et al 2009; Mi et al., 2005), bone morphogenic factors (BMPs) (Fuller, et al. 2007) and transforming necrosis factor (TNF) (Su, et al 2011) are released by reactive astrocytes and have been shown to inhibit neurite outgrowth and/or OL differentiation. Furthermore PGs within the glial scar are able to block axonal migration, as well as induce growth cone collapse *in vitro*. Axon outgrowth into lesions may be dependent upon clearance of the glial scar as degradation of PGs and GAGs within the scar can

[1] Portions of this chapter were adapted from a previously published work. Preston MA. and Sherman LS. (2011). Neural stem cell niches: Roles for the hyaluronan-based extracellular matrix. *Frontiers in Bioscience*. Review. (Schol Ed). 2011 Jun 1;3:1165-79.

promote axonal sprouting and regeneration following spinal cord crush in rodents (for an extensive review see Sherman & Back, 2008). More recent data suggests that PGs inhibit OPC migration and differentiation in spinal cord injury (Siebert, et al. 2011) and that GAG accumulation prevents OL maturation in CNS lesions (Back, et al. 2005).

A major GAG that accumulates in glial scars is Hyaluronan, also known as Hyaluronate and Hyaluronic acid (HA). Karl Meyer and John Palmer first isolated this high-molecular weight polysaccharide from the vitreous humor of the bovine eye in 1934 and named it Hyaluronic acid, for hyaloid (vitreous) + uronic acid. (reviewed in Simoni et al. 2002). HA is a ubiquitous molecule found in the ECM of all tissues, where it provides structural support, hydration and cushioning, and serves as a backbone for organization of large proteoglycan complexes in the ECM. HA is a large, unbranched, non-sulfated GAG, composed of repeating disaccharide units of N-glucuronic acid and N-acetylglucosamine. While simple in composition, HA polymers can reach upwards of 2,000-25,000 disaccharide units, with molecular weights in the 10^7 Dalton range, and are capable of organizing into complex secondary and tertiary structures. Different sizes of HA appear to have distinct physiological functions, including hydration of tissues, providing elasticity to tissues, and creation of cell free spaces for cell migration. These functions are primarily regulated through transmembrane HA receptors, whose activation depends on the size and organization of the sugar both intra- and extracellularly. Growing evidence suggests specific and critical roles for HA in the healthy and damaged nervous system, including the regulation of neural stem cell niches and control of neural progenitor proliferation, migration, and differentiation following injury.

Physiological relevance of HA size and receptors in HA-mediated biological activities.

Unlike most other GAGs, which are processed and extensively modified in the Golgi apparatus, HA is synthesized at the inner face of the plasma membrane and secreted as a linear, undecorated polysaccharide directly into the extracellular space by a family of transmembrane proteins known as HA synthases (HASs). The mammalian genome codes for three such synthases, HAS1, HAS2 and HAS3, each thought to generate distinct molecular weights of HA. HAS3 generates intermediate sizes of low-molecular weight (LMW) HA of ≤ 2.5 x 10^5 Daltons, while HAS1 and HAS2 are thought to preferentially secrete high-molecular weight (HMW) forms of HA, ranging from 2.0 x 10^6 to ≥ 4.0 x 10^6 Daltons (reviewed in Toole, 2004). HAS activity generates large intact linear molecules of HA that are rapidly incorporated into the ECM surrounding cells or may be retained at the cell surface through HA binding receptors and interacting proteins. HA may also be tethered to the cell surface by interactions with HAS proteins themselves (reviewed in (Spicer and Tien, 2004; Stern, 2008; Toole, 2004)). HASs have been reported to have distinct temporal and spatial expression patterns (Tien and Spicer, 2005), supporting the hypothesis that while the synthases are capable of generating similar sizes of HA, various HASs are probably not functionally redundant in tissues. Consistent with this idea, the transcription of different HAS genes is differentially regulated by distinct growth factors and cytokines (Itano and Kimata, 2002).

While relatively simple in composition the size of HA molecules can range from HA oligomers composed of small numbers of disaccharide units to giant high molecular

weight polysaccharides of 25,000 or more disaccharide units, stretching upwards of 20 micrometers in length. Multiple reviews have covered the significance of size for the biological activity of HA *in vivo and in vitro* (Itano, 2008; Stern, 2005; Stern et al., 2007). Importantly it has been shown that distinct sizes of HA activate distinct groups of receptors, leading to distinct physiological effects in cells.

The aggregation and structure of HMW species of HA has been shown to be highly flexible, with large molecules folding and aggregating spontaneously into coils, fibers, and nets (Spicer and Tien, 2004). Due to its high negative charge, HMW HA is capable of holding 10-10,000 times its weight in water, and it is proposed that hydration of the HA-rich ECM creates cell free pores for migration of cells (Toole, 2004). The structure of HMW HA in the nervous system is likely dependent on binding with HA binding proteins such as hyalectins (Lemons et al., 2005), brain-enriched HA binding protein (BEHAB) (Jaworski et al., 1996), and other HA-binding proteins (Deepa et al., 2006). A large body of research has explored the function of HA and HA-binding proteins in large proteoglycan complexes during development and repair of the CNS (for more information see Kwok et al., 2008; Sherman and Back, 2008).

HMW HA has also been implicated in the clustering of receptor tyrosine kinases (RTKs), as well as influencing the constitutive activation of multiple growth-associated receptors (Misra et al., 2006). In particular, several studies have implicated HA in the activation of the erbB2 and erbB3 receptor tyrosine kinases in Schwann cells (Gorlewicz et al., 2009; Sherman et al., 2000), and erbB1 (the epidermal growth factor receptor) and erbB4 in

other cells types via interaction with the transmembrane HA receptor CD44 (Ghatak et al., 2005; Palyi-Krekk et al., 2008; Tsatas et al., 2002). HA-CD44 interactions have also been implicated in regulating cell proliferation by regulating the phosphorylation of the merlin tumor suppressor protein in Schwann cells (Morrison et al., 2001).

LMW species of HA show varying binding affinities for a variety of HA receptors, as well as strong angiogenic properties and may function as a signal for CNS tissue damage by inducing pro-inflammatory signaling cascades (Gao et al., 2008). Small fragments of HA (4-8mers) have been shown to induce anti-apoptotic cascades via activation of NFkB and heat shock proteins (Stern, 2004). Additionally, various breakdown products of HA have been shown to activate toll-like receptors (TLR-2 and TLR-4) (Campos et al., 2004; Gariboldi et al., 2008) and these LMW HA products may be responsible for activation of local microglia and astrocytes in response to pathogens and CNS infection (Gurley et al., 2008). Small fragments (<12 disaccharides) or HA oligomers have been used to disrupt HMW and LMW HA signaling *in vitro* by directly competing with the intact sugar for binding sites in HA receptors and binding proteins (reviewed in (Stern et al., 2006)). Whether HA oligomers produced by endogenous hyaluronidases are capable of competing with large polymers of HA *in vivo* is still unknown.

Activation of HA receptors has been implicated in tumor metastasis, migration and differentiation of cells, and the modulation of signaling cascades associated with the growth and homeostasis of cells in tissues and following injury. Such a multitude of cellular responses reported for HA is likely related to the “where, when and how” a cell

encounters HA. The biological activities of HA in the nervous system are largely mediated by transmembrane receptors, including TLR2, TLR4, CD44 and RHAMM. An isoform of the HA receptor for endocytosis (HARE) is also expressed in the brain, but its function there is not known (Kyosseva et al., 2008). Among these receptors, CD44 and RHAMM have been the most extensively characterized in the CNS and PNS.

Multiple isoforms of CD44 are created both by alternative mRNA splicing of 20 exons present in the CD44 gene and extensive posttranslational modification of CD44 proteins, including N- and O-linked glycosylation, the addition of heparan sulfate side chains, and the incorporation of chondroitin sulfate side chains, among other modifications (see Ponta et al., 2003). Different modifications occur depending on the presence of particular variant exon-encoded sequences. HA binds to CD44 via an extracellular domain related to cartilage link protein. The binding affinity of HA to CD44 depends on the receptor isoform expressed and the extent of post-translational receptor modifications. In general, CD44 preferentially binds HMW HA, although lower MW forms of HA may also signal via CD44. CD44 is expressed predominantly by astrocytes and microglia throughout both the central and peripheral nervous systems (Gorlewicz et al., 2009; Liu et al., 2004). CD44 signaling has been shown to influence multiple cellular behaviors, including proliferation, survival, and migration via receptor interactions with a variety of signaling molecules, such as ErbB receptors (Ghatak et al., 2005; Liu et al., 2004), SRC family kinases (Ilangumaran et al., 1998; Jurzynski et al., 2007; Wong et al., 2008), RHO GTPases (Shi et al., 2001) and proteins in the Ezrin/Radixin/Moezin (ERM) family (Harrison et al., 2002). Additionally, HA uptake and degradation by a CD44-

dependent mechanism has been proposed which may also influence tissue integrity, cell growth and homeostasis (Stern, 2004).

The receptor for HA-mediated motility (RHAMM) is also expressed at the cell surface, exists in multiple isoforms, and is capable of binding various sizes of HA (Lynn et al., 2001a; Zhang et al., 1998a). Like CD44, RHAMM expression has been reported in neurons and glial cells (Casini et al., 2010). Extracellular binding of HA to RHAMM has been reported to influence cell migration and growth by activating molecules such as focal adhesion kinase (FAK), and by inducing changes in actin and microtubule dynamics (Evanko et al., 2004; Hall and Turley, 1995; Hall et al., 1994; Lokeshwar and Selzer, 2000). HA binding to RHAMM has also been shown to activate a variety of signaling molecules such as protein kinase C (Hall et al., 2001) and PI3K (Goueffic et al., 2006). Interestingly RHAMM is also found in the cytoplasm and is associated with nuclear and mitochondrial membranes (Lynn et al., 2001b), perhaps mediating intracellular signaling by internalized HA (see Entwistle et al., 1996).

Finally, HA has been proposed as a ligand for Toll-Like Receptors (TLRs), a family of pattern recognition receptors activated by pathogenic molecules such as bacterial cell wall components, LPS, DNA and RNA. To date, 10 mammalian TLRs have been identified (see Fraser et al., 2004). Signaling through TLRs leads to activation of the innate immune system via MyD88-mediated signaling pathways, resulting in the nuclear translocation of NFkB and the subsequent upregulation of inflammatory chemokines and cytokines (reviewed in Sloane et al., 2010b). The endogenous ligands for TLRs are still being

investigated, but HA oligosaccharides have been proposed as ligands for TLR2 and TLR4 in a variety of cells, such as macrophages (Ernens et al., 2010; Iwata et al., 2009), dendritic (Termeer et al., 2002), and endothelial cells (Voelcker et al., 2008). TLR2 and TLR4 expression has been reported in neural stem cells (Rolls et al., 2007), microglia, astrocytes (Gurley et al., 2008) and OL lineage cells (Sloane et al., 2010a).

Hyaluronidases could significantly modulate the microenvironment of CNS lesions.

Increased HA synthesis and accumulation is seen in a variety of diseases and injuries such as rheumatoid arthritis, chronic obstructive pulmonary disease (COPD), and renal failure, where HA is likely secreted as an immune regulator (for an extensive review see Jiang et al., 2011). In the CNS, increased HA synthesis is reported in MS lesions (Back et al., 2005b), spinal cord injury (SCI) (Sakayama et al., 2006; Saxena et al., 2011), and following stroke (Al Qteishat et al., 2006; Al'Qteishat et al., 2006). It has also been reported that HA accumulates in the aged non-human primate brain (Cargil, et al. in press) and in patients with Alzheimer's disease (Sakayama et al., 2006). Dense accumulations of HA and the HA receptor CD44 are found within large chronically demyelinated lesions in both MS patients and rodents with EAE (Back et al., 2005b). Reactive astrocytes are thought to be the major source of HA in demyelinated lesions, where HA is likely secreted to minimize the spread of inflammatory cytokines and chemokines released from damaged tissue. Of particular interest to remyelination failure, it has been shown that HMW HA is able to block remyelination in the mouse corpus callosum following lysolecithin-induced chemical demyelination (Back et al.,

2005b; Sloane et al., 2010a). Importantly it has also been shown that HMW HA reversibly blocked embryonic and adult rodent OPC maturation into oligodendrocytes *in vitro* (Back et al., 2005b; Sloane et al., 2010a). Intriguingly, OL maturation failure appears to be dependent on HA size, as LMW species failed to block remyelination *in vivo* or OPC differentiation *in vitro*, consistent with the notion the size of HA is critically important to cellular responses to the sugar. *These data collectively support the hypothesis that HMW HA is contributing to the inhibition of oligodendrocyte maturation within the microenvironment of the MS lesion and glial scar.*

Prior to the identification of HA, it was noted in the 1930's that tissue extracts (particularly testis) contained a "spreading factor" which increased the diffusion of dyes and drugs (Hoffman and Duran-Reynals, 1931) . Karl Meyer went on to coin the term hyaluronidase, once it was discovered that the substrate of this "spreading factor" was HA (Hobby et al., 1941). Initially, hyaluronidases were grouped into three distinct classes of enzymes based on simple biochemical assays (Meyer, 1950), and today classification is based on the enzyme's mechanism of action. One group of hyaluronidases includes the vertebrate enzymes, which cleave the sugar by hydrolysis to generate saturated products and are classified as endo-β-N-acetylhexosaminidases. A second group of bacterial enzymes cleave the sugar in a hydrolysis-independent mechanism through formation of an unsaturated double bond and are classified as lyases or β-elimidases. The final group contains primarily annelid and crustacean enzymes, which are endo-β-glucuronidases and share more similarity with the vertebrate enzymes than the bacterial enzymes (for an extensive review see Stern and Jedrzejas, 2006).

Characterization of the vertebrate hyaluronidases has lagged behind the bacterial hyaluronidases, primarily due to difficulties in isolation and purification of the enzymes, which are highly unstable and expressed at very low levels in tissues (Frost et al., 1997). Mammals possess six hyaluronidase genes, named HYAL1-5 and PH20 (SPAM1) (Csoka et al., 2001). Humans, which lack the rodent-specific HYAL5, also possess a psuedogene name PHYAL1. HYAL genes lie in two separate chromosomal clusters (3p21.3 and 7q31.3) in humans (Csoka et al., 1999). In general, the mammalian hyaluronidases fall into two distinct groups, the somatic hyaluronidases (HYAL1-3) and the testicular hyaluronidases (HYAL4, PH20 or PHYAL1) (reviewed in Csoka, et al, 2001). While all the HYALs share a similar mechanism of cleavage, each enzyme is thought to have a distinct cellular localization and pH optima, and to produce distinct HA breakdown products (Figure 1.1) (compiled from data in Hofinger et al., 2008).

PROTEIN	pH OPTIMA	PRODUCT	LOCALIZATION
Hyal1	3-4	$HA_4 – HA_6$	Lysomal
Hyal2	4 and 7.5?	HA_{50} (~20kD)	GPI-linked
Hyal3	3.5? and 7.5?	??	Lysosomal? Secreted?
Hyal4	??	??	??
Hyal5	4 and 7.5	??	Cell surface?
PH20 (SPAM1)	4 and 7.5	HA_{20}	GPI-linked Secreted

β-N-acetylglucosaminidase and β-glucoronidase required to degrade HA into monosaccharides

? Unknown or conflicting data

Figure 1.2. A comparison of mammalian hyaluronidase proteins. Each HYAL is though to have an optimal pH for activity, usually correlated to its cellular localization, and generate distinct HA degradation products.

Despite the estimation that approximately 30% of the body's total HA is turned over every day (Stern, 2004), little is known about the expression, activity, and control of HYALs, especially in the nervous system. HA degradation begins with the extracellular processing of the large polysaccharide into smaller cleavage products of about 20 to 50 disaccharides by the GPI-anchored HYAL2 (Rai et al., 2001). HA breakdown products are then internalized for further degradation into disaccharides by HYAL1 in lysosomes (Stern, 2004), while N-acetylglucosaminidase and N-glucuronidase are required to fully degrade the sugar into monosaccharides. Little is known about HYAL3, and it is still unclear whether HYAL3 is a functional hyaluronidase (Atmuri et al., 2008), although evidence suggests it may regulate the activity of HYAL1 (Hemming et al., 2008). Similarly, HYAL4 is has no predicted hyaluronidase activity (Csoka et al., 2001). HYAL5, not present in humans, is thought to be a functionally redundant with PH-20 (see below) in the testes of rodents (Kim et al., 2005; Kimura et al., 2009).

The PH20 hyaluronidase is of particular interest because of its biochemical properties. PH20, also known as sperm adhesion molecule-1 (SPAM-1), was first discovered in the testes, where it is localized to the heads of mature sperm and used to penetrate the HA-rich zona pellucida during fertilization (Zheng and Martin-Deleon, 1999). There is, however, growing evidence that PH20 is expressed in many other locations, including the female reproductive tract (Griffiths et al., 2008; Zhang and Martin-DeLeon, 2003b), kidney (Sun et al., 1998) and numerous cancer cell types (Delpech et al., 2002; Godin et al., 2000; Wang et al., 2004). PH20 is unique among the HYALs in that it is reported to be

active at both acidic and neutral pH (Zhang and Martin-Deleon, 2003a). Additionally, it can be tethered to the extracellular side of the plasma membrane or it can be released into the ECM following cleavage of its GPI anchor (Zhang and Martin-Deleon, 2003a). As such, PH20 is capable of modulating HA signaling both at the cell surface and in the surrounding ECM. Recent work has shown that OL lineage cells express a variety of hyaluronidases including HYAL1-3 and PH20 (Sloane et al., 2010a).

Repair and remyelination of CNS lesions could be dependent on clearance of HMW HA within the glial scar, once inflammation has subsided. Hyaluronidases may also be involved in the initial processing of GAG side chains from the protein cores of chondroitin sulfate proteoglycans (CSPGs), another inhibitory class of GAGs commonly found in the glial scar (Gilbert et al., 2005; Tan et al., 2005). Alteration of endogenous HYAL expression and activity is presumed to be necessary in order to generate and maintain the dense accumulation of HMW species of HA and other GAGs within the glial scar. New data suggests that hyaluronidase activity impairs remyelination via TLR2/MyD88 signaling (Sloane, et al. 2010a) though these findings have yet to be reproduced by other labs. *Currently there is very little known about the role of hyaluronidases in neural progenitor proliferation or differentiation within either the normal or diseased CNS.* Given the known roles of HA in cell cycle regulation, progenitor specification, migration and differentiation (described above) altering the delicate balance between HA synthesis and degradation could profoundly influence the speed and course of regeneration and repair in the central nervous system.

HYPOTHESIS and RATIONALE

Given the fact that previous work identified HMW HA as potential inhibitory signal to OL maturation that is found in demyelinated lesions**, I hypothesized that clearance of HMW HA by hyaluronidases from the demyelinated lesion would promote OL maturation and remyelination.** To test this hypothesis I developed a novel culture system to generate mouse OPCs, and utilized animal models of demyelination to investigate the role of hyaluronidases in oligodendrogliogenesis. The work outlined in this thesis supports the notion that degradation products of HMW HA, rather than the intact sugar, contribute to the remyelination failure seen in large chronic demyelinated lesions. This work also shows that a single hyaluronidase, PH20, is sufficient to inhibit OPC differentiation and is up regulated in EAE and MS lesions. Overall, this thesis supports the notion that PH20 influences the proliferation and differentiation of OPCs, both processes required for remyelination, and as such identifies PH20 as promising therapeutic target for promoting the repair and remyelination of damaged nervous tissue.

CHAPTER 2: GENERAL METHODS

Reagents. HMW HA (100 μg; 1.59 x 10^6 Da, Seigaku) was dissolved in sterile PBS and HA fragments were generated with addition of Bovine Testicular Hyaluronidase (BTH, Sigma, 100 U/ml), Streptomyeces (StrepH, Sigma, 1-10 U/ml) or PBS vehicle for 1 hour at 37°C then incubated at 95-100°C for 30 minutes to heat inactivate enzymes. HA fragments were analyzed by electrophoresis on a 0.5% agarose gel, followed by detection of HA using the cationic dye Stains-All (Sigma) as previously described (Lee and Cowman, 1994). 4-MU (Sigma) was dissolved in PBS at 37°C and added to cultures at a final concentration of 1 mM. VCPAL (Sigma) was dissolved in DMSO at a concentration of 100 mM and further diluted to a working concentration of 25μM for cell culture experiments and for co-injection into lysolecithin lesions. Turbidity assays for VCPAL activity and IC50 calculations were performed as previously described (Botzki et al., 2004).

Lentiviral Construction and Infections. The open reading frame of PH20, HYAL1, HYAL2 and HYAL5 were cloned in front of the CMV promoter of a vector plasmid and packaged into a third generation lentiviral vector (see Supplementary methods and Supplementary Table 1). Cells were plated at 4-5 x 10^4 cells per coverslip and infected overnight using 2.5 -5.0 x 10^5 Transforming Units (MOI 1:50-1:100).

Viral constructs. The transgene expression vector (LV-intron -GFP) used for cloning is from Gregory A.Dissen (Dissen GA et al, Methods 49 (2009) 70-77). PH20 and HYAL5

cDNA were from Stephan Reitinger (extracellular Matrix Research Group, Institute for Biomedical Aging Research, Austrian Academy of Sciences, Innsbruck, Austria) Hyal 1 cDNA was from Barbara L. Triggs-Raine (Atmuri V et al, Matrix Biol 27 (2008) 653-660). Hyal 2 was obtained by RT-PCR using the forward primer: 5'-GAGTTCCTGAGCTGCTACCA-3' and the reverse primer: 5'-AGGGGGAGAGATCCCTCATA-3'.

Table 2.1: PCR Primers and Products

	Forward Primer	Reverse Primer	Amplicon
HYAL1	5'-GCACCCTCCAACTGGGGCAG-3'	5'-CTGGGCTGCACTCTCCCCCA-3'	387 bp
HYAL2	5'-GGCGTCCTCCGTACACAGCC-3'	5'-CGTCTTCCGAGTCGCCCCAG-3'	227 bp
HYAL5	5'-AAAGACCCTCGAAATCCAC-3'	5'-CCACATACCATTCCAGAGG-3'	144 bp
PH20	5'-TGGGATGCTATGAGTTTAGC-3'	5'-CAAAGTGTTTGGCTGCACAT-3'	124 bp
PH20	5'-ATGGAAGGAACTTTACACCT-3'	5'-GCTAAACTCATAGCATCCCA-3'	440 bp
PH20	5'-TGGGATGCTATGAGTTTAGC-3'	5'-CCAAATTACTGAGGCCTGCA-3'	588 bp

Table 2.2: MS Patient Information

#	age (yrs)	gender	duration of disease (yrs)	diagnosis
1	53	M	10	relapsing/remitting*
2	65	F	35	relapsing/remitting**
3	55	F	4	relapsing/remitting
4	45	F	18	relapsing/remitting
5	70	F	15	primary progressive

*presumed diagnosis; records unclear
**relapsing/remitting disease that had become secondary progressive

Cell Culture. Neural stem cells were isolated from the medial and lateral ganglionic eminences of embryonic day 13.5 mouse (C57BL/6) embryos and expanded in epidermal growth factor and fibroblast growth factor-2 (10 ng/ml) as neurospheres for one week as previously described (Zhang et al., 1998b). Neurospheres were dissociated into single cells in trypsin (0.05%, Invitrogen), washed in DMEM plus 10% fetal bovine serum and plated at 5 x 10^6 cells/ml on uncoated polystyrene plates in DMEM/F12 media containing 0.1% BSA, PDGF-AA and FGF2 (20 ng/ml each), B27 supplement minus vitamin A (GIBCO), N1 supplement (Sigma) and D-Biotin (10 nM, Sigma) for conversion into OPCs. Small adherant spheres of cells formed and were passaged once a week after dissociation with accutase (Invitrogen). After 2-3 weeks mouse 'oligospheres' were transferred to poly-L-ornithine-coated 100 mm dishes. After 2-4 passages, highly enriched populations (>95%) of PDGFRα+Olig2+O4- OPCs (as assayed by immunocytochemistry) were obtained and further propagated for *in vitro* experiments. For maturation experiments, OPCs were plated at 4-5 x10^4 cells per coverslip and differentiated in DMEM/F12, 0.1% BSA, plus triiodothyronine (T3, 30 nM, Sigma) and N-acetyl-L-cysteine (NAC, Sigma) as previously described (Zhang et al., 1998b).

Lysolecithin Lesions. All animal experiments were approved by the Institutional Animal Care and Use Committee at the Oregon Health & Science University. Demyelination was induced in the rostral corpus callosum of 3-4 month old C57BL/6J mice by injection of lysolecithin (4% in PBS; Sigma) as previously described (Back et al., 2005b) mixed with either vehicle (PBS), HMW HA, degraded HA, or VCPAL. Five days post-injection, the

same volumes of PBS, HA, and VCPAL were re-injected. Brains were harvested at 8-days post-injection, fixed and processed for immunohistochemistry as previously described (Back et al., 2005b).

Induction of EAE. EAE was induced in female C57BL/6J mice as previously described (Back et al., 2005b). Animals were anesthetized with isofluorane and perfused transcardially with 100 U/ml sodium heparin (Sigma-Aldrich) containing saline followed by 4% paraformaldehyde in PBS. Spinal cords were dissected and processed for immunohistochemistry as described below.

Immunohistochemistry. Cells were fixed for 30 minutes at room temperature in 4% paraformaldehyde and washed 3 times in PBS. Lumbar spinal cord tissue from mice with EAE was immersion fixed for 12-16 hrs in 4% paraformaldehyde at 4°C, rinsed three times in PBS at room temperature, then cyroprotected in 30% sucrose overnight at 4°C. Tissues were embedded in Optimal Cutting Temperature (OCT) medium, rapidly frozen on dry ice and cryosectioned at a thickness of 10-12 µm. Cells and tissues were pre-blocked in 10% heat-inactivated fetal bovine serum for 45 minutes. Primary antibodies were diluted in blocking buffer and cells or EAE tissue were incubated overnight at 4°C, rinsed in blocking buffer three times, then incubated with the appropriate species-specific fluoro-conjugated secondary antibodies (Alexa546 or Alexa488, Molecular Probes Inc.) for 45 minutes. Antibodies used were: rat anti-PDGFR-α (1:250, BD Pharminagen) mouse anti-O4 (1:500, Millipore) mouse anti MBP (1:1000, Sternberger

Monoclonal) rabbit anti GalC (1:100, Millipore) rabbit anti GFAP (1:1000, DAKO) rabbit anti MAP2 (1:1000, Millapore) rabbit anti OLIG2 (1:500, Millipore) Mouse anti BrdU (1:50). Polyclonal rabbit antisera to PH20 were generously provided by James Overstreet (PH20, 1:1000) and Patricia DeLeon (msSPAM, 1:400). HA was visualized by probing cells or tissues with biotinylated HA Binding Protein (HABP, 1:200, Seikagaku) followed by avidin-conjugated Cy3 (1:1000, Molecular Probes Inc.). Myelin was visualized by a 30 minute incubation of EAE sections with Fluoromyelin (1:400 in PBS, Invitrogen) following primary and secondary antibody application. Cell nuclei were visualized by DAPI staining (Hoeschst 33342, 1:15,000; Molecular Probes). Sections from mice with lysolecithin lesions were analyzed for MBP reactivity as previously described (Back et al., 2005b).

Image processing and cell counts were performed using Photoshop 3.0 and ImageJ, respectively. For cell counts, 10 fields were randomly selected and at least 500 cells counted per coverslip (3 coverslips per group). Mean cell numbers and standard deviations were calculated for each group. Student's t-test was used to determine statistical significance at ≤0.05% confidence.

Proliferation assays mOPCs were plated onto poly-ornithin coated coverslips in PDGF-aa and FGF2 to induce proliferation and treated with PBS (control) or BTH (100U/mL) or infected with GFP (control), HYAL1, HYAL2, HYAL5, PH20 (see above). After 48 hours

cells were given an hour pulse of Bromodeoxyuridine (5-bromo-2'-deoxyuridine, BrdU, 1:200 of 10uM stock) to label proliferating cells, fixed and processed for ICC (see above).

Characterization of MS patients. The use of tissues from individuals with MS was approved by the Human Subjects Committee at the University of Washington. For the analysis of MS patient lesions, paraffin sections from 5 MS patients (mean duration of disease: 16.4 years; mean age: 57.6 years; see Supplementary Methods and Supplementary Table 2) were deparaffinized in xylene and then rehydrated in graded alcohols. Endogenous peroxidase activity was blocked with 0.3% (v/v) hydrogen peroxide in methanol before washing the slides in water. Slides were heated in citrate buffer (10 mmol/L, pH 6.0) for 5 min in a microwave for antigen retrieval. Sections were then pre-blocked in 5% normal goat serum. Primary antibody incubation was the same as indicated above, except that rabbit anti PH20 (J.Overstreet) was used at a 1:500 dilution. Following biotinylated goat anti-rabbit secondary antibody binding, the avidin–biotin–peroxidase complex technique (Vectastain ABC kit and NovaRED, Vector Laboratories Inc.) was used for visualization. The substrate reaction was stopped by washing the slides in running water. Finally, the slides were dehydrated, and mounted with a permanent mounting medium. In separate experiments, sections were also double-labeled with anti-PH20 (1:300) and either anti-MBP (1:500), anti-O4 (1:400), or anti-GFAP (1:50, DAKO) followed by fluorescence-conjugated secondary antibodies as above. The lesions analyzed all had substantial myelin loss (as assessed by Luxol fast blue staining and anti-

MBP immunohistochemistry) and evidence of astrogliosis (as assessed by GFAP immunohistochemistry).

RT-PCR and qRT-PCR. Total RNA was isolated from cells or tissues using Trizol (Invitrogen) following the manufacturer's instructions. mRNA was reverse transcribed into cDNA using random hex or oligo d(t) primers and a reverse transcriptase kit (Promega). mRNA sequences for each hyaluronidase were downloaded from the NCBI website (http://www.ncbi.nlm.nih.gov) and primers for RT-PCR were designed manually (supplied by Intergrated DNA Technologies). See Supplementary Table 1 for primers used. RT-PCR was performed using GoTaq or Superscript DNA transcriptase (Promega or Invitrogen) in a Mastercycler Gradient following the manufacturer's protocols. RT-PCR reaction products were analyzed by electrophoresis on a 1% agarose gel and amplicons visualized following staining with ethidium bromide. Quantitative Real Time PCR (qRT-PCR) was preformed using predesigned primer and probe sets (Taqman Assays, Applied Biosciences; HYAL1: Mm00476206; HYAL2: Mm0477731; and PH20 (SPAM1): Mm00486329), with an AB 7900HT fast PCR system using SDS 2.4 software. Data Analysis was performed using Microsoft Excel.

CHAPTER 3: Products of the PH20 hyaluronidase in demyelinating lesions inhibit remyelination[2].

ABSTRACT

Oligodendrocyte progenitor cells (OPCs) recruited to lesions in patients with multiple sclerosis (MS) and other demyelinating conditions often fail to mature into oligodendrocytes (OLs) that can remyelinate spared axons. The glycosaminoglycan hyaluronan (HA) accumulates in demyelinating lesions and has been implicated in the inhibition of remyelination but the mechanism by which HA contributes to remyelination failure is unclear. We now report that HA degradation by the PH20 hyaluronidase but not other hyaluronidases inhibited OPC differentiation into OLs. In contrast, inhibiting HA synthesis did not influence OPC differentiation. These findings support the notion that HA degradation by PH20 within demyelinating lesions but not HA itself blocks OPC differentiation. Consistent with this hypothesis, PH20-generated HA degradation products were distinct from those generated by another hyaluronidase. Furthermore, HA-degradation products generated by PH20 inhibited remyelination while inhibition of hyaluronidase activity lead to increased OPC differentiation and promoted remyelination in mice with lysolecitin-induced demyelinating lesions. PH20 expression was elevated in OPCs and reactive astrocytes in demyelinating lesions from mice with experimental autoimmune encephalomyelitis and patients with MS. Pharmacological inhibition of PH20 may therefore be an effective way to promote remyelination in MS and related conditions.

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[2] Submitted to Journal of Neuroscience, in review at time of dissertation publication.

INTRODUCTION

The destruction of myelin sheaths that surround axons in the central nervous system (CNS) causes conduction deficits in affected neurons that can lead to motor, sensory and cognitive deficits. Demyelination occurs following numerous insults to the CNS and is the hallmark of multiple sclerosis (MS). Pre-myelinating oligodendrocytes (OLs) and OL progenitors (OPCs) are recruited to demyelinated lesions of MS patients and of mice with experimental autoimmune encephalomyelitis (EAE), a demyelinating disease that mimics features of MS pathology (Nait-Oumesmar et al., 2008). OPCs can mature into OLs that remyelinate demyelinated axons. However, OPCs often accumulate at chronic demyelinated lesions and fail to give rise to myelinating OLs (Chang et al., 2000; Chang et al., 2002; Maeda et al., 2001; Scolding et al., 1998a; Wolswijk, 1998b, 2002). Strategies that promote OPC maturation within demyelinated lesions therefore have the potential to promote remyelination and functional recovery in affected individuals.

High molecular weight (HMW) forms of the glycosaminoglycan hyaluronan (HA) accumulate in demyelinating lesions and are linked to remyelination failure (Back et al., 2005b; Sloane et al., 2010a). HA is a glycosaminoglycan synthesized by transmembrane synthases and composed of multiple disaccharide units of glucuronic acid and *N*-acetylglucosamine. HA molecules range in size from $\leq 2.5 \times 10^5$ Da to $\geq 4 \times 10^6$ Da. Different molecular weight forms of HA have distinct functions in the nervous system including inducing cell motility, regulating cell growth, and regulating cell differentiation (reviewed by (Sherman and Back, 2008)). During peripheral inflammatory responses,

activated fibroblasts or other cells secrete hyaluronidases that generate HA oligosaccharides that act as immune regulators (Jiang et al., 2011). Reactive oxygen species at sites of inflammation further promote this degradation (Soltes et al., 2006). Although hyaluronidases are expressed in the CNS (Al'Qteishat et al., 2006; Sloane et al., 2010), it is unclear if HA is similarly degraded during CNS inflammation where astrocytes are the principle source of HA (Marret et al., 1994).

HMW HA blocks OPC maturation and prevents remyelination following lysolecithin-induced demyelination (Back et al., 2005b; Sloane et al., 2010a). Degradation of HA in astrocyte-OPC co-cultures with *Streptomyces* hyaluronidase results in increased OL maturation and HMW HA pre-treated with *Streptomyces* hyaluronidase does not prevent remyelination suggesting that HMW HA itself signals the inhibition of OPC maturation (Back et al., 2005b). In contrast, treatment of OPCs with HA degraded by a mammalian hyaluronidase followed by b-glucuronidase blocked OPC maturation *in vitro* (Sloane et al., 2010a). It is possible, therefore, that specific HA degradation products in demyelinated lesions rather than HMW HA cause remyelination failure. Here, we show that a specific hyaluronidase, called PH20, is expressed by OPCs and astrocytes in demyelinated lesions and blocks OPC maturation. Degradation products of this hyaluronidase but not another hyaluronidase prevent remyelination, while inhibition of hyaluronidase activity promotes OPC maturation and remyelination. All together these data suggest that agents that block PH20 activity or expression could promote remyelination in demyelinated lesions with spared axons.

RESULTS

Degradation of endogenous HA by a mammalian hyaluronidase blocks OPC maturation *in vitro.*

We optimized a culture system in which mouse neural stem cells are differentiated *in vitro* into mouse OPCs that can subsequently be differentiated into pre-OLs and mature OLs (Figure 3.1).

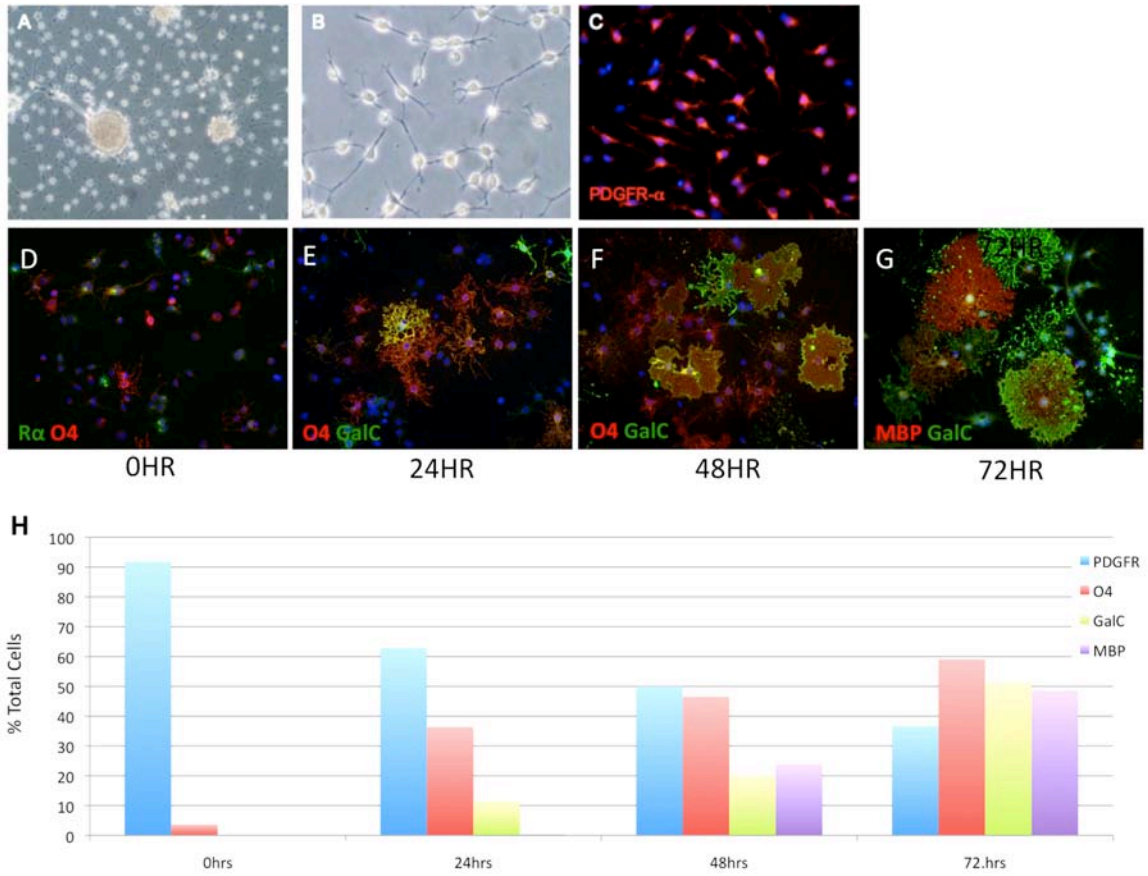


Figure 3.1. Characterization of mouse OPC cultures. (A) Phase contrast photomicrograph of oligospheres derived from neurospheres. (B) Morphology of individual cells that migrate away from oligospheres. (C) Expression of PDGFRa (red) in OPC cultures. Cells were counterstained with DAPI (blue) to label nuclei. To examine OPC maturation, OPCs were grown in the presence of T3 and NAC, then examined for the expression of OPC and OL markers immediately after plating (D), then 24 hours (E), 48 hours (F), and 72 hours (G) later. (H) Quantification of typical OL lineage marker expression from one experiment.

After 72 hours in media conditions that promote OPC maturation (see methods), less than 60% of cells in these cultures express myelin basic protein (MBP), a marker of mature OLs. By 12 hours post-plating, we observed elevated HA synthesis in OPC cultures that increased over time (Figure 3.2).

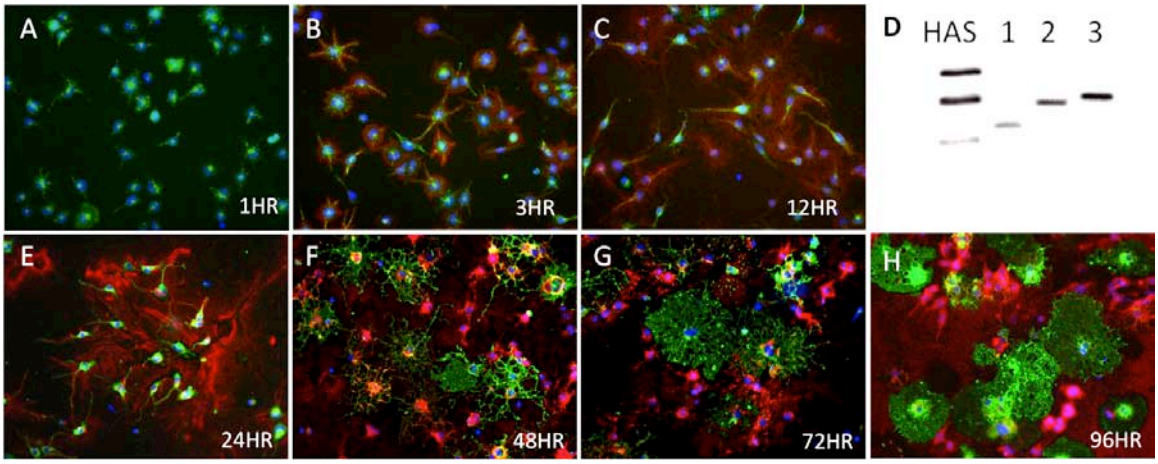


Figure 3.2 Accumulation of HA in proliferating and differentiating OPC cultures. OPCs were plated in serum-free media and fixed 1 (A), 3 (B), or 12 (C) hours later, then labeled with an anti-PDGFRa antibody (green) and biotinylated-HABP (red). Cells were counterstained with DAPI (blue) to label cell nuclei. (D) RT-PCR for different hyaluronan synthases (HAS) showing that all three enzymes are present in OPCs *in vitro*. To test how HA synthesis changes as OPCs mature into OLs, cells were grown in the presence of T3 and NAC for 24 (E), 48 (F), 72 (G) and 96 (H) hours, then stained with HABP (red) and either O4 (green, E, F) or MBP (green, G, H).

Given that HA can block OPC maturation, we tested if the prevention of HA synthesis or the removal of HA from these differentiating OPC cultures could promote OL maturation. To block HA synthesis, OPCs were switched to media containing the HA synthase inhibitor 4-methylumbelliferone (4-MU, 1mM). Unexpectedly, we found that inhibiting HA synthesis with 4-MU had no significant effect on OL maturation compared to controls despite the nearly complete absence of HA in these cultures (Figure 3.2*A*, *B*, *D*). Surprisingly, degradation of HA using bovine testicular hyaluronidase (BTH) potently

inhibited OPC maturation (Figure 3.3*A*, *C*, *D*). This inhibitory activity was reversed by heat inactivation of the enzyme prior to HA digestion (data not shown). We tested if the inhibitory effects of BTH on OPC maturation were the specific result of HA degradation as opposed to a more general breakdown of glycosaminoglycans in the cultures. We compared OPC maturation in cultures treated with BTH (as above), with chondroitinase ABC (CS'ase; which degrades chondroitin sulfate into unsaturated disaccharides) or with *Streptomyces* hyaluronidase (StrepH), each at concentrations that were optimal for their substrates (data not shown). OPC maturation was only inhibited in the BTH-treated cultures (Figure 3.3*E*).

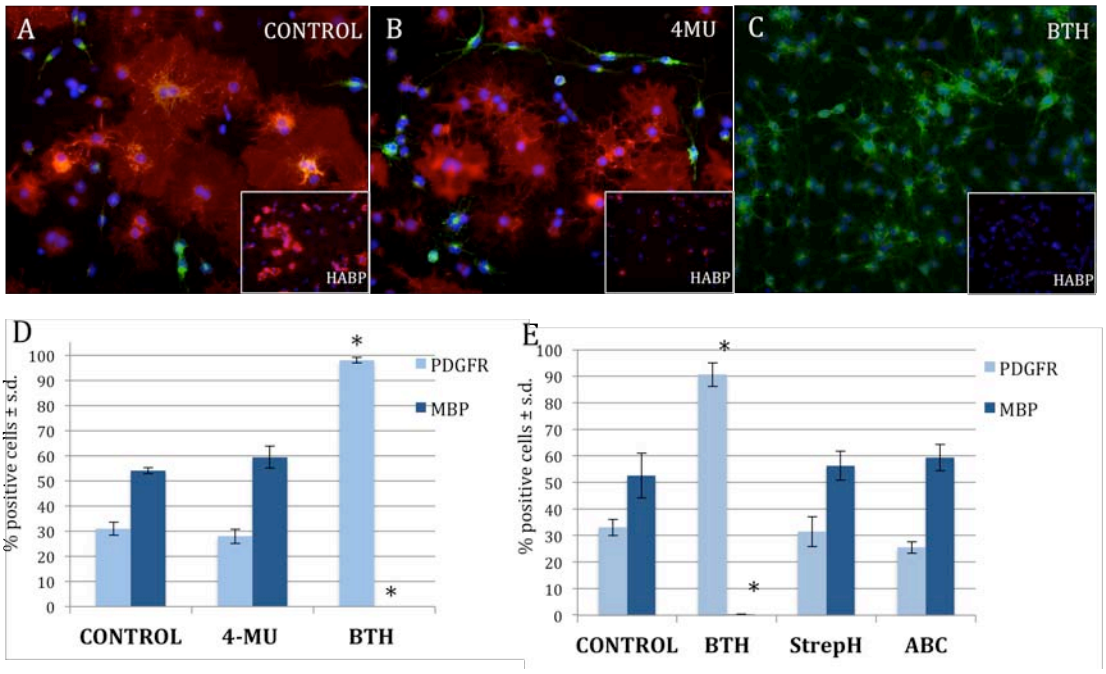


Figure 3.3 Bovine testicular hyaluronidase inhibits OPC maturation, *in vitro.* OPCs were differentiated in (A) T3/NAC alone or in the presence of (B) the HA synthase inhibitor 4-MU or (C) BTH for 72-96 hours, fixed and stained for the OL lineage markers: MBP (red) and PDGFRα (green), with DAPI (blue) to visualize nuclei. The total percentage of PDGFRα+ and MBP+ cells are quantified in (D). Insets in (A-C) show representative levels of HA (as assayed using HABP) for each treatment group. BTH treatment significantly inhibited OL maturation as assayed by decreased MBP expression compared to controls (<0.5% v. 54.1% p = 0.00008) and increased PDGFRα expression compared to controls (97.8% v. 30.9%, p=0.00028). Inhibiting HA synthesis did not significantly alter OL maturation (B, D). Furthermore, OPCs differentiated in the presence of chondroitinase ABC or the bacterial-derived *Streptomyces* hyaluronidase showed no significant changes in OL maturation as compared to controls (E).

HA degradation products are sufficient to inhibit remyelination *in vivo*

Taken together, our data support the hypothesis that specific HA degradation products generated by BTH but not StrepH inhibit OPC maturation. To determine if these enzymes generated distinct HA degradation products, we analyzed HMW HA that had been treated with either enzyme using the quantitative and sensitive SEC-MALLS technique to characterize the HA products in these two digests (Figure 3.4, *A*). The weight-average sizes of the BTH (63.9 ± 0.5 kDa) and StrepH (2.4 ± 0.4 kDa) digests were very different. More importantly, the two distributions of HA product sizes in the two

digests were essentially non-overlapping. Only about 2% of the products in both digests were in the same size range (e.g. 98% of the BTH products ranged from 5.5 to 227 kDa and 98% of the StrepH products were < 4.4 kDa). We next determined whether the HA breakdown products generated by BTH activity were capable of blocking remyelination. We induced focal demyelination in the corpus callosum of mice using lysolecithin as previously described (Back et al., 2005b). After 4 days, a second injection of either vehicle, HMW HA that had been incubated with vehicle, BTH-degraded HMW HA, or StrepH-degraded HMW HA was delivered into the original lesion site and mice were allowed to recover for 6 days (n ≥ 6 per group). OPC maturation and remyelination were assessed by analysis of MBP immunoreactivity in sections through lesions as previously described (Back et al., 2005b). Compared to vehicle controls (Figure 3.4, *B*), lesions injected with HMW HA (Figure 3.4*C* shows typical example) or BTH-degraded HA (Figure 3.4, *D*) failed to remyelinate as shown by the lack of MBP immunoreactivity at the injection site, while animals treated with StrepH-degraded HA remyelinated to the same degree as vehicle controls (Figure 3.4*E*). Specific HA-degradation products produced by BTH are therefore capable of blocking OL maturation in demyelinated lesions where HA accumulates.

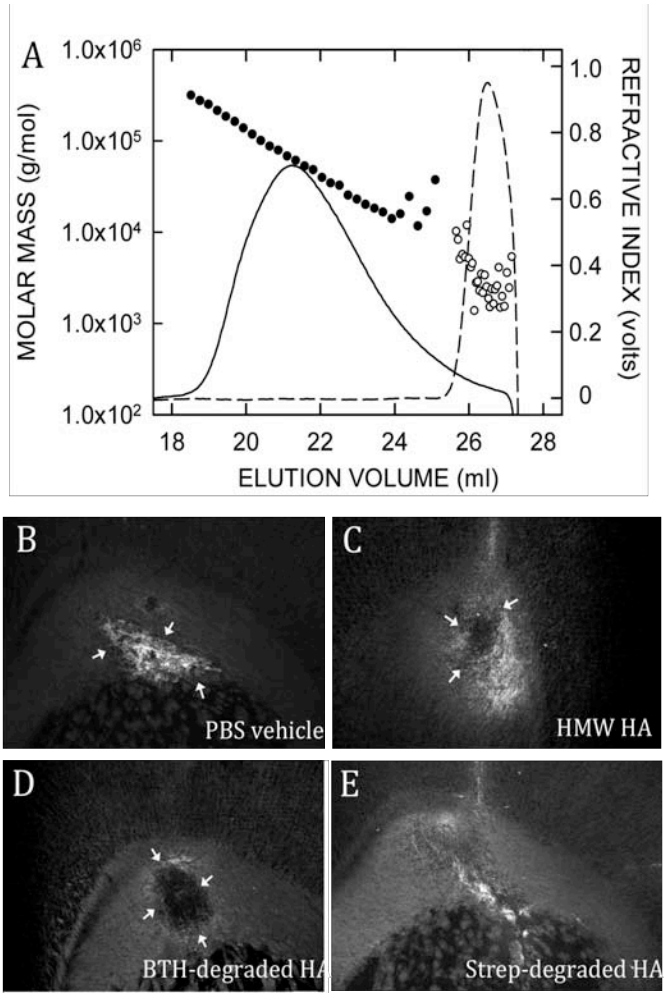


Figure 3.4. BTH breakdown products of HA block remyelination. (A) HA samples were treated with BTH (solid line and black circles) or StrepH (dashed line and white circles) and analyzed by SEC-MALLS to determine the amount (refractive index, solid and dashed lines) and the molar masses based on light scattering (black and white circles) of HA products present. The weight-average masses of the BTH and StrepH samples were 63.9 ± 0.5 kDa and 2.4 ± 0.4 kDa, respectively. Overlap of the two digests was ≤ 2% (e.g. 98% of the BTH products ranged from 5.5 to 227 kDa and 98% of the StrepH products were < 4.4 kDa). (B) Vehicle alone, (C) HMW HA (100μg/mL), (D) BTH-degraded HA (100μg/mL), or (D) StrepH degraded HA (100μg/mL) was stereotactically co-injected with lysolecithin (LYS) into corpus callsoum, and the agents were re-injected into demyelinated lesions 5 days following LYS injections. Remyelination was determined by immunolabeling for MBP. Arrows in (A) indicate the typical pattern of enhanced MBP immunoreactivity (white) in areas of apparent remyelination. Arrows in (B) and (C) indicate regions of persistent LYS-induced demyelination.

OPCs degrade HA and express hyaluronidases

The majority of the hyaluronidase activity in BTH is attributed to PH20 (also called sperm adhesion molecule-1 or SPAM1) with only very low activity from other hyaluronidases including Hyal1 and Hyal2 (Meyer et al., 1997). A previous study using

only immunohistochemistry with single antibodies reported that OPCs express multiple hyaluronidases, including PH20 (Sloane et al., 2010a). We therefore hypothesized that OPCs that are recruited to demyelinating lesions express hyaluronidases that then degrade the HMW HA synthesized by reactive astrocytes in the lesion microenvironment. We isolated total RNA from mouse testes (as a positive control for testicular hyaluronidases), OPCs grown *in vitro*, and from adult mouse corpus callosum, then performed RT-PCR using primers specific for the hyaluronidases with known hyaluronidase activity in mouse testes (Hyal1 Hyal2, Hyal5 and PH20). We found that Hyal1, Hyal2 and PH20 but not the testes-specific Hyal5 were expressed by OPCs and in white matter (Figure 3.5*A*). Hyal1, Hyal2 and PH20 transcripts were also amplified from RNA isolated from whole brain, cortex and spinal cord (data not shown).

We next determined if OPCs are capable of degrading HA. OPCs were plated onto coverslips uniformly coated with HMW HA (approximately 1.59 MDa) and allowed to differentiate for 24 or 72 hours, then fixed and labeled with an anti-O4 antibody and a biotinylated HA-binding protein (HABP). We found that OL lineage cells were capable of degrading HA. There was progressive loss of HABP reactivity around the cell bodies and processes of O4+ cells at 24 hours *in vitro* (Figure 3.5*B*,*C*). A more pronounced reduction in the HA-coated surface was seen after 72 hours (Figure 3.5*D*) that corresponded to the presence of O4+ membranes (Figure 3.5*E*). Collectively, these data indicate that OL-lineage cells express multiple hyaluronidases and are capable of degrading HMW HA.

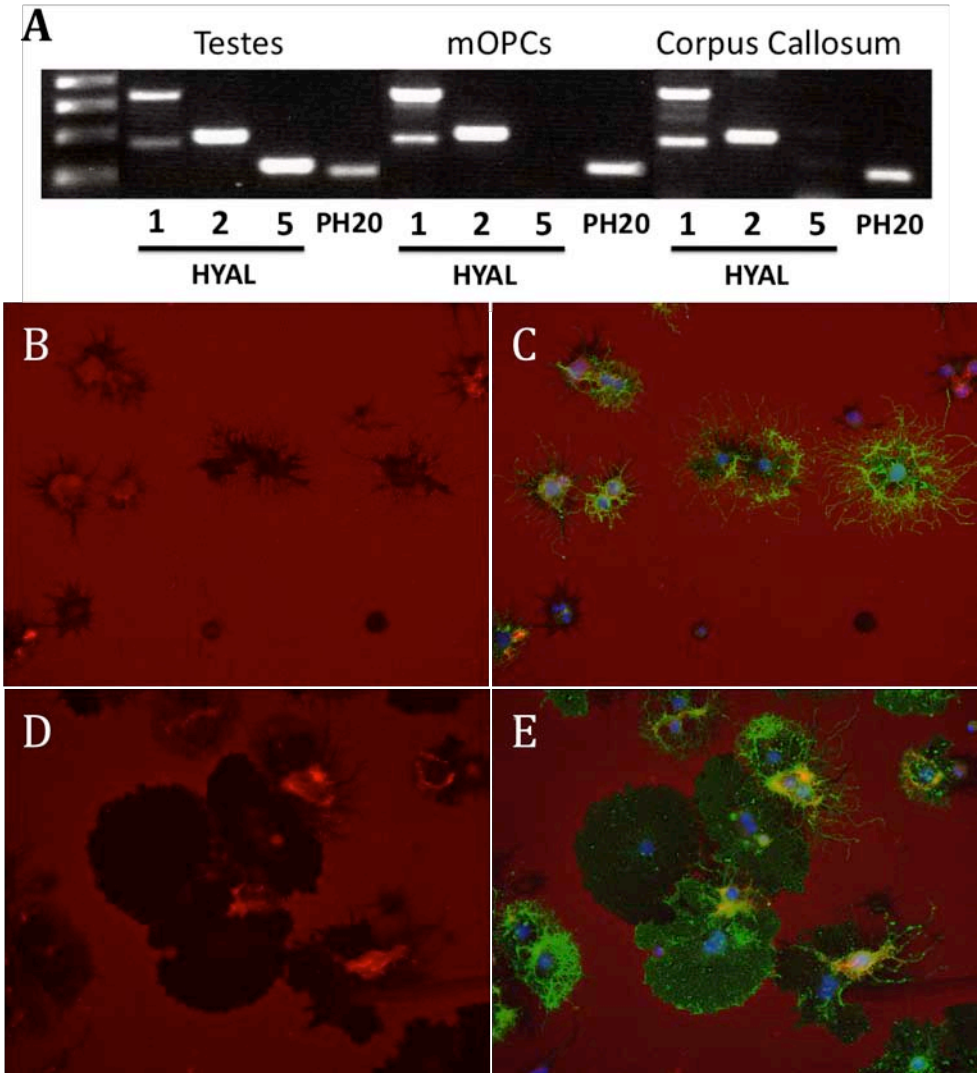


Figure 3.5. OPCs express hyaluronidases and are capable of degrading HA as they mature. (A) Total mRNA was isolated from mouse testes (as a positive control), OPCs and adult mouse corpus callosum, reverse transcribed and subjected to RT-PCR. OPCs express *Hyal1*, *Hyal2* and PH20 but not the testes-specific *Hyal5*. The same patterns of hyaluroidase expression were found in the corpus callosum. (D-E) OPCs were plated onto coverslips coated with HA, allowed to mature for 24 (B-C) or 72 (D-E) hours and stained with O4, HABP and DAPI. Degradation of HA (B), as assayed by loss of HABP immunoreactivity, was seen around cell bodies and extending O4+ processes (C) at 24 hours. By 72 hours HA staining was lost (D) in areas corresponding to the presence of O4+ membranes (E).

Pharmacological inhibition of hyaluronidase activity promotes OL maturation and remyelination

Given that OPCs express multiple hyaluronidases and that degradation products of BTH inhibit OPC maturation, we reasoned that blocking the activity of hyaluronidases, and

therefore the generation of inhibitory HA breakdown products, would promote OL maturation. We chose to inhibit hyaluronidase activity in OPC cultures with the hyaluronidase inhibitor 6-O-Palmitoyl-L-ascorbic acid (VCPAL) for 72-96 hours. Consistent with previous studies, we found that VCPAL inhibited BTH activity with an IC_{50} of 25-35 μM (Figure 3.6*A*). We examined treated and control (vehicle) cultures for changes in the expression of the OL lineage markers PDGFRα to label OPCs and MBP to label mature OLs. VCPAL treatment prevented HA degradation (data not shown) and significantly increased the proportion of cells that became mature OLs, assayed as the total percentage of cells expressing MBP as compared to cultures treated with vehicle alone (Figure 3.6*B-D*).

To assess whether inhibition of hyaluronidase activity is sufficient to promote remyelination, we co-injected VCPAL with HMW HA into lysolecithin-induced corpus callosum lesions. In animals with lysolecithin-induced lesions, VCPAL resulted in elevated MBP immunoreactivity, overcoming the inhibitory effects of HMW HA, while there was reduced MBP immunoreactivity in animals treated with HMW HA and vehicle (Figure 3.6*E-F*). These findings support the hypothesis that the hyaluronidase activity of OPCs or other cells in demyelinating lesions contributes to the impairment of OL maturation, contributing to the remyelination failure seen in chronically demyelinated MS plaques. These data further suggest that hyaluronidase inhibition may be an efficacious strategy to promote remyelination.

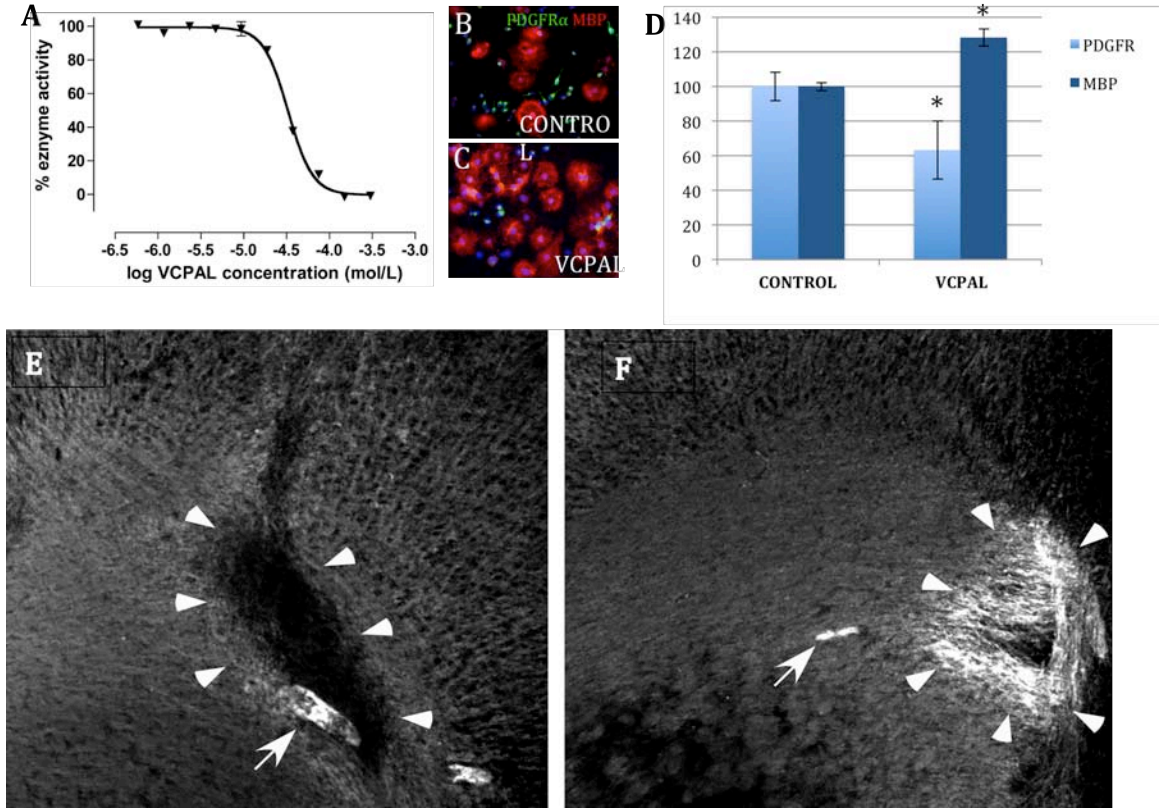


Figure 3.6. Inhibiting endogenous hyaluronidase activity promotes OL maturation *in vitro* and remyelination *in vivo.* (A) Inhibitory activity of increasing concentrations of the pan hyaluronidase inhibitor VCPAL (6-O-Palmitoyl-L-ascorbic acid) on BTH as determined using a turbidimetric assay. The final BTH concentration was 100 U/ml. Each point represents the mean±SEM of 4 replicate experiments. The VCPAL IC_{50} was approximately 33 µM. (B) OPCs were grown in OL differentiation medium alone or (C) with 25µM VCPAL for 72-96 hrs and stained with MBP, PDGFRα and DAPI. Total MBP+ and PDGFRα+ cells are quantified in (D). Consistent with the hypothesis that hyaluronidases expressed by OPCs generate HA fragments which inhibit OL maturation, blocking HYAL activity with VCPAL increased the total percentage of MBP+ cells compared to controls (69.31% v. 54.05% p= 0.00505) while decreasing the total percentage of PDGFRα+ cells (19.61% v. 30.98% in control, p=0.01719). (E, F) MBP immunoreactivity (white) in lysolecithin lesions treated with HMW HA and vehicle (E) or HMW HA with 25 µM VCPAL (F). Arrowheads indicate the borders of the lesions; arrows indicate non-specific staining of adjacent blood vessels.

PH20 is the hyaluronidase expressed by OPCs that blocks OPC maturation

We used a gain-of-function strategy to assess which of the hyaluronidases found in BTH inhibit OPC maturation. The cDNAs from *Hyal1*, *Hyal2*, *Hyal5* and PH20 were cloned into bicistronic lentiviral expression vectors carrying the cDNA for enhanced green

fluorescence protein (EGFP). OPCs were then infected with lentiviruses carrying these vectors and analyzed for changes in OPC maturation as described above. Cells infected with the PH20-carrying viruses demonstrated a pronounced and significant ($p<0.0009$) inhibition of OPC maturation, as assessed by quantification of MBP+ cells, compared to cultures infected with a virus carrying only the EGFP cDNA (Figure 3.7*A*-*C*). Infection with the *HYAL1*-bearing virus had no significant effect on OL maturation while cells infected with viruses carrying *HYAL2* and *HYAL5* were able to only weakly inhibit OL maturation ($p<0.002$ and $p<0.02$, respectively; Figure 3.7*C*). Cells infected with hyaluronidase vectors demonstrated reduced levels of pericellular HA (e.g. inset, Figure 3.7*B*) which was not observed in cells infected with EGFP alone (e.g. inset, Figure 3.7*A*). Taken together these results support the notion that overexpression of PH20, the major component of BTH, generates HA breakdown products capable of blocking OL maturation.

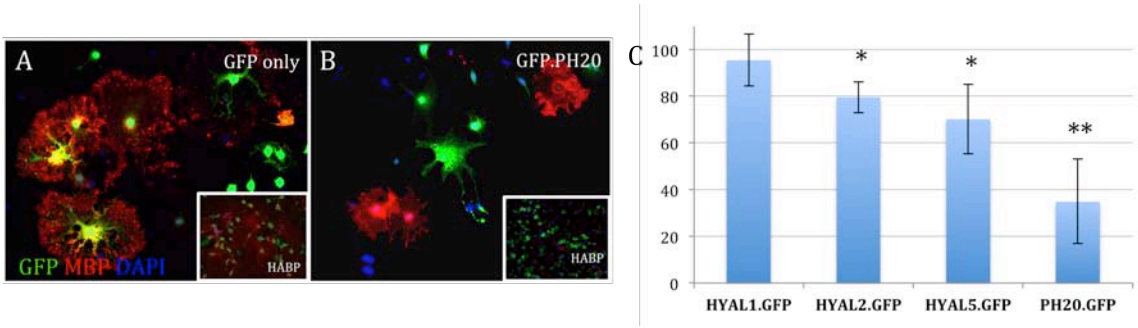


Figure 3.7. Elevated expression of PH20, the major hyaluronidase in BTH, blocks OL maturation *in vitro*. OPCs were infected with lentiviruses carrying GFP only (control; A), HYAL1.GFP, HYAL2.GFP, HYAL5.GFP (not shown) or PH20.GFP (B), allowed to differentiate for 72-96hrs and stained for GFP and MBP. Results are quantified in (C). PH20 overexpression (B) robustly inhibited OL maturation compared to GFP only (A) as assayed by coexpression of MBP and GFP (35.91% of control, $p=0.0008$). Insets in (A, B) show complete degradation of HA in OPCs overexpressing PH20. HYAL2 overexpression partially inhibited OL maturation (78.48% of control, $p=0.00148$). HYAL5 overexpression also partially inhibited OL maturation (70.08% of control, $p=0.0183$) but is not expressed by OL lineage cells *in vitro* or *in vivo* and is thus unlikely to have any physiological relevance to remyelination.

PH20 expression is elevated in demyelinating lesions

Previous reports had indicated that PH20 was localized to testes but not other tissues (e.g. (Jones et al., 1995; Jones et al., 1996; Phelps and Myles, 1987; Zheng and Martin-Deleon, 1997)). To verify that the transcripts we had amplified by RT-PCR were indeed mouse PH20, three distinct sets of primers were used to amplify separate regions of PH20 mRNA isolated from adult mouse brain and were sequenced, confirming that OPCs were expressing bona fide PH20 RNA (data not shown).

Given that PH20 transcripts are expressed by OPCs and in the corpus callosum, and that elevated PH20 expression is sufficient to inhibit OPC maturation, we examined PH20 expression in OL lineage cells at different stages of differentiation. PH20 protein expression was assayed in proliferating OPCs and maturing OLs by immunocytochemistry using two separate PH20 antibodies (generous gifts of P.DeLeon and J. Overstreet) in combination with the OL lineage specific markers PDGFRα, O4 or MBP. Consistent with the notion that OL lineage cells are capable of degrading HA as they mature, PH20 immunostaining localized both to the cell body and processes of immature PDGFRα+ OPCs (Figure 3.8*A*-*D*) but was restricted to the cell body of mature MBP+ OLs (Figure 3.8*E*-*H*). Additonally, PH20 expression was observed in the tips of O4 positive processes (Figure 3.8*E*-*H*) leading to the speculation that maturing OPCs use PH20 to modify the extracellular space during process extension. All together, these data indicate that the distribution of PH20 is maturation-dependent and becomes more restricted as OPCs mature into OLs.

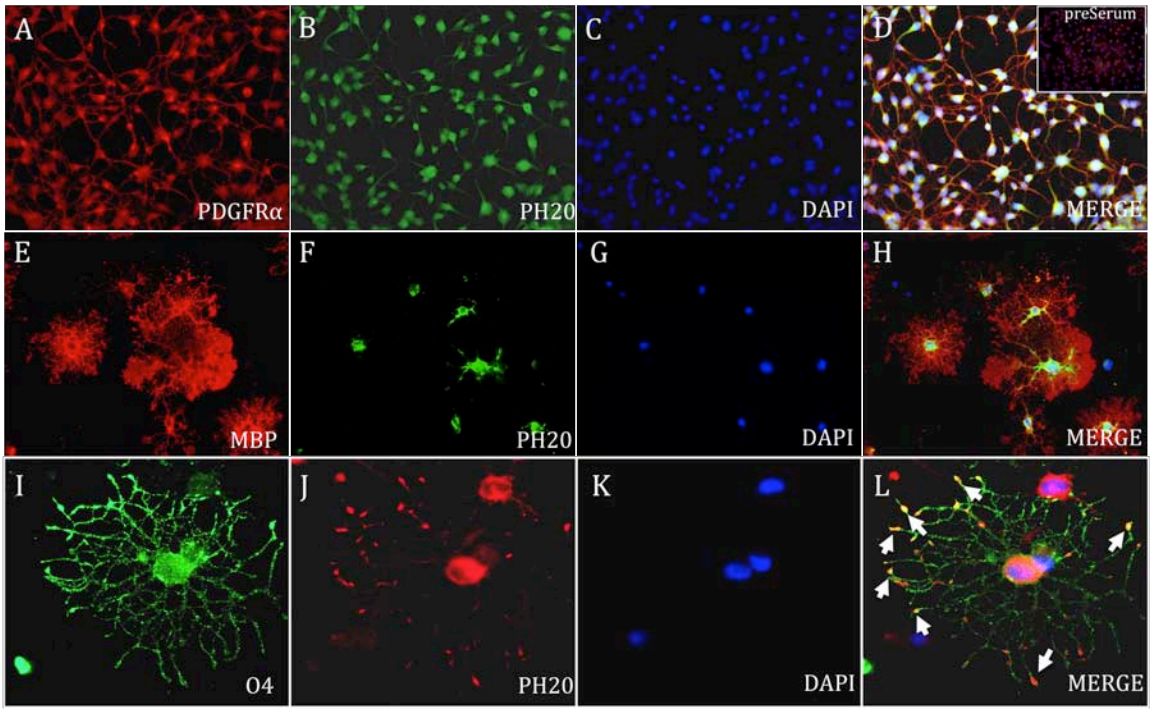


Figure 3.8. PH20 ***expression is differentially localized on proliferating OPCs compared to mature OLs in vitro.*** PH20 expression was confirmed by immunocytochemistry in OPCs (A-D) and in maturing OLs (E-H). In OPCs, PH20 expression (B) was seen in cell bodies and processes of PDGFRα+ OPCs (A). PH20 expression became confined to the cell body (F) of MBP+ OLs (E). Inset in panel (D) shows lack of staining in OPCs incubated with preimmune serum. DAPI (C, G) was used to identify cell nuclei. Merged images are shown in D and H. (I-L) Distribution of PH20 (J, red) in O4+ (I, green) OPCs at premyelinating stages during maturation into MBP+ OPCs. As seen clearly in the merged image (L) PH20 expression is observed in the tips of O4 positive processes (arrows). Cells were counterstained with DAPI (K).

Given that HMW HA accumulates in chronic demyelinated lesions, that PH20 can degrade HMW HA, that PH20 breakdown products can inhibit OPC maturation and remyelination, and that PH20 is only found at low levels in the mature brain, we hypothesized that PH20 expression may be upregulated in demyelinated lesions. In mice with EAE, we observed that PH20 was elevated in demyelinated spinal cord lesions (Figure 3.9*A*-*D*) where it was expressed by both reactive astrocytes and occasionally by OPCs (data not show). Furthermore in chronically demyelinated plaques of MS patients, PH20 immunoreactivity was enriched at the borders of lesions (Figure 3.9 *E*, arrows). Numerous cells expressed PH20 at the lesion borders (Figure 3.9 *F*-*G*), while small

numbers of cells with the morphologies of reactive glia expressed PH20 in areas of complete demyelination (Figure 3.9*H*). Double labeling immunohistochemistry demonstrated that the majority of the PH20 staining in MS patients originated from reactive astrocytes and from O4+ OPCs (Figure 3.9*I-L*). These data indicate that PH20 expression is elevated in demyelinating lesions.

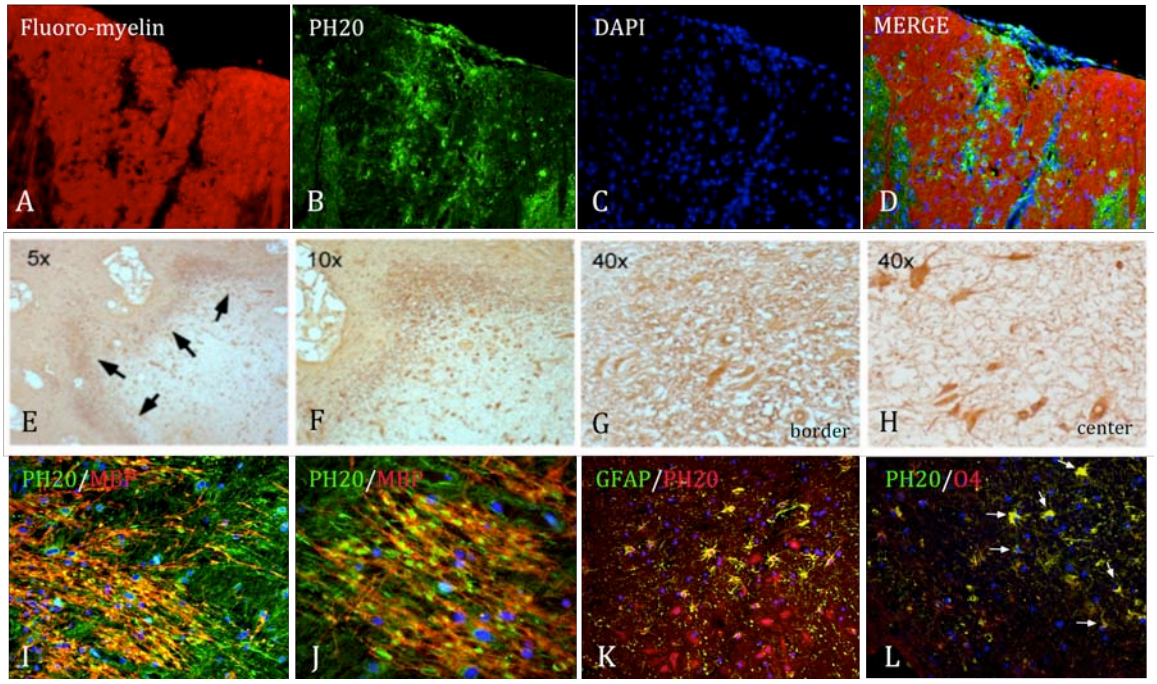


Figure 3.9. PH20 expression in demyelinated lesions. (A-D) Sections of lumbar spinal cord from mice with EAE, 21 days post-inoculation. PH20 immunoreactivity (green; B, D) was elevated in areas where there was demyelination, identified by loss of fluoromyelin (red) staining (A, D) and increased DAPI labeling (C, D). Merged image is shown in D. (E-H) PH20 is also expressed by glial cells at the borders of chronic, cortical MS patient lesions. PH20 immunoreactivity was enriched at the borders of lesions (arrows, E). Numerous cells expressed PH20 in the lesion borders (F, G), while small numbers of cells with the morphologies of reactive glia expressed PH20 in areas of complete demyelination (H). (I-L) Double-labeling of MS patient lesions with antibodies against PH20 and glial markers. (I) Lesion border, showing MBP immunoreactive OLs (red) weakly expressing PH20 (green). (J) A higher power (40x) image of an area from (I) showing that PH20 has a similar cellular localization on MBP+ OLs *in situ* as compared with OPC cultures. (K) Expression of PH20 (red) by GFAP-immunolabeled reactive astrocytes (green). (L) Co-localization of PH20 (green) and O4 (red). Cells where both proteins co-localize are yellow. Sections were counterstained with DAPI (blue) to label cell nuclei.

DISCUSSION

We have demonstrated for the first time that the PH20 hyaluronidase is elevated in astrocytes and OPCs in demyelinating lesions from MS patients and rodents with EAE; that elevated expression of PH20 by OPCs leads to the generation of HA breakdown products that inhibit OPC maturation; and that inhibiting hyaluronidase activity leads to enhanced OPC maturation and remyelination *in vivo*. Previous studies have demonstrated that HA and HA receptors, including CD44, are elevated coincident with astrogliosis following a variety of CNS insults including demyelinating lesions in MS patients and mice with EAE (Back et al., 2005b), traumatic spinal cord injuries (Struve et al., 2005), and ischemia (Wang et al., 2011). HA is also elevated in the brains of aged rodents (Jenkins and Bachelard, 1988a) and in patients with Alzheimer's disease and age-related vascular brain injury (Jenkins and Bachelard, 1988b; Suzuki et al., 1965). Myelination disturbances occur in each of these conditions. Our data therefore support a model for remyelination failure in which HMW HA synthesized by reactive astrocytes and other cells within CNS lesions is degraded into specific classes of HA oligosaccharides by PH20 expressed by glial cells in the lesion microenvironment as well as OPCs recruited to lesions. These oligosaccharides in turn inhibit OPC maturation. Thus, inhibiting hyaluronidase activity or blocking signaling by hyaluronidase-generated HA fragments are potentially efficacious strategies for promoting remyelination in numerous conditions.

HA fragments may influence OPCs through a number of mechanisms. A study in Xenopus tadpoles demonstrated that glycogen synthase kinase-3b (GSK3b), a serine/threonine protein kinase that is part of the Wnt signaling cascade, is activated by HA signaling (Contreras et al., 2009). The Wnt signaling cascade and GSK3b in particular has been implicated in regulating OPC maturation (Fancy et al., 2009; Feigenson et al., 2009; Azim and Butt, 2011; Tawk et al., 2011). Inhibition of GSK3b stimulates remyelination in adult mice (Azim and Butt, 2011). It is possible therefore that HA fragments inhibit OPC maturation at least in part through the activation of GSK3b. HA fragments may also signal through toll-like receptor-2 or -4 (Termeer et al., 2002; Taylor et al., 2004; Jiang et al., 2005; Scheibner et al., 2006; Shimada et al., 2008), both of which can also influence GSK3b activation (Kim et al., 2010; Zhang et al., 2009). Toll-like receptors are expressed by OPCs and one study has suggested that HA-mediated inhibition of OPC maturation is dependent on toll-like receptor 2 (Sloane et al., 2010). The contributions of other HA receptors, including CD44 and the receptor for hyaluronan-mediated motility, in regulating OPC maturation have yet to be elucidated.

HA fragments likely act in concert with other signals in demyelinated lesions to prevent remyelination. Like HA accumulation, many of these signals appear to be linked to reactive astrogliosis. Reactive astrocytes in spinal cord injuries increase their expression of bone morphogenetic proteins that inhibit OPC differentiation with concurrent promotion of astrocyte differentiation (Wang et al., 2011). Similarly, the Notch ligand Jagged1 is elevated on reactive astrocytes in demyelinating lesions and activates Notch

signaling on OPCs, preventing their maturation (John et al., 2002). It is possible that HA, which is synthesized predominantly by reactive astrocytes, acts on OPCs in concert with these and other inhibitory mechanisms including the NOGO receptor interacting protein LINGO-1 (Mi et al., 2007) to prevent remyelination.

Although we have demonstrated that inhibiting hyaluronidase activity is sufficient to promote remyelination in lesions where HA accumulates, such broad inhibitors are not likely to be useful as therapeutic agents. Hyal and Hyal2, for example, are widely distributed in many different tissues (Stern and Jedrzejas, 2006). *Hyal1*-null mice develop osteoarthritis (Martin et al., 2008) while humans with *HYAL1* mutations develop a lysosomal storage disorder, mucopolysaccharidosis (MPS) IX (Triggs-Raine et al., 1999). *Hyal2*-null mice develop skeletal and hematological abnormalities (Jadin et al., 2008). In contrast, although PH20 mRNA has been detected at high levels in testis and at low abundance in a limited number of other tissues, PH20-null mice do not display any significant pathological phenotypes (Baba et al., 2002). Our finding that PH20 is specifically upregulated in demyelinating lesions and is sufficient to block OPC maturation suggest that agents that target PH20 and not other hyalurondiases may be both safe and efficacious as long-term therapies for the promotion of remyelination.

CHAPTER 4: Additional Data: The role of somatic hyaluronidases in OL maturation and inflammatory demyelination.

BACKGROUND & RATIONAL

While it is estimated that 30% of the body's total HA is turned over everyday surprising little is know about the role of hyaluronidases in normal tissue homeostasis. Given the prominent role HA plays in tissue and wound repair, it is even more surprising how little is known about the role of hyaluronidases following injury or disease. The extracellular accumulation and clearance of HA following injury is likely dependent upon alterations in both HA synthesis and degradation. HYAL expression has been confirmed in almost all tissues including the lungs, liver, kidneys, heart and reproductive organs, yet it remains controversial which HYALs are expressed in the mammalian brain. Several labs report a conspicuous absence of HYAL2 in the brain (Lepperdinger et al., 2001; Strobel et al., 1997) and no gross abnormalities in the CNS or PNS are reported in the HYAL2 null mouse (Jadin et al., 2008). However, a GPI-linked hyaluronidase such as HYAL2 would most likely be needed to degrade HA at the cell surface. Similarly, HYAL3 mRNA is expressed in the brain (Csoka et al., 2001) but conflicting reports exist on the nature of this gene's function. Addditionally, brain abnormalities in HYAL3 or HYAL1 null mice have not been reported (Atmuri et al., 2008; Martin et al., 2008).

My work and others (Sloane et al., 2010a) have shown that OL lineage cells express hyaluronidases. I have also reported that one particular hyaluronidase, PH20, generates HA breakdown products inhibitory to OPC maturation *in vitro* and to remyelination *in*

vivo (Preston, et. submitted). Previous work in this thesis shows that PH20 is expressed in MS patients and animals with EAE. Furthermore, degradation of HWM HA by an enzyme preparation primarily composed of PH20 inhibits OL maturation *in vitro* and remyelination *in vivo.* PH20 has largely been considered a testes-specific enzyme (Jones et al., 1995; Jones et al., 1996; Meyer et al., 1997; Phelps and Myles, 1987) and thus its role in remyelination failure represents a highly novel finding. The studies described here were undertaken to characterize the expression profiles of the somatic HYALs in OL maturation *in vitro*, during cortical development and in EAE disease progression. Here, I present further evidence that HA degradation by the major somatic hyaluronidases, HYAL1-3, are unlikely to influence OL differentiation during development of the brain or following inflammatory demyelination, further supporting the importance of identification of PH20 activity as mediating HA-induced remyelination failure.

MATERIALS & METHODS

For detailed description of experimental methodology refer to Chapter 2: General Methods. Briefly, HYAL2 expression in OPCs was confirmed using *in situ* hybridization. To investigate the role of the major somatic hyaluronidases in oligodendrogliogenesis, I collected RNA during growth factor-induced differentiation of OPCs and used quantitative real time polymerase chain reaction (qRT-PCR) to analysis the expression profiles of HYAL1 and HYAL2, *in vitro*. Additionally, to characterize HYAL1 and HYAL2 expression profiles *in vivo*, I isolated RNA for qRT-PCR analysis from the developing cortex of embryonic and postnatal mice during known periods of OPC genesis and

maturation. Additionally, immunocytochemistry was used to study the expression of HYAL3 in OL lineage cells and the role of HYAL3 in OL maturation was tested using viral-mediated enhanced expression of the protein during growth factor-induced OL differentiation. Finally to study the expression profiles of HYAL1-3 in inflammatory demyelination, RNA from the lumbar spinal cords of MOG peptide-induced EAE mice ($n \geq 3$ per group) was collected during pre-symptomatic (day 7 post inoculation), acute (day 16 post inoculation) and chronic (day 26 post inoculation) periods of inflammatory demyelination for qRT-PCR analysis.

RESULTS

OPCs express transcripts for the major somatic hyaluronidases, HYAL1 and HYAL2.

Previously, I have reported that OPCs contain mRNA transcripts for HYAL1 and HYAL2 (see Chapter 3, Figure 3.5.a) and that HYAL1 and HYAL2 protein expression in OL lineage cells is reported by others (Sloan, et al. 2010a). Commercially available antibodies raised against rat and primate HYAL1 and HYAL2 failed to cross-react with the mouse proteins *in vitro* or *in vivo*. Therefore, to confirm expression of these HYALs in cultured OPCs, RT-PCR primers were used to isolate 200-400bp DNA fragments (see Table 2.2) of HYAL1 or HYAL2 transcripts from cultured OPCs. Specificity of the primers for either HYAL1 and HYAL2 mRNA was confirmed by independent sequencing of the purifed amplicons at the Molecular Biology Core Facilities at Oregon National Primate Research Center (data not shown). Digoxygen-labeled antisense probes were synthesized for *in situ* hybridization against the anti-sense strand of either the HYAL1 or HYAL2 (sense

synthesiszed as control). Hyal2 expression was confirmed in OPC cultures (Figure 4.1). Confirmation of HYAL1 expression is still underway.

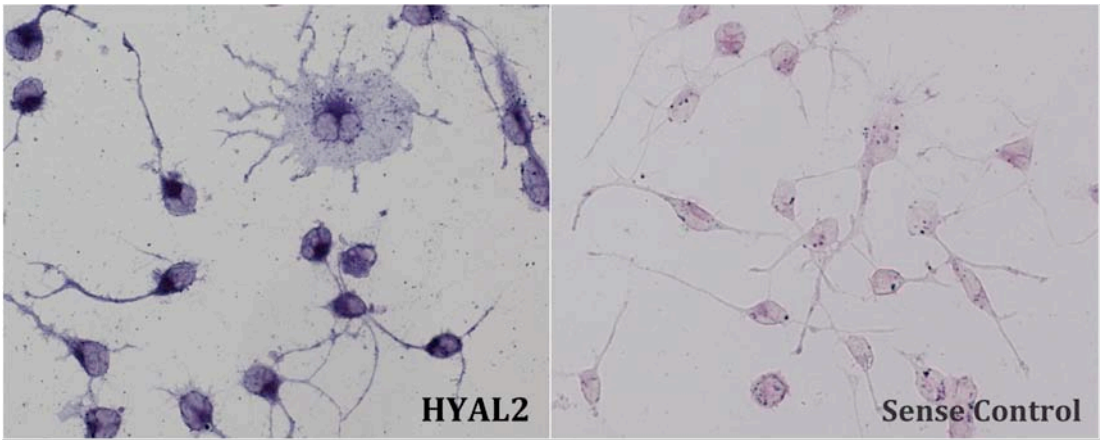


FIGURE 4.1. *In situ* hybridization confirms HYAL2 expression in cultured OPCs. Anti-sense RNA probes for HYAL2 where generated to confirm the expression of HYAL2 in NSC-derived OPCs (left panel). Nonspecific binding for HYAL2 probe tested by incubating cells with a nonspecific probe (sense RNA of HYAL2).

HYAL1 and HYAL2 expression is largely unchanged during growth factor-induced OL maturation.

While it has been confirmed that OL lineage cells express HYAL1 and HYAL2 (Sloane et al., 2010a) the expression profiles of these hyaluronidases during OPC differentiation and OL maturation is unknown. Therefore, I characterized the expression of these hyaluronidase during oligodendrogliogensis *in vitro.* NSC-derived OPCs were plated and given T3/NAC to induce differentiation. Total RNA was collected prior to addition of growth factors (start differentiation, 0hr) or after 24, 48 , 72 or 96 hour exposure to growth factors, reverse transcribed into cDNA and subjected to qRT-PCR analysis for HYAL1 and HYAL2 expression. While OPCs appear to upregulate HYAL2 appoximately 8-

fold upon plating and removal of proliferative growth factors (i.e. PDGF-AA and FGF2) only very modest changes in HYAL1 (approximately 2-fold beginning 24 hours after GF addition) and HYAL2 (less then 1-fold beginning 24 hours after GF addition) were observed during OL maturation (Figure 4.2). Taken together these results indicate that significant changes in HYAL1 and HYAL2 expression do not appear to occur during T3/NAC-induced OL maturation.

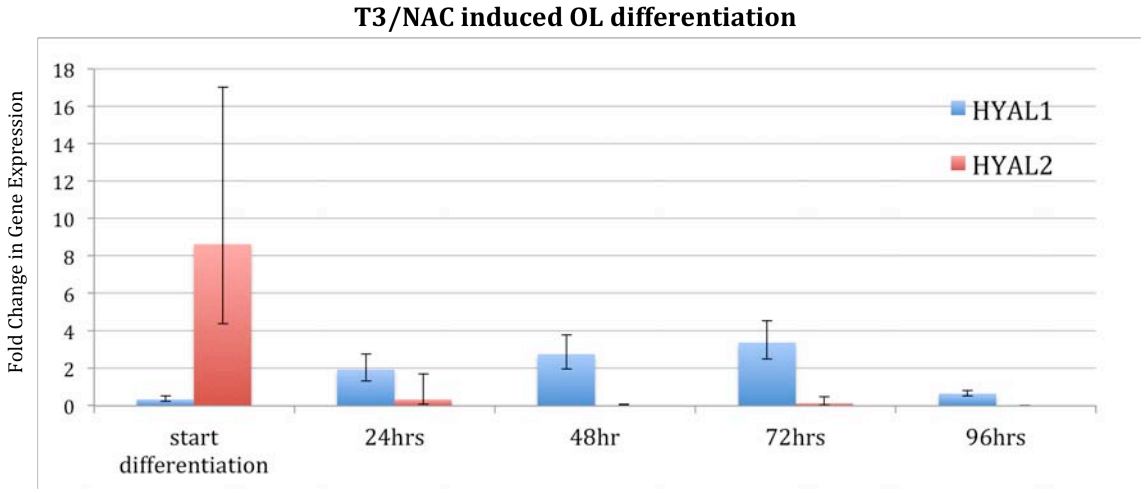


Figure 4.2 HYAL1 and HYAL2 expression during OL maturation. OPCs were plated in growth factor-free media and given T3/NAC to induce differentiation. Total RNA from differentiating OPCs was collected prior to growth factor (GF) addition (Ohrs, start differentiation) or 24, 48, 72 or 96hrs after addition of GFs. Quantitative real time polymerase chain reaction (q-RT-PCR) was performed and gene expression analyzed (see general methods). A 8-fold increase in HYAL2 expression was seen upon plating OPCs (start differentiation) but no changes in gene expression for HYAL1 or HYAL2 were seen during GF-induced differentiation. N=3 plates where collected per time point.

HYAL1 and HYAL2 expression is largely unchanged during developmental oligodendrogliogenesis.

As described in detail previously (Chapter 1) oligodendrogenesis begins in the embryonic brain with the first wave of OPCs arising from the MGEs at embryonic day 12.5 (e12.5) followed by a second wave of OPCs from the LGEs at embryonic day 15.5 (e15.5). Proliferation and migration of GE-derived OPCs into the nascent cortex continues until birth (P0). Postnatally, a third wave of OPCs arises from the venticular zone of the expanding cortex. Changes in HYAL1 and HYAL2 gene expression during cortical maturation have not been reported. Therefore to characterize the expression of the HYALs during periods of *in vivo* oligodendrogliogenesis, total RNA from the cortices of day 14.5, day 16.5 and day 18.5 embryonic and day 0 (birth) day 4, and day 16 postnatal mice (n=4) was isolated, transcribed into cDNA and subjected to q-RT-PCR for HYAL1 and HYAL2 expression. Consistent with q-RT-PCR analysis of GF-induced OL maturation (Figure 4.2), HYAL1 and HYAL2 expression remains relatively stable (less than 1-fold change in gene expression, Figure 4.3) during either embryonic or postnatal cortical expansion.

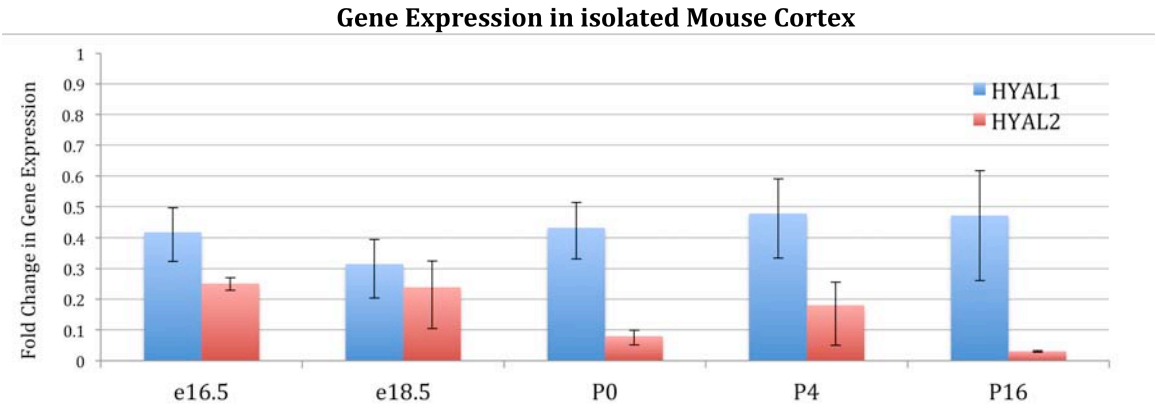
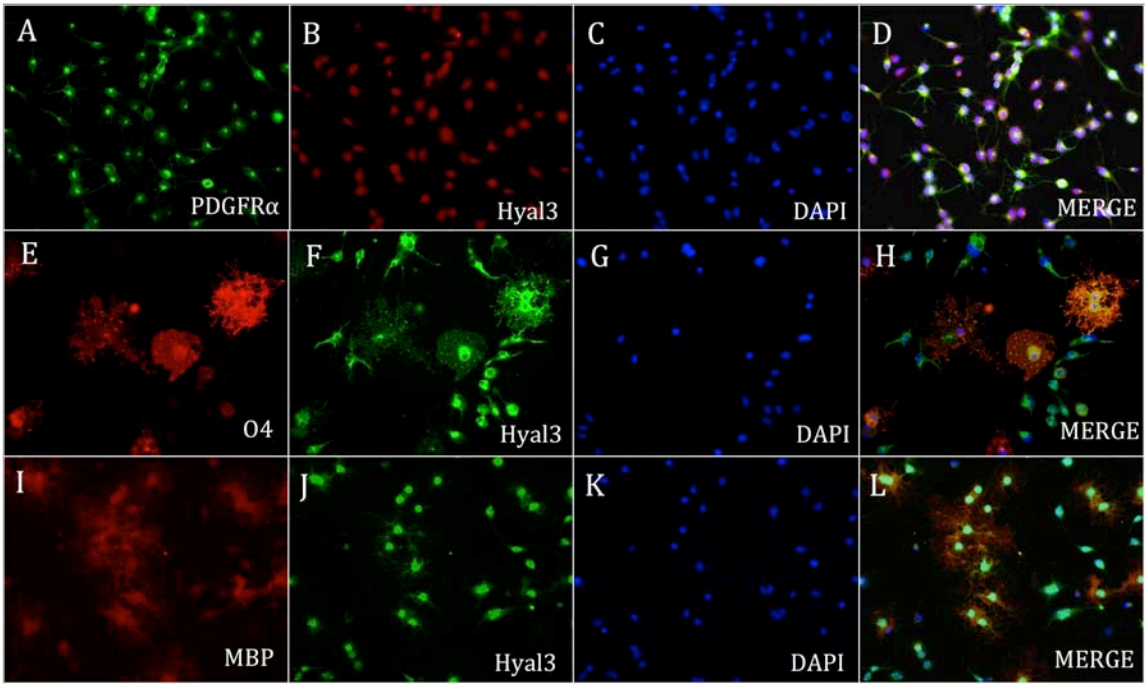


Figure 4.3. HYAL1 and HYAL2 expression during periods of oligodendrogliogensis in the developing mouse cortex. Total RNA from developing cortices from embryonic day 14.5, 16.5, 18.5 and postnatal day 0, 4 and 16 was isolated and q-RT-PCR preformed and gene expression analyzed. Expression of HYAL1 and HYAL2 decreased from e14.5 to e16.5 by 0.4 and 0.25-fold; respectfully, and remained mostly constant throughout embryonic and postnatal cortical development. N≥4 animals collected per group.

OL lineage cells express HYAL3 and viral-mediated over expression of HYAL3 inhibits OL maturation, *in vitro*.

In addition to HYAL1 and HYAL2 it has been reported that OL lineage cells also express HYAL3 (Sloan, et al. 2011). HYAL3 expression is also seen in the brain but the hyaluronidase function of this protein remains controversial (Atmuri et al., 2008; Hemming et al., 2008; Reese et al., 2010). To confirm expression of HYAL3 in OL lineage cells, OPCs, immature and mature OL were fixed and stained with antibodies to HYAL3 and PDGFRα, O4 or MBP. The expression of HYAL3 was observed in the soma and processes of PDGFRα positive OPCs (Figure 4.4, a-d) and O4 positive immature OLs (Figure 4.4, e-h); but was only seen in the soma of MBP positive OLs (Figure 4.4, i-l). Next, I used a gain-of-function strategy to test the role of HYAL3 in OL maturation. The open reading frame of HYAL3 was subcloned into a bicistronic expression vector carrying the cDNA for enhanced green fluorescence protein (EGFP). OPCs were then

infected with a lentivirus containing either the EGFP vector or EGFP and HYAL3, differentiated for 72-96 hours in T3/NAC then fixed and stained with antibodies to MBP and GFP. OL maturation was analyzed as described previously (see Figure 3.7). Cells infected with the HYAL3 virus displayed impaired maturation as assayed by MBP expression compared with cells infected with EGFP alone. (51.34% of control; $p = 0.0004$). Intriguingly, viral-enhanced expression of HYAL3 had no effect on extracellular HA levels (as assayed by HABP staining, data not shown) indicating that HYAL3 induced OL maturation failure might occur by a HA-independent mechanism.



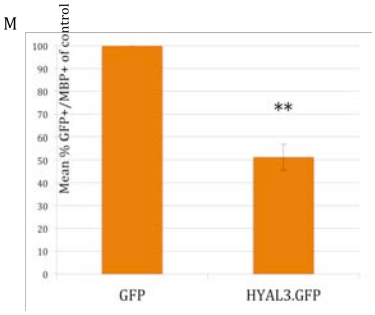


Figure 4.4. **Expression HYAL3 significantly blocks OL maturation**. Immunocytochemistry shows that proliferating (a-d), immature (e-h) and mature OLs (i-l) express Hyal3. OPCs were infected with lentivirus containing either GFP (control) or GFP and HYAL3, allowed to differentiate for 72-96hrs, then fixed and stained for MBP and GFP (data not shown). Total cells number of cells co-expressing GFP and MBP were counter and compared to cells infected with GFP only (control). HYAL3 over expression significantly inhibited OL maturation (51.34% of control; p = 0.0004).

HYAL3 expression is largely unchanged during acute and chronic periods of inflammatory demyelination.

Enhanced HYAL1 expression does not impair OL maturation and enhanced HYAL2 expression only weakly impairs OL maturation (see Figure 3.7). In contrast, enhanced

HYAL3 expression is able to significantly impair OL maturation. Therefore, I asked if HYAL3 expression is increased during periods of chronic inflammatory demyelination as seen in late stages of EAE disease course. To test this hypothesis, total RNA was isolated from the lumbar spinal cord of mice with MOG-induced EAE during pre-symptomatic (day 7 post inoculation), acute (day 16 post inoculation), or chronic (day 26 post inoculation) disease course, reverse transcribed into cDNA and subjected to qRT-PCR analysis for HYAL1-3. HYAL1 expression was decreased during pre-symptomatic disease (~0.4 fold as compared to CFA only inoculated mice, control) and slightly elevated in acute and chronic disease (1.5-fold and 0.9-fold, respectively). HYAL2 expression was slightly elevated during all periods of disease (1.2 to 2.5-fold increase). Interestingly, HYAL3 expression was increased more than 3-fold during pre-symptomatic disease but only slightly increased during periods of acute and chronic disease (0.6-fold and 1.2-fold). Collectively, these data do not support a significant role for HYAL3, other the other somatic hyaluronidases, in remyelination *in vivo*, which is largely confined to acute and chronic periods of disease course.

Lumbar Spinal Cords (EAE animals vs. CFA only control animals)

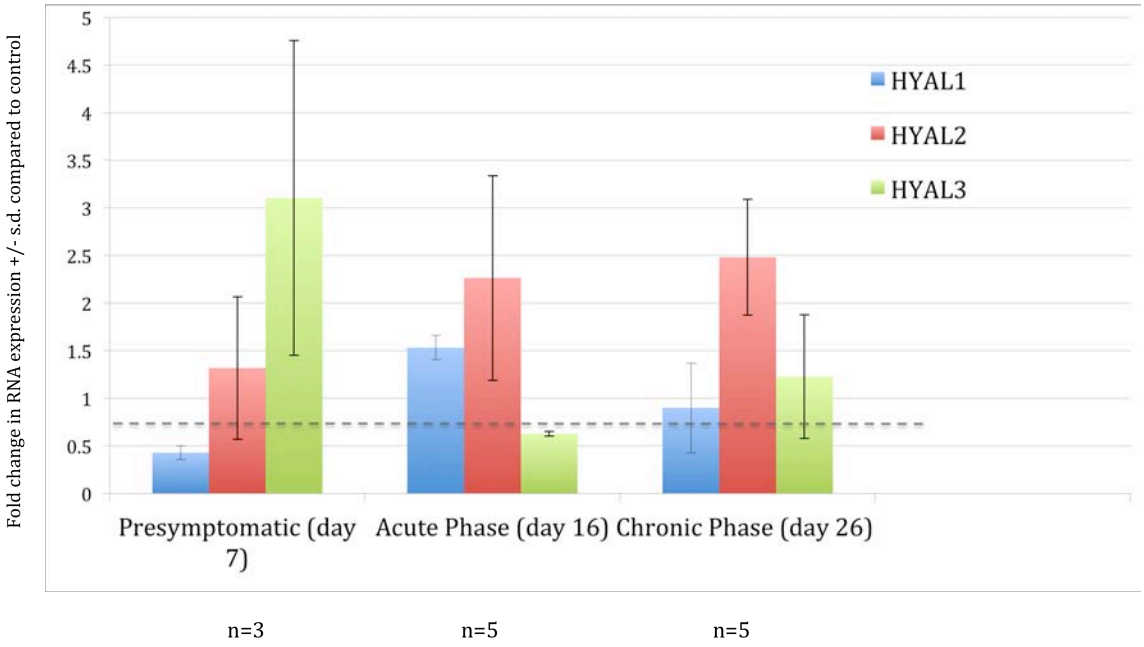


Figure 4.5 Small changes in HYAL expression is seen in the lumbar spinal cords of mice during EAE disease course. Mice were inoculated with MOG-peptide in CFA and allowed to survive for 7, 16 or 26 days. RNA from the lumbar spinal cord was isolated, reverse transcribed and subjected to q-RT-PCR. Expression of HYAL1-3 was compared to animals receiving CFA with no peptide (control). During pre-symptomatic disease phase HYAL 1 expression was reduced compared to controls while HYAL3 expression was elevated. During acute and chronic phases, Hyal2 was elevated while HYAL1 showed only a slight elevation during the acute phase. N≥3 animals collected per group.

CONCLUSIONS & DISCUSSION

Previous studies indicate that while OL lineage cells express multiple hyaluronidases, PH20 plays a unique role in inhibiting OL maturation and in HA-mediated remyelination failure (see Chapter 3). Here, I confirm that while OPCs express the major somatic hyaluronidases HYAL1 and HYAL2, the expression of these genes does not fluctuate significantly during growth factor (GF)-induced differentiation or during periods of oligodendrogliogenesis in the embryonic or postnatal brain. Recent data indicates that HYAL3 may be able to functionally substitute for PH20 in the testes of rodents (Reese et

al., 2010). Consistent with this observation, I find that viral-mediated expression of HYAL3 during OPC maturation is able to significantly inhibit OPC maturation, yet I saw no evidence of HA degradation following viral-enhanced expression of the HYAL3 enzyme. Additionally, immunohistochemical analysis of normal (i.e. uninjured) spinal cord tissue showed that HYAL3 expression was largely confined to neurons in the gray mater and only weakly expressed in the white mater of the lumbar spinal cord (data not shown). Finally, no changes in the expression of HYAL3 were observed in cells in or around demyelinated lesions during acute or chronic periods of EAE disease course (data not shown). Collectively, these data do not support the notion that HYAL1-3 are contributing to in HA-mediated remyelination failure.

Proliferating OPCs synthesize HA and HA continues to accumulate during all stages of OL-genesis, *in vitro* (see Chapter 3, Figure 3.2). HA has been shown to be a major component of the neural ECM (Baier et al., 2007; Bignami and Asher, 1992; Bignami et al., 1993; Deepa et al., 2006) which undergoes extensive reorganization during postnatal development (Margolis et al., 1975). The morphological transformation of OPCs from simple bipolar progenitors to complex multipolar cells capable of synthesizing huge volumes of myelin membrane likely requires extensive physical remodeling of the neural ECM to accommodate cell body and process outgrowth. The expression of the HASs and HYAL1-3 in OL lineage cells leads to the speculation that modification of the HA-rich neural ECM (via changes in HA synthesis and degradation) is a normal part of OL-genesis. Therefore, HA degradation products generated by these enzymes would not be expected to significantly impact progenitor maturation. In contrast, PH20 expression is

not found in the developing brain and therefore is unlikely to play a role in developmental myelination. However, upregulation of this enzyme in injured neural tissue could generate distinct HA degradation products not seen by OL lineage cells during normal OL-genesis. Collectively, the experiments presented here offer further support to the notion that PH20 activity drives HA-mediated OL maturation failure and is a promising target for therapies aimed at promoting remyelination.

CHAPTER 5: CONCLUSIONS AND FUTURE DIECTIONS

A role for PH20 in remyelination failure.

Multiple mechanisms likely underlie remyelination failure in chronic demyelinated lesions in diseases, such as MS, and following ischemia, traumatic injury and other insults to the CNS. While great strides have occurred in the basic biology of OPC generation, proliferation and maturation in the developing nervous system (see Chapter 1), mechanisms inhibiting OL maturation in the injury environment of the demyelinated lesion have lagged behind. It is becoming clear that the local environment of the glial scar contains molecules that profoundly impact the repair and replacement of damaged myelin. Previous work has shown that HMW species of HA accumulate in chronic demyelinated lesions and inhibit the maturation of OLs into myelin producing cells (Back et al., 2005b and Sloan, et al. 2010a). Data presented in this thesis shows that mouse OPCs synthesize HA and express transcripts for the major *HAS* genes, yet inhibition of HA synthesis has no effect on OL maturation. Recent evidence suggests that the digestion of HA by hyaluronidases, rather than the intact sugar itself, inhibits intracellular signaling cascades in OPCs, preventing OL maturation (Sloan, et al. 2010a). The work presented in this thesis expands upon these observations by identifying one specific hyaluronidase, PH20, as being the primary enzyme responsible for HA-mediated OL maturation failure *in vitro* and a candidate for HA-mediated remyelination failure.

In support of this statement, I have described experiments showing that hyaluronidase preparations composed primarily of PH20, but not bacterial-derived hyaluronidases or

other glucuronidases, are able to block OL maturation *in vitro*. Treating HMW species of HA with this PH20-rich hyaluronidase preparation generates HA degradation products, which are inhibitory to the remyelination of lysolecithin induced-lesions. Furthermore, inhibiting hyaluronidase activity promotes OL maturation and prevents HA-mediated remyelination failure in chemically demyelinated lesions. This work confirms that OPCs express multiple hyaluronidases including HYAL1-3 and PH20 as previously suggested (Sloan, et al. 2010a), and demonstrates that OPCs are capable of degrading HMW species of HA similar in size to HA species secreted by reactive astrocytes and in chronic demyelinated lesions. Viral-enhanced expression of PH20, but not HYAL1 or HYAL2, robustly inhibited OL maturation of NSC-derived OPCs. These experiments support the notion that each hyaluronidase generates specific HA degradation products, and that the digestion products produced by PH20 are primarily responsible for HA-mediated remyelination failure. Importantly, I have also shown for the first time that PH20 is expressed by OPCs and astrocytes in demyelinated lesions of patients with MS and in mice with EAE. Collectively, these data support a model where PH20, but not other hyaluronidases, expressed by OPCs and astrocytes in chronic demyelinated lesions, degrades HMW species of HA generating HA degradation products that inhibit OL maturation (Figure 5.1).

Degradation of HMW HA impairs OL Maturation and Remyelination.

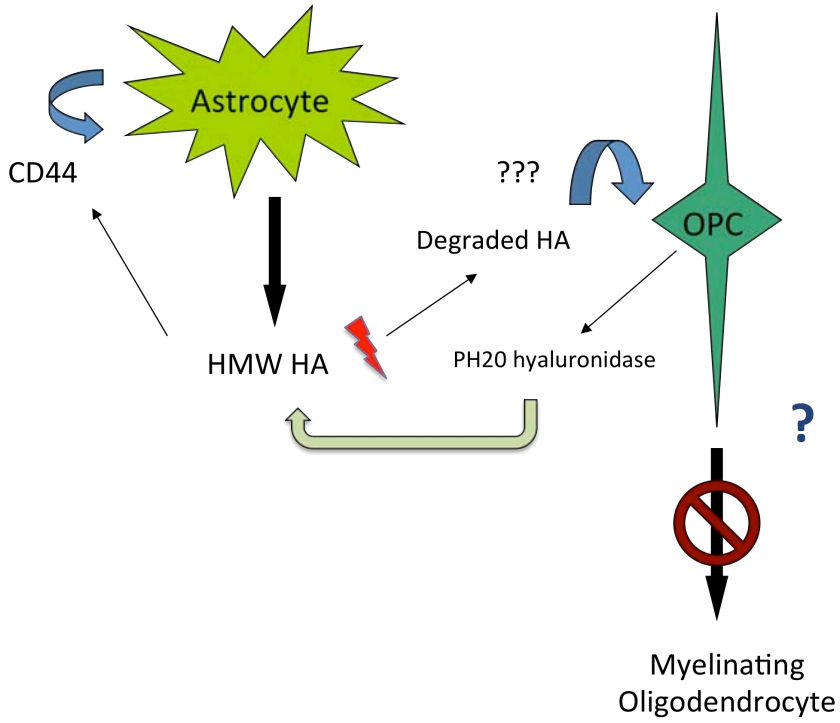


Figure 5.1. A model for OL maturation inhibition in demyelinated lesions. In this model reactive astrocytes express CD44 and secrete HMW HA in response to local inflammation. HMW HA can bind to CD44 or be degraded by the PH20 hyaluronidase, which is expressed by OPCs (and other glia) within the lesion. Degraded HA then signals through an unidentified receptor impinging on intercellular signaling cascades necessary for OL differentiation and remyelination.

The identification of PH20 in demyelinated lesions represents a significant contribution to our understanding of the processes underlying remyelination failure. PH20 is expressed at such low levels in the spinal cord and brain of normal rodents that it is almost undetectable yet it is seen in demyelinated lesions of human MS patients and the spinal cord of mice with EAE. PH20 has been regarded as a testes-specific hyaluroidase for decades and historically the brain has been used as a negative control for PH20 expression. However, mounting evidence suggests that PH20 is also expressed in a variety of tissues including the female reproductive tract, kidneys, and several types

of cancers. The last observation is particularly interesting as it suggests that expression of PH20 expression might be tightly regulated in normal tissue to prevent cells from remodeling their ECM or degrading local HA-induced growth inhibition cues, thus maintaining cellular homeostasis and preventing unchecked growth. During CNS injury, cells are exposed to a variety of pro- and anti-inflammatory molecules within the glial scar and demyelinated lesions. It has been proposed that hyaluronidases are upregulated in response to a variety of inflammatory molecules, including members of the tumor necrosis factor (TNF) and interleukin (IL) family of cytokines (Flannery, et al. 1998, Monzón, et al. 2008, and Motte, et al., 2009). These hyaluronidases, PH20 included, may be transiently induced in glia in the CNS injury environment to promote clearance of the HA-rich scar, thus removing a physical barrier to progenitor cells. However, the clearance of HA may inadvertently generate HA degradation products associated with growth regulation, thereby activating signaling cascades that prevent remyelination.

Further support for a key role for PH20 in remyelination failure comes from examination of the expression and activity of other hyaluronidases (HYAL1-3) in OL lineage cells. HYAL1 and HYAL2 are unlikely to contribute to HA-mediated remyelination failure, as viral-enhanced expression of these hyaluronidases either fails to or only weakly inhibits OL maturation, respectively. Furthermore, HYAL1 and HYAL2 expression remain relatively stable during OL maturation *in vitro*, during the course of oligodendrogliogenesis in the cortex or during EAE disease progression in the spinal cord (see Chapter 4). The role of HYAL3 in HA-mediated remyelination failure is less clear.

While viral-enhanced expression of HYAL3 partially blocks OL maturation (by approximately 50%) it does not appear to be via the extracellular degradation of HA (see Chapter 4). The enzymatic activity of HYAL3 continues to be controversial with one group reporting that HYAL3 codes for a non-functional hyaluronidase and another reporting it to be an acid-active hyaluronidase capable of functionally substituting for PH20 in the testes. The latter is particularly intriguing, as this leads to the speculation that the inhibition of OL maturation seen in HYAL3 gain-of-function studies is due to a PH20-like activity of HYAL3. This interpretation is largely speculative, as no changes in extracellular HA levels are observed in cells over expressing HYAL3. However, it is possible that focal changes in HA degradation occurred that were beyond the resolution of the techniques used.

Paradoxically, while enhanced HYAL3 expression in cultured OPCs inhibits OL maturation, HYAL3 expression appears to be largely confined to neurons in the gray matter of the spinal cord and no appreciable changes in HYAL3 expression levels were observed in mice with EAE in or around demyelinated lesions. Additionally, only modest changes in HYAL3 expression levels (less than 1-fold) were seen in the spinal cord during EAE progression especially during periods associated with remyelination of EAE lesions. Collectively, these data indicate that HYAL3 is not likely to be an enzyme that contributes to HA-mediated remyelination failure. Overall, a more detailed analysis of hyaluronidase (including PH20) expression patterns in the nervous system following demyelinating events (i.e. EAE, SCI and TBI), particularly in OL lineage cells, is needed to elucidate the role these enzymes play in remyelination failure. Strategies to knock down

PH20 expression via RNA interference are currently underway to test the hypothesis that specifically inhibiting PH20 will promote OL maturation. Future experiments should include chemical demyelination and/or EAE induction in HYAL1-3 and PH20-null animals to test the role each hyaluronidase plays in remyelination and MS-like disease. Also, a comparison of hyaluronidase transcript expression patterns in isolated OL lineage cells between healthy and injured CNS tissue will aid in determining the specific contribution of each enzyme to injury pathology and remyelination failure.

The cellular receptors and intracellular signaling cascades driving OL maturation failure have not been determined in this study. It has been reported that hyauronidase-mediated remyelination failure activates TLR2-dependent MyD88 signaling cascades (Sloan, et al. 2010a). However, in collaboration with the laboratory of Dr. Stephen Back, we have been unable to replicate these findings. Sloan et al. report TLR2 and TLR4 expression (via immunocytochemistry) on OL lineage cells. I have not been able to confirm that these receptors are expressed in NSC-derived OPCs, immature or mature OLs using the same commercially available antibodies reported in their studies. Furthermore, injection of HMW HA or BTH degraded HMW HA products still inhibited the remyelination of lysolecithin-lesions in TLR2-null animals ($n \geq 4$, three separate experiments, data not shown). Taken together these results do not support a TLR2-mediated mechanism for HA-induced inhibition of OL maturation or remyelination failure. Whether these discrepancies in experimental findings represent technical issues or subtle differences in lysolecithin-induced demyelination protocols remains to be seen and is being actively pursued.

An alternative candidate for HA-mediated remyelination failure is the HA receptor for endocytosis (HARE), the primary scavenger receptor for LMW species of HA (Pandey et al., 2008). HARE mediates the clearance of HA and chondroitin sulfates (CS's) from lymphatic and vascular tissue via endocytosis of the sugar (Harris et al., 2007). HA binding to HARE has been shown to increase phosphorylation of the HA receptor and activate members of the mitogen-activated protein kinase (MAPK) pathway, namely extracellular signal-regulated kinase 1 and 2 (ERK1/2) (Kyosseva et al., 2008). HARE expression in the brain has been reported, but the function of this receptor in the nervous system remains unknown and it has yet to be shown if OL lineage cells express HARE (Kyosseva et al., 2008). Similarly, RHAMM-mediated HA signaling has been implicated in a plethora of cellular activities including migration and differentiation via activation of multiple intracellular signaling cascades such as PI3K and PKC (see Chapter 1). RHAMM expression has been identified on OL lineage cells in the developing and adult brain (Lynn et al., 2001a) and an upregulation of RHAMM mRNA in OLs is seen during development corresponding to periods of progenitor migration (Lynn et al., 2001a). The role of HA-RHAMM interactions in OPC behavior following injury has not been studied. Future studies in the Sherman lab are planned to test the role of these HA receptors in HA-mediated remyelination failure.

Alternatively, a series of studies have recently identified *wingless* (Wnt)-mediated signaling cascades as being a key player in the inhibition of OL genesis during development (Feigenson et al., 2009; Langseth et al., 2010; Tawk et al., 2011; Zhong et al., 2011) and in the demyelinated lesion environment (Fancy, et al, 2009). Stabilization

of Axin2, which inhibits Wnt cascades by degrading b-catenin, promoted maturation of OPCs after hypoxia and demyelinating injury (Fancy et al., 2011). Similarly, glycogen synthase kinase 3β (GSK3β) inhibitors were able to override the inhibitory effects of Wnt3 on OL maturation and promote remyelination following chemical demyelination (Azim and Butt, 2011). Keratinocytes grown on HA-coated plates upregulate transcription of several members of the Wnt family of proteins (Wnt4 and Wnt6) (Nagira et al., 2007) and Wnt-inducible signaling protein 1 (wisp-1), a Wnt target gene, is upregulated in a hyaluronan- and MyD88-dependent manner in myofibroblasts during epithelial injury (Heise et al., 2011). As such, it would be interesting to test if HA-mediated OL maturation failure occurs in a Wnt/b-catenin dependent manner or via TLR2-indepedent activation of MyD88 signaling cascades.

HA catabolism may influence cellular behavior in NSC niches[3].

Repairing CNS damage following injury or disease is dependent upon the proper mobilization of neural stem/progenitor cells (NSPCs) to sites of tissue damage and their maturation within the post injury environment (Horner et al., 2000). In addition to influencing OL maturation and remyelination in the injured brain, HA synthesis and catabolism may also play critical roles in regulating NSPC proliferation, differentiation and migration in the neural stem cell niches as well. It is still unclear what signals regulate activation of NSPC niches to generate progenitors for repair. Under non-

[3] Portions of this section were adapted from a previously published work. Preston MA. and Sherman LS. (2011). Neural stem cell niches: Roles for the hyaluronan-based extracellular matrix. *Frontiers in Bioscience*. Review. (Schol Ed). 2011 Jun 1;3:1165-79.

pathological conditions these neurogenic niches predominantly produce neurons that, in the subventircular zone, migrate to the olfactory bulb and, in the case of cells that arise in the subgranular zone of the hippocampus, play a critical role in learning and memory. However experimental evidence also shows that NSPC niches are activated in response to nearby injury to generate OPCs and astrocyte progenitors in rodents and humans. The mechanisms underlying niche activation are currently unknown. In addition to data presented in this thesis supporting a role for PH20 in HA-mediated OL maturation and remyelination failure, changes in HA synthesis and catabolism may also be one mechanism used to alter the ECM surrounding NSPCs in the niche to allow for the generation and proliferation of OPCs for repair and remyelination.

The subventricular zone (SVZ) is a neural stem cell niche organized along the lateral walls of the lateral ventricles of the postnatal and adult cortex. This niche is characterized by a thin area adjacent to the ependymal cell layer that contains infrequently dividing NSPCs (also known as the GFAP-positive B cell), rapidly dividing so-called “transit amplifying precursors” derived from the stem cells and neuroblasts which generate new neurons for the olfactory glomeruli. This niche also contains endothelial cell/pericyte-derived blood vessels which are contacted by the endfeet of astrocytes and a large variety of interactions between these cells types have been implicated in regulating the behaviors of NSPCs within the niche (see Goldberg and Hirschi, 2009; Kriegstein and Alvarez-Buylla, 2009; Lathia et al., 2007). Like the adult SVZ, the subgranular zone (SGZ) of the hippocampal dentate gyrus is a neurogenic niche comprised of multiple cell types. NSPCs in the SGZ divide only occasionally and produce

immature granule neurons through an intermediate progenitor stage. Other cells within this niche include astrocytes, mature granule cell layer neurons, and the endothelial cells and pericytes of nearby blood vessels (Palmer et al., 2000). SGZ NSPCs may also produce glial cells although it is unclear under what conditions they do so. Neurons born in the SGZ migrate within the granule cell layers until they reach a final position and integrate into hippocampal circuits (Seri et al., 2004). Both SGZ neurogenesis and the integration of these new neurons in to the hippocampus are required for certain forms of learning and memory (for an extensive review see (Deng et al., 2010).

Another proposed NSC niche in the CNS is the central canal of the spinal cord. Extensive characterization of the central canal has lagged behind that of the SVZ and SGZ but recent reports indicate that the organization of the central canal shares many of the key features of the SVZ and SGZ including GFAP-positive progenitors and high expression of neural stem cell/progenitor markers such as vimentin, nestin and SOX2 (Hamilton et al., 2009; Sabourin et al., 2009). However, in contrast to the SVZ and SGZ, the central canal lacks a well-defined subependymal layer of cells. Proliferating cells are primarily observed in the dorsal portion of the ependymal layer rather than in an adjacent subependymal layer (Hamilton et al., 2009). Committed progenitors expressing OPC, astrocyte and neuronal markers are also found adjacent to the ependymal layer but appear to arise directly from ependymal cells rather than from a transient amplifying progenitor population (Hamilton et al., 2009; Sabourin et al., 2009). There is also speculation that these committed progenitors influence the behavior of the ependymal stem cells directly.

Although cell intrinsic (e.g. epigenetic changes) and growth factor-related cues have been implicated in the maintenance and the differentiation of NSPCs, both cellular and extracellular elements within NSPCs microenvironments are critical for regulating NSPC behavior both during embryogenesis and in adults (Marthiens et al., 2010). It is thought that a highly specialized ECM is one of the defining features of stem cell niches. The cell bodies and processes of NSPCs in the SVZ and SGZ are surrounded by a variety of ECM interacting molecules such laminin chains (Campos et al., 2004; Hunter et al., 1992; Lathia et al., 2007), the glycoprotein tenascin-C (Garcion et al., 2004), and a number of proteoglycans (von Holst et al., 2006). The complex ECM of the neural stem cell niche has been shown to direct the proliferation and differentiation of NSPCs. For example, mice lacking laminin γ1, integrin α6 or integrin β1 have a number of deficits including ectopic growth in the cortical marginal zone and retraction of radial glial cell basal endfeet (Beggs et al., 2003; Georges-Labouesse et al., 1998; Niewmierzycka et al., 2005). Interfering with b1 integrin in 'neurosphere' cultures, an *in vitro* model of NSPCs niches, using function-blocking antibodies or genetic ablation resulted in reduced MAPK activity, leading to compromised NSC maintenance (Campos et al., 2004).

HA is an established component of the hematopoietic stem cell niche where it is concentrated around hemopoietic stem cells (HSCs) in the endosteal region of bone marrow (Ellis et al., 2009). HA is thought to be a key molecule in the homing of transplanted HSC to bone marrow niches and it has been demonstrated that interfering with HA interactions in the bone marrow reduces HSC proliferation and granulocyte differentiation (Nilsson et al., 2003). HA may act to slow proliferation of stem or

progenitor cells in NSC niches as demonstrated by a recent report that shows mesenchymal stem cells have increased G0/G1 length when plated on HA coated coverslips leading to slowed proliferation (Liu et al., 2008). Furthermore, HAS activity, intracellular HA concentrations and RHAMM mRNA synthesis have been shown to fluctuate during mitosis (Evanko et al., 2004; Prehm, 1989; Sohr and Engeland, 2008). There are several reports linking HA and RHAMM to the formation of the mitotic spindle apparatus suggesting that intercellular binding of HA may directly affect mitosis (Evanko et al., 2004; Maxwell et al., 2003; Maxwell et al., 2008). HMW HA-CD44 interactions at the cell membrane have also been shown to organize mitotic spindle orientation and thereby influence asymmetrical versus symmetrical division, a process strongly linked to self-renewal or differentiation of NSPCs *in vivo* (Fujiwara et al., 2008). Collectively, these data suggest that HA may be serving as a quiescent signal in the ECM of the NSC niche where it inhibits proliferation and differentiation NSPCs.

HA localizes adjacent to GFAP positive B cells, the proposed neural stem cell (Ihrie and Alvarez-Buylla, 2008), in the SVZ (K.Feistel, unpublished data) and SGZ of the dentate gyrus (Su W., et al. submitted). When neural stem cells are isolated from the embryonic SVZ, SGZ or ganglionic eminences (GEs) and grown as free-floating neurospheres (see Figure 5.2), they contain both HA and hyaluronidases (as assayed by immunocytochemistry and RT-PCR, unpublished data). PH20 positive cells can also be seen in and around the SVZ of the adult mouse cortex (Figure 5.3, arrows) leading to the speculation that changes in HA catabolism (i.e. the upregulation of hyaluronidases to degrade extracellular HA) may be one mechanism used by NSCs to escape the niche.

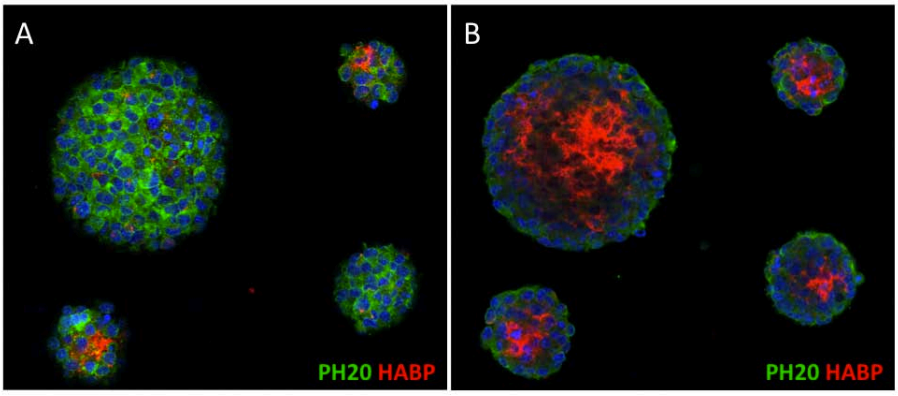


Figure 5.2. Ganglionic Eminence-derived "Neurospheres" contain both PH20 and HA. NSPCs were isolated from the GEs of e13.5 and grown as free-floating neurospheres. PH20 expression is localized to cells found on the outside of the spheres (green, A), while HA accumulates adjacent to the PH20 positive layer of cells and inside the core of the spheres (red, B). Images generated from merged confocal Z-stacks.

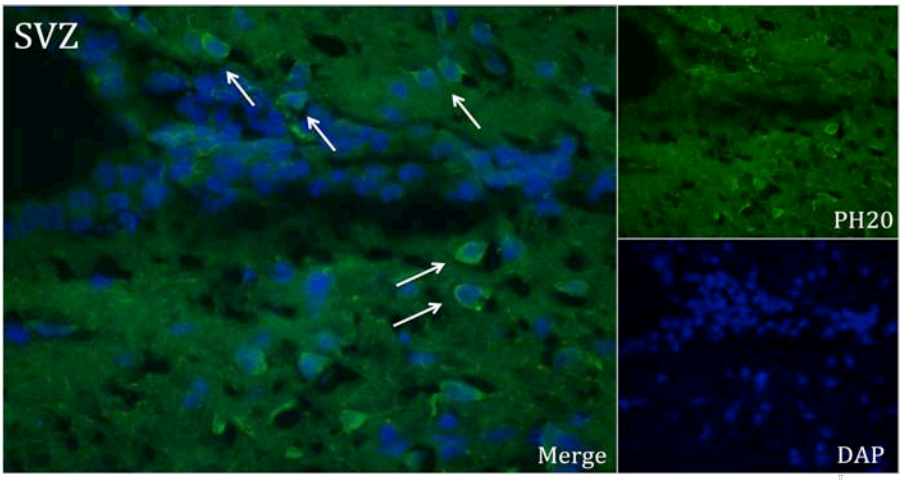


Figure 5.3 PH20 positive cells are seen in and adjacent to the SVZ in the adult mouse brain. 12uM sections of the adult cortex were fixed and stained with antibodies against PH20 (green) or DAPI (blue) to visualize cell nuclei. PH20 positive cells can be seen in and around the SVZ (arrows).

HA is also found adjacent to the ependymal layer of the central canal (M. Preston, unpublished data). Cells in the ependymal layer of the central canal are largely quiescent in the adult spinal cord (Sabourin et al., 2009; Sevc et al., 2011), yet following

injury it has been shown that ependymal cells generate astrocytes and OPCs for repair and remyelination (Attar et al., 2005; Cizkova et al., 2009; Matsumura et al., 2010; Mothe and Tator, 2005). The mechanisms underlying activation of the central canal are not known. Degradation of HA (via upregulation of PH20) may be one mechanism used to activate NSPCs in niches following demyelinating injuries. Consistent with this hypothesis, PH20 expression in the normal (non-pathological) central canal is largely absent (Figure 5.4, a), and only rarely are PH20 positive cells seen within or near the ependymal layer (Figure 5.4. a, arrows). However, PH20 expression appears to be elevated in the lumen of the central canal in mice with EAE (as assayed by total immunofluorescence, Figure 5.4, b-c) and numerous PH20 positive cells can be found adjacent to the ependymal cell layer (Figure 5.4, b-c, arrows).

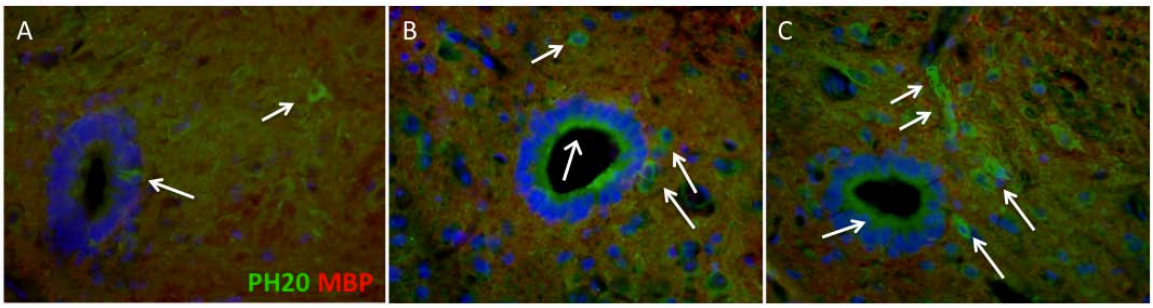


Figure 5.4 PH20 immunoreactivity is elevated in the central canal of EAE mice. 10uM sections of lumbar spinal cord of either wild type (naïve, control, A) or EAE (day 21 post inoculation, B,C) mice were stained with PH20 (green), MBP(red) and Dapi (blue). In naïve (wild type) mice only very rarely were PH20 positive cells observed within or adjacent to the ependymal layer of the central canal (A. arrows). However, Increased PH20 immunoreactivity was seen in the central canal of mice with EAE and numerous PH20 positive cells were also observed adjacent to the central canal (B,C. arrows).

While it is still necessary to establish which cell types (i.e. ependymal, GFAP positive B cells or committed progenitors) express PH20, it is tempting to speculate that increased PH20 expression in this niche mediates NSPC proliferation and differentiation and

contributes to the recruitment of progenitors for repair. It will be interesting to see if elevated PH20 expression in the central canal correlates to the proximity of demyelinated lesions in mice with EAE or other CNS insults such as SCI. Similarly, it remains to be seen if increasing PH20 expression in the central canal and other NSPC niches induces the *de novo* generation of committed progenitors such as OPCs and astrocytes. Future experiments, such as the injection of BTH into the SVZ combined with BrdU delivery or the pharmacological inhibition of hyaluronidase activity by VCPAL will be necessary to establish how HA synthesis and catabolism drive NSPC quiescence, proliferation and differentiation in these neurogenic niches.

HA degradation increases the proliferation of neural cells.

In addition to being highly enriched in neural stem cell niches, HA is also widely distributed throughout the ECM of the developing rodent brain including the cortex, cerebellum, striatum, and subpallidian structures (Bignami and Asher, 1992; Yasuhara et al., 1994). Hydration of the HA-rich ECM of the developing CNS may create a 'loose' cell-free matrix to promote neural precursor migration. Consistent with this hypothesis, 90% of HA is associated with water in the developing brain and is expressed in a diffuse global pattern when many cells are moving both tangentially and radially into the rapidly expanding cerebrum (Margolis et al., 1975). Total HA content of the developing CNS declines to approximately 25% of embryonic HA levels by 2 weeks after birth in the rodent (Margolis et al., 1975) and becomes re-organized into dense nets in the corpus callosum and cerebellum, both sites of extensive postnatal progenitor migration (Baier

et al., 2007; Delpech et al., 1989; Kappler et al., 2009). It is unknown if such an extensive reorganization of HA in the ECM of the CNS is mediated by decreased HA synthesis or increased HA degradation by neural progenitors; however, it is becoming clear that HA degradation induces the differentiation of NSPCs and proliferation of a wide variety of neural progenitors.

Hyaluronidase-induced degradation of HA, or the disruption of HA-CD44 interactions in the SGZ increases the generation and proliferation of immature neurons (Su W., et al. submitted); while viral enhanced expression of PH20 induces SVZ-derived NSPCs to take on the histological and morphological appearance of radial glia (K.Feistel, unpublished data). Taken together these observations support the hypothesis that altering HA dynamics can lead to the generation of committed progenitors from NSPC niches. Furthermore, enzymatic degradation of HA induces proliferation of O-2A (oligodendrocyte – type2 astrocyte) progenitors isolated from rat brain and in confluent primary rat astrocyte cultures (Marret et al., 1994). HA has also been shown to inhibit Schwann cell proliferation via CD44 and Merlin (Morrison et al., 2001). Finally, HA degradation has been shown to induce the proliferation of quiescent astrocytes and GFAP positive cells in the rat spinal cord (Struve et al., 2005). These data suggest that controlled degradation of HA may be required to allow for proper expansion of progenitor cells from proliferative niches during development and in response to nervous system damage.

Experimental evidence also indicates that PH20 plays a role in OPC proliferation, distinct

from its role in OL maturation. I find that enhanced expression of PH20 (either exogenously with BTH or via lentiviral-mediated enhanced expression of PH20) induces a robust proliferation of OPCs (as assayed by BrdU incorporation) (Figure 5.4). Furthermore, gain-of-function experiments show that only PH20, but not HYAL1-3 or HYAL5 (data not shown), increased OPC proliferation.

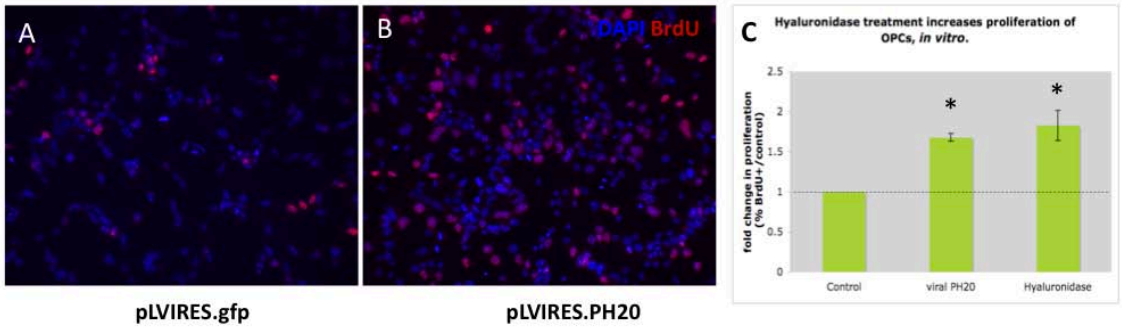


Figure 5.5 Enhanced PH20 expression drives proliferation of OPCs. BTH treatment increased proliferation of OPC (as assayed by incorporation of Bromodeoxyuridine, 5-bromo-2'-deoxyuridine, BrdU) by 1.82 fold as compared to PBS treated groups (quantified in Figure 5.3, c). To confirm that PH20 is the primary hyaluronidase responsible for BTH-induced proliferation, OPCs were infected with lentivirus containing EGFP (control Figure A), HYAL1.EGFP, HYAL2.EGFP, HYAL5.EGFP (data not shown) and PH20.EGFP (Figure B). Consistent with the observation that BTH, which is primarily composed of PH20, induces OPC proliferation, viral over expression of PH20 in OPCs induced a 1.67 fold increase in cell proliferation while over expression of HYAL1-3 and HYAL5 had no significant effect (data not shown).

Therefore, PH20 activity may also play a critical role in the expansion of adult OPC populations to generate a sufficient number of progenitors for replacement of damaged myelin. It is likely that OPCs must exit the cell cycle to initiate myelination programs (Sun et al., 2011; Swiss et al., 2011; Tanner et al., 2011) particularly during remyelination (Caillava et al., 2011; Simon et al., 2011). Given the hypothesized role of HA-RHAMM interactions in mitosis (see above), PH20 expression in adult OPCs may be beneficial to induce proliferation of cells in neurogenic niches but becomes detrimental to OL

maturation by altering cell cycle dynamics in OPCs once they enter the HA-dense glial scar. This hypothesis has yet to be tested. Given that remyelination is dependent on the generation, proliferation, and maturation of OPCs, understanding how HA dynamics drive lineage commitment and oligodendrogliogenesis is critical for developing therapeutic interventions to promote the repair of damaged myelin.

THESIS SUMMARY

The work described in this thesis adds to a large body of research showing that the generation of specialized structures in the body depends not only on cell-intrinsic mechanisms, but also the extensive interaction of cells with molecules in their extracellular environment. HA is a major constituent of the neural ECM during development and is secreted in large volumes following CNS injuries. The HA-rich ECM likely provides both structural shape and mechanical support to the developing and adult nervous system; as well as, acts as core scaffolding for the incorporation of a variety of ECM molecules, growth factors, and signaling molecules known to influence cellular growth and homeostasis. Large molecules of HA are capable of hydration, cushioning and facilitation of cellular migration, as well as preventing the spread of inflammation in the glial scar. HA has been found to signal directly through a variety of receptors, based on size and presentation, and to influence a diverse collection of cellular behaviors including progenitor proliferation, differentiation, migration, and maturation. A more detailed analysis of HA synthesis, degradation, and signaling in the brain and spinal cord is needed to understand how such a simple molecule is able to

modulate such a large variety of complex signaling cascades and generate a plethora of cellular responses.

Collectively, the data presented in this thesis supports a model in which the matrix of NSC niches is enriched with HA, where HA maintains NSC quiescence and prevents their differentiation. Following injury, neurogenic niches are activated and NSPCs upregulate PH20, and possibly other hyaluronidases, to escape the HA-rich niche. Degradation of HA relieves extracellular cues that inhibit the proliferation and differentiation of progenitor cells and/or generates distinct HA products that promote proliferation, differentiation and possibly migration through a variety of HA receptors. If these cells later encounter a HA-rich matrix at a site of CNS injury, their differentiation and maturation are again inhibited, preventing them from contributing to nervous system repair. Identification of the PH20 hyaluronidase in demyelinated lesions and characterization of its role in OPC proliferation and OL maturation highlights the notion that the controlled degradation of HA may be required for proper generation, proliferation, and maturation of OPCs, and other progenitors, needed for repair of the damaged nervous system. A better understanding how HA dynamics in the lesion environment drive lineage commitment, OL genesis, and myelin synthesis is critical for developing therapeutic interventions to promote the repair of damaged myelin, to protect axons and to maintain and restore neurological function following demyelinating insult.

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