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ROLES OF FERRITIN RECEPTOR AND ITS LIGANDS IN OLIGODENDROCYTES:

IMPLICATIONS FOR OLIGODENDROCYTE DEVELOPMENT AND SURVIVAL IN DISORDERS OF MYELINATION

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by

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ABSTRACT

Oligodendrocyte progenitor cells (OPCs) originate in the subventricular zone (SVZ) and populate the developing brain, proliferating and differentiating into myelin producing oligodendrocytes in early post-natal period. In adult brain, OPCs are the principal cell type involved in myelin repair and remyelination. Therefore, understanding factors that govern development and survival of oligodendrocytes is important not only for a complete understanding of normal brain development, but also may aid in elucidating pathogenesis and identify therapeutic targets of dysmyelinating disorders. Hence, the purpose of this thesis project was the identification of Tim-2 protein as novel cellular receptor for the uptake of extracellular H-ferritin in developing oligodendrocytes, and characterization of how this molecular system may play a physiologic role in iron acquisition and pathologic relevance in oligodendrocyte dysfunction and destruction in two demyelinating disorders: perinatal vanadium toxicity and multiple sclerosis.

In support of the thesis, we demonstrate that Tim-2 is expressed on cells of oligodendroglial lineage in rodent Central Nervous System (CNS) both in vivo and in vitro, that the pattern of Tim-2 expression overlaps with pattern of H-ferritin binding in oligodendrocytes, and that Tim-2 expression can be regulated by iron in the oligodendrocyte CG4 line. Blocking antibodies to the extracellular domain of Tim-2 nearly completely abate specific binding of H-ferritin to primary OPCs and CG4 oligodendrocyte cell line, suggesting that binding and uptake of H-ferritin in oligodendrocytes is dependent on Tim-2. In vitro, we demonstrate that H-ferritin stimulates differentiation of OPCs, and can replace iron and support growth and survival
of OPCs in the absence of transferrin, which was previously considered to be an essential growth factor of oligodendrocytes. Collectively, this part of the thesis suggests that transferrin and H-ferritin, with their respective receptors, are complementary and alternative iron acquisition systems in oligodendrocytes. Because the H-ferritin delivered iron in oligodendrocytes can be red-ox active, and because of intrinsic vulnerability of OPCs to oxidative stress, we evaluated the contribution of the H-ferritin iron delivery pathway to vanadium toxicity, because the chief neurological consequence of early postnatal exposure to this metal is hypomyelination. We show that vanadium exposure in the early post-natal period results in selective destruction of OPCs in vivo and in vitro, an effect which is accompanied by cytotoxicity, oxidative stress and increase of apoptotic indices of OPCs. Vanadium cytotoxicity on OPCs was increased by iron and H-ferritin treatment, and was decreased by the iron chelator desferroxamine (DFO), suggesting an interaction between H-ferritin-delivered iron and vanadium. Using a supercoiled DNA relaxation assay, we demonstrate iron-dependent synergistic effect of vanadium and ferritin on DNA nicking, which suggests that vanadium interaction with ferritin may promote unregulated release of red-ox active iron with consequent oxidative stress and apoptosis. Lastly, we demonstrate that the other reported ligand of Tim-2 in the immune system, class IV semaphorin Sema4A, is overexpressed within active MS plaques as compared to normal white matter. Sema4A primarily immunolocalized within infiltrating lymphocytes and activated microglia within the plaques. We demonstrate that recombinant Sema4A-Fc chimera collapses processes and causes apoptosis of oligodendrocyte progenitors in culture, which carries significant implications for pathogenesis of remyelination failure within MS plaque. We also found that Sema4A
preferentially binds to OPCs over astrocytes, but we find no evidence of Sema4A binding dependence on Tim-2, suggesting that Sema4A acts on oligodendrocytes through an alternative receptor, most likely of the Plexin family.

In summary, we demonstrate a novel molecular pathway for iron assimilation involving H-ferritin and its receptor Tim-2, which appears to play important physiological and pathological role developing oligodendrocytes. Identifying regulatory mechanisms of this pathway as well as human orthologue of this pathway are important future research directions.
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LIST OF ABBREVIATIONS

OLGcs (oligodendrocytes)
OPCs (oligodendrocyte progenitor cells)
Tf (transferrin)
apoTf (apo-transferrin)
TfnR1 (transferrin receptor 1)
HF (H-ferritin)
rHF (recombinant H-ferritin)
FGF (fibroblast growth factor)
PDGF (platelet derived growth factor)
IGF-1 (insulin-like growth factor 1)
MBP (myelin basic protein)
MAG (myelin associated glycoprotein)
PLP (proteolipid protein)
CNPase (2',3'-cyclic nucleotide 3'-phosphodiesterase)
MOG (myelin oligodendrocyte glycoprotein)
PND (post-natal day)
O4 and O1 (markers of pro-oligodendrocytes)
A2B5 and NG2 (markers of oligodendrocyte progenitor cells)
ATP (adenosine triphosphate)
Tim-2 (T-cell immunoglobulin mucin domain 2)
Fe$^{+2}$ (ferrous iron)
Fe$^{+3}$ (ferric iron)
V (vanadium)
Olig2 (oligodendrocyte transcription factor, olig2)
Olig1 (oligodendrocyte transcription factor, olig1)
Sox10 (oligodendrocyte transcription factor, Sox10)
IRE (iron responsive element)
ROS (reactive oxygen species)
t-booh (tert-butyl hydroperoxide)
H$_2$O$_2$ (hydrogen peroxide)
BBB (blood brain barrier)
BRECs (bovine retinal epithelial cells)
SVZ (subventricular zone)
PVL (periventricular leukomalacia)
MS (multiple sclerosis)
EAE (experimental autoimmune encephalomyelitis)
NGS (Normal goat serum)
Min (minute)
Hr (hour)
PFA (paraformaldehyde)
HBSS-CM (Hank’s balanced salt solution supplemented with Mg and Ca)
% (percent v/v unless indicated otherwise)
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Chapter 1 (Introduction)

Oligodendrocytes and Myelination: the Role of Iron and Iron Transport Proteins

ABSTRACT:

Iron is an essential trophic factor that is required for oxygen consumption and ATP production. Thus iron plays a key role in vital cell functions. Although the brain has a relatively high rate of oxygen consumption as compared to other organs, oligodendrocytes are the principal cells in the CNS that stain for iron under normal conditions. The importance of iron in myelin production has been demonstrated by studies showing that decreased availability of iron in the diet is associated with hypomyelination. The timing of iron delivery to oligodendrocytes during development is also important because hypomyelination and the associated neurological sequelae in humans persist long after the systemic iron deficiency has been corrected. Therefore, identifying the molecular roles of iron in oligodendrocyte development and myelin production, and the mechanisms and timing of iron acquisition are important prerequisites to developing effective therapies for dysmyelinating disorders. The purpose of this introductory chapter is to provide a comprehensive overview of the available literature on role of iron in oligodendrocytes and the mechanisms of iron acquisition and intracellular homeostasis.
Oligodendrocytes (OLGs) are the glial cells in the CNS that produce myelin. A number of trophic factors have been identified that support oligodendrocyte development and myelinogenesis including FGF, PDGF, IGF-1, thyroid hormone, to name a few. The focus of this chapter is to review the current understanding of the role of iron in oligodendrocyte development and myelin production. Oligodendrocytes stain for iron more robustly than any other cell in the normal adult brain (Benkovic and Connor 1993; Connor and Menzies 1996; Connor et al. 1995). Iron-enriched oligodendrocytes appear in rows in the white matter (Figure 1.1). The cell body of oligodendrocytes is clearly visible following iron staining, but processes can also be detected (Figures 1.1, 1.3). Iron-enriched oligodendrocytes are found in lower animals forms such as chickens and flys (Erb et al. 1996). Thus, even animals with minimal myelin, oligodendrocytes accumulate iron. Indeed, in mammalian brains, in those areas identified as iron enriched (cerebellar nuclei, the substantia nigra, striatum), the principal cells that stain for iron are oligodendrocytes (Figure 1.2) (Benkovic and Connor 1993; Dwork et al. 1988; Hill 1989). These data suggest that iron accumulation by oligodendrocytes contributes more to the function of these cells than just metabolic support for myelination, but data in areas other than myelination are not available. Therefore this introductory chapter focuses on the relationship between iron and myelination. Because oligodendrocytes do not synthesize iron but must acquire it, the iron acquisition mechanisms will also be reviewed.

According to the World Health Organization, iron deficiency is the most common nutrient deficiency in the world. On a global scale, the economic consequences of iron deficiency are second only to tuberculosis (Connor 1994); (2001). The most common
neurological signs of iron deficiency include poor school performance, decreased
cognitive abilities, and behavior problems in children (Grantham-McGregor and Ani
2001; Oski and Honig 1978; Oski et al. 1983). Several studies have shown that most of
these clinical outcomes in iron-deficient children can be traced to hypomyelination
(Lozoff et al. 2006; Oski et al. 1983). Furthermore, these neurological sequelae persist
even after iron supplementation (Beard 2007; Lozoff et al. 2006; Lozoff and Georgieff
2006). Thus, understanding the role of iron in myelination and iron acquisition
mechanisms by oligodendrocyte is important to developing effective intervention
strategies. It is therefore the purpose of this introduction to review the current literature
on the physiological role of iron in myelination and the mechanisms of iron import into
oligodendrocytes.

**Iron in the Normal Brain and Myelination**

In the brain, white matter stains more strongly for iron than does gray matter
(LeVine and Macklin 1990). The iron distribution in the white matter, although unique to
oligodendrocytes, is not homogeneous, but rather occurs in patches (Connor and Menzies
1996). The functional significance of the patchy iron staining (Figure 1.4, 1.5) is not
clear, but it suggests an epigenetic influence. Previous studies have shown that in the
developing brain, iron is first detected in oligodendrocytes proximal to blood vessels and
subsequently in oligodendrocytes farther from the blood vessels with the blood vessel
remaining near the center of the patch (Burdo, et al. 1999). During the second postnatal
week, the distribution of iron-positive oligodendrocytes colocalizes with the
myelinogenic foci, suggesting the functional relationship between iron accumulation and
myelin production (Connor and Menzies 1996). This observation is consistent with evidence that the highest period of iron uptake in the CNS coincides with the peak myelination (Connor and Menzies 1996; Taylor and Morgan 1990). At this time, immature, iron-positive oligodendrocyte progenitors are clearly visible (Figure 1.3). Functionally, the iron acquisition by oligodendrocytes at the peak of myelination is likely linked to their energy metabolism. At this stage of oligodendrocyte development, glucose is primarily metabolized via the pentose-phosphate shunt, which provides reducing equivalents (NADPH) for synthesis of myelin fatty acids and also requiring iron as cofactor for its key enzymes (i.e. glucose-6-phosphate dehydrogenase) (Cammer 1984). After peak myelination is passed, pentose-phosphate decreases to 25% of total cellular metabolism, which is also followed by decreased iron consumption and uptake (Cammer 1984; Taylor and Morgan 1990), but not by decreased iron staining or apparently iron content of oligodendrocytes. For example, oligodendrocytes synthesize transferrin, the iron mobilization protein, and the brain is the only organ in which transferrin mRNA increases postnatally (Bloch et al. 1985). This increase, as discussed later in this chapter, is dependent on oligodendrocytic maturation (Bartlett et al. 1991).

Clearly, iron is important for optimal oligodendrocyte function. This argument is further reinforced by observations that chronic severe iron deficiency during development leads to hypomyelination, which has been documented both in human and animal studies. In human studies, an increased latency of auditory brain stem potentials and visual-evoked potentials (indirect markers of myelination) have been reported in iron-deficient children (Roncagliolo et al. 1998); (Algarin et al. 2003). In several studies using the rat model of iron deficiency, restriction of dietary iron during gestation and the early post-
natal period resulted in a decrease in myelin proteins (MBP and PLP), lipids (galactolipids and phospholipids) and cholesterol (most significantly changed) in iron-deficient animals as compared to controls (Ortiz et al. 2004; Yu et al. 1986).

Interestingly, placing rats on an iron-restricted diet after the post-weaning period (P21-63) also produced a significant decrease in myelination indices (including MBP, phospholipids, CNPase and cytochrome oxidase activity) in the hindbrain and cerebrum (Beard et al. 2003). These data suggest that the oligodendrocytes of the adult brain are metabolically active and that iron deficiency was somehow able to compromise oligodendrocyte iron status resulting in their dysfunction and consequent hypomyelination. Continued brain iron accumulation with age, especially if neurons begin to acquire substantial amounts of iron, could be associated with oxidative stress resulting in neurodegeneration and subsequent myelin breakdown as observed in such as Alzheimer’s, Huntington’s and Parkinson’s disease (Connor, 2002; Bartzokis, et al, 2007a; Bartzokis, et al, 2007b; Bartzokis, et al., 2004; Todorich and Connor, 2004).

Thus, the delivery mechanisms and regulation of those mechanisms are critical for normal brain function throughout all stages of development. In reference to the various studies on myelin and iron deficiency, Larkin and Rao (1990) suggested that the common thread among animal studies of iron deficiency shows that even though globally decreased, the relative ratio of myelin components is in fact normal (Larkin 1990). These data further indicate that the effect of iron restriction is associated with a general decline of myelin production from a metabolic compromise and not a loss of one particular myelin component that is uniquely dependent on or regulated by iron. Overall, the iron deficiency effects on myelin production suggest that iron requirements for myelin are
related to metabolic processes underlying general myelin production rather than limited to lipid or protein biosynthesis.

To date, it is not clear whether iron deficiency leads to global brain hypomyelination by affecting oligodendrocyte number, or their differentiation state, or both. To begin to address this question, Morath and Mayer-Proschel (2001) showed that supplying increased iron concentrations in the form of ferric ammonium citrate to the media of glial restricted precursor cells *in vitro* (tripotential astro-oligodendrocytic precursors) isolated from E13 rat spinal cord resulted in an increased generation of GalC+ oligodendrocytes in Fe-treated media compared to controls (Morath and Mayer-Proschel 2001). These data showed that iron availability may result in an accelerated differentiation of GRPs into oligodendrocytes. On the other hand, increasing iron availability in O2A oligodendrocyte progenitors (OPCs) altered oligodendrocyte proliferation without stimulating them to differentiate in their model (Morath and Mayer-Proschel 2001). Although these data clearly show that iron can modify oligodendrocyte proliferation and differentiation, they illustrate that the timing of iron delivery is critical for its effects on myelination.

So, what do we know about the molecular mechanisms by which decreased iron availability in oligodendrocytes leads to decreased myelin? The best documented biological consequences of iron deficiency are post-translational effects on the iron-requiring proteins involved in energy metabolism and myelin synthesis (Beard JL 1996). Iron is needed as cofactor for cytochromes a, b and c, including the Fe-sulfur complexes of the oxidative chain and hence is indispensable for ATP production (Glinka 1999). It has been reported that oligodendrocytes are the most metabolically active cells in the
brain, supporting myelin membranes that can exceed 100x the weight of an average oligodendrocyte cell (Cammer 1984). Consequently, oligodendrocytes must generate a relatively high supply of ATP, which could explain their sensitivity to iron deprivation. Many of the enzymes involved in ATP production, cholesterol and fatty acid synthesis, which are precursors of myelin are iron-dependent. These enzymes include HMG-CoA reductase, squalene epoxidase, succinic dehydrogenase, NADH dehydrogenase, dioxygenase and glucose-6-phosphate dehydrogenase. Most of the enzymes are enriched in oligodendrocytes as compared to other cells in CNS (Connor and Menzies 1996; Pinero 2000). Lipid saturase and desaturase enzymes, which are involved in increasing and decreasing number of double bonds in fatty acids of lipids also have iron as a cofactor (Lange and Que 1998). Iron deficiency leads to impaired function and degradation of these iron-dependent proteins, which then impairs energy production leading to a decrease in myelin production (2001). Several studies reinforce, albeit indirectly, the molecular connection between iron and myelin. In humans, tellurium causes peripheral neuropathy by inhibiting squalene epoxidase, a key enzyme in the synthesis of cholesterol that requires Fe as cofactor (Wagner-Recio et al. 1991). The mechanism of that inhibition was determined to be tellurium mimicking iron at a key site of the enzyme and inhibiting its function (Harry et al. 1989; Wagner-Recio et al. 1991). In addition, exposure to ethanol during the development results in a number of pathological effects including a delay in myelination (Harris et al. 2000); (Ozer et al. 2000; Zoeller et al. 1994). Exposure to ethanol in utero results in iron deficiency in the brain. Specifically, pups of mothers administered ethanol during gestation had decreased amounts of iron, transferrin, and ferritin in cerebral cortex, subcortical forebrain, and
brainstem at different stages in development suggesting that ethanol produced a delay in iron acquisition by the brain (Miller et al. 1995). Ethanol exposure reportedly decreases transferrin synthesis in hepatocytes (Jeejeebhoy, et al., 1972) and could be expected to have a similar effect on oligodendrocytes. Therefore, the common thread in the various developmental models is that limiting iron availability to oligodendrocytes results in adverse outcomes on oligodendrocyte function, development and myelination.

Iron transport proteins in the brain

Clearly, iron is essential for myelination. Thus understanding the mechanisms of iron import by oligodendrocytes is a necessary prerequisite for developing therapies for demyelinating disorders that are associated with iron dyshomeostasis. There are three main proteins that have been identified that deliver iron to the brain: transferrin, H-ferritin and lactoferrin (Leveugle et al. 1996). Transferrin and ferritin are discussed in detail because they appear to be most important for oligodendrocyte development.

a. Transferrin-- role in iron import and myelination

Transport of iron into the brain across the blood-brain barrier (BBB) is traditionally considered as mediated by transferrin, although other putative mechanisms of iron BBB transport have been described (Fisher et al. 2007). We have recently reviewed the mechanisms of brain iron transport (Connor 2002) and offered significant challenges to this long held belief that are beyond the scope of this chapter. In addition to the BBB transport, the choroid plexus secretes transferrin, which could
be an iron source for pro-oligodendrocytes (Malecki et al. 1999), but the transfer of iron from CSF to the brain parenchyma is reportedly limited (Moos and Morgan, 1998a; Moos and Morgan, 1998b). The latter study, however, investigated bulk flow and did not consider diffusion that follows the microvasculature in the Virchow-Robbins space or how delivery of a bolus of iron differs from the steady, slow secretion that likely occurs from the choroid plexus.

On the other hand, stoichiometrically most of transferrin in the CNS is produced by the oligodendroglial cells (OLGs), but this transferrin is reportedly not secreted (de Arriba Zerpa et al. 2000). The functional importance of the intracellular transferrin in oligodendrocytes is unclear. Nevertheless, the evidence for the importance of transferrin in oligodendrocyte development and myelination is compelling. Tf gene expression in the brain (Bloch et al. 1985); (Espinosa de los Monteros et al. 1994) is predominantly present in OLGs and the appearance of Tf in OLGs is key in the formation of myelin (Espinosa de los Monteros et al. 1989); (Connor et al. 1993). The brain is the only organ in which Tf mRNA expression increases after birth. This increase in expression is directly related to the maturation of oligodendrocytes (Bartlett et al. 1991). Transgenic mice overexpressing Tf (hypertransferrinemic mice) have increased levels of myelin components, including galactolipids, phospholipids, and proteins (CNPase, MBP, MAG) as compared to littermate controls (Saleh, et al., 2003). The hypermyelinating effect of transferrin overexpression had no effect on mouse locomotion, cognitive and emotional abilities, and electron microscopic analysis showed myelin of normal thickness and compaction (Sow et al. 2006). Using the same mouse model, Sow et al. (2006) showed that OPCs
cultured from the brains of these mice show accelerated differentiation (expression of O4 and PLP markers) as well as increased expression of Sox 10 and Olig1 mRNA and protein. These observations suggest that the overexpression of transferrin in oligodendrocytes in this model stimulates accelerated differentiation (i.e. earlier production of normal myelin), but not necessarily more myelin. The intracellular Tf may be important for mobilization of iron within the oligodendrocytes, although mice deficient in Tf (Hpx mice) still myelinate normally and their oligodendrocytes acquire iron (Beard et al. 2005; Ortiz et al. 2004).

In addition to the intracellular effects, the role of extracellular Tf in iron acquisition by oligodendrocytes has also been studied fairly extensively. Developing oligodendrocytes express receptors for Tf as shown by binding studies (Espinosa de los Monteros and Foucaud 1987) and by immunocytochemistry (Giometto et al. 1990); however, Tf receptor expression in OLGcs reportedly diminishes as the animal ages and is not detected in the white matter in adults in either rodents (Hulet, et al., 1999; 2002) or humans (Hulet et al. 1999b). Emerging evidence suggests there is an alternative (non-transferrin mediated) Fe import to oligodendrocytes involving secreted H-ferritin may replace transferrin iron delivery to developing oligodendrocytes and this system is also in humans (Espinosa-Jeffrey et al. 2002; Hulet et al. 1999a; Hulet et al. 2000; Hulet et al. 2002; Hulet et al. 1999b). This topic will be discussed later in this chapter.

Since 1994, numerous studies designed to define the role of extracellular transferrin as a trophic factor on myelin production have been performed by the Pasquini group. These investigators have found that the intracranial injection (ICI) of a single dose of Tf in 3-day-old rats produces an increase in the levels of the MBP and
CNPase mRNA and protein without affecting those of PLP (Escobar Cabrera et al. 1994; Escobar Cabrera et al. 1997). Subsequently, it was demonstrated that in the brain of rats receiving ICI with Tf there is an increase in the mRNA of tubulin and actin, as well as in various microtubule-associated proteins (MAPs) (Cabrera et al. 2000). These results appeared to indicate that Tf acts on pro-oligodendrocytes and induces their rapid differentiation. However, these effects occurred only when the animals were injected with transferrin at days 2-7 of age, suggesting that there was a “developmental temporal window” during which Tf was effective (Marta et al. 2000). In another study, run off transcription experiments showed that the MBP mRNA was significantly increased at the nuclear level, but that the PLP mRNA was unaffected. These results seemed to indicate that Tf selectively regulates MBP at the transcriptional level to enhance the maturation and myelinogenic properties of oligodendrocytes in myelin-deficient rats as well as in controls (Espinosa-Jeffrey et al. 2002). The mechanism by which Tf would result in upregulation of only MBP mRNA is not known and is presumably indirect.

In congruence with the biochemical studies, a morphologic evaluation of Tf-injected rats showed increased deposition of myelin in the optic nerves and the corpus callosum. Specifically, the ultrasturctural analysis showed that the intracranial treatment with Tf resulted in increased myelin decompaction, enlargement in the distance between adjacent major dense lines, a decreased density of the intraperiod line, and an increase in the electron density of the major dense line, accompanied by a significant increase in its width (Marta et al. 2003). Quite probably, the impaired myelin compaction found in both brain and optic nerves from Tf-treated rats is
directly related to the abnormal myelin composition that has been previously described in these animals (Escobar Cabrera et al. 1994). Myelin Basic Protein and PLP are fundamental ultrastructural constituents of the dense and intraperiod lines respectively, and observations showing that the electron density and the width of the major dense line was markedly increased, while the intraperiod line was difficult to observe, could be a direct consequence of the significantly higher MBP/PLP ratio present in the myelin membrane of Tf treated rats.

To determine whether neonatal intracranial injection of Tf has behavioral effects in rats, 3-day-old rats were intracranially injected with 350 ng of Tf and tested at 25 and 60 days of age. An anxiolytic-like behavior was observed in Tf-treated rats, evidenced by an increase in the exploration of open arms in the plus maze test without changes in the locomotor activity. Intraperitoneal injection of picrotoxin (GABA$_A$ receptor channel antagonist) abolished this anxiolytic-like behavior, indicating that neonatal Tf induces a long-lasting increase in GABA$_A$ receptor functionality (Viola et al. 2001). These data suggest that the Tf injections may have a direct neuronal effect in addition to the effect of these injections on myelin development.

Because the majority of Tf receptors are expressed on neurons, a direct neuronal effect is therefore not surprising and raised the possibility that some of the myelin effects could be secondary to neuronal activity. Therefore, the effects of Tf on OLGcs differentiation in *in vitro* experiments were also studied and yielded similar results to those obtained *in vivo*. When treated with increasing concentrations of Tf, OLGcs in culture developed a more multipolar morphology and showed an increased expression of MBP and myelin-associated glycoprotein (MAG) as well as enhanced
reactivity to O4 and O1 as compared to controls. Migration studies using the agarose drop assay showed that addition of Tf strongly inhibited oligodendrocyte precursor cell migration. This effect was abolished with an antibody against the transferrin receptor. These results suggest that Tf added in vitro to cultured OPCs inhibits first their migration and then enhances their differentiation (Paez et al. 2002). The differentiation process is mediated by the transferrin receptor (TfR1) and by different signal transduction molecules, with the participation of the cytoskeleton. Increase in cAMP and CREB phosphorylation occurs when Tf is added to OLGcs in culture and/or when transferrin is intracranially injected into newborn rats (Marta et al. 2002); (Garcia et al. 2003).

As is mentioned before in this chapter, Tf has been shown to increase the expression of different components of the myelin cytoskeleton. When OLGcs in culture are treated with Tf, this glycoprotein has a punctate distribution pattern along the OLGcs processes (Ortiz et al. 2005). Treatment of cultured oligodendrocytes with colchicine, cytochalasin, or taxol induced a displacement of the immunoreactivity of Tf towards the OLGcs soma. Analysis of the effects of Tf on the cell distribution of tyrosinated and detyrosinated tubulin and STOP (stable tubule only polypeptide), showed Tf promoted changes suggesting a stabilizing effect on the microtubules (MT) at the tip of the processes. Kinesin and dynein were found to colocalize with the Tf, suggesting that these two motors participate in the transport of the added glycoprotein. Moreover, after treatment with Tf, clathrin immunoreactivity was displaced from the OLGcs body toward the cell processes. The results indicated that Tf seems to be transported in clathrin-coated vesicles from the cell body to the tips of
the OLGcs processes where it promotes their stabilization. This mechanism may be of importance in the increased formation of the myelin membrane induced by Tf (Ortiz et al. 2005)

The mechanisms involved in the effect of Tf on OLGcs are complex and are not completely elucidated. In studies in which the progression of the cell cycle was analyzed in primary cultures of OPCs treated with Tf and/or with different combinations of mitogenic factors, Tf decreased the number of BrdU+ cells and increased the cell cycle time, while the number of cells in the S phase was decreased. The cell cycle inhibitors p27\(^{kip1}\), p21\(^{cip1}\) and p53 were increased, and in agreement with these results, the activity of the complexes involved in G1-S progression (cyclin D/CDK4, cyclin E/CDK2), was dramatically decreased. Transferrin also inhibited the mitogenic effects of PDGF and PDGF/IGF on OPCs, but did not affect their proliferation rate in the presence of bFGF, bFGF/PDGF or bFGF/IGF (Paez et al. 2006b). These results indicated that the inhibition of the progression of the cell cycle of OPCs by Tf, even in the presence of PDGF, leads to an early beginning of the differentiation program, as evaluated by different maturation markers (O4, GC and MBP) and by morphological criteria. The modulation by Tf of the response of OPCs to PDGF supports the idea that this glycoprotein might act as a key regulator of the OLGcs lineage progression (Paez et al. 2006b).

The studies on Tf suggest that Tf should be considered as a possible useful factor which either alone or in combination with other growth/ differentiation factors, could promote remyelination in demyelinating pathological processes. As an example of this concept, Adamo, et al. (2006) recently demonstrated that feeding Wistar rats a
diet containing cuprizone caused histological and biochemical evidence of marked demyelination. Treatment of these animals with a single intracranial injection of Tf (350 ng) at the time of cuprizone withdrawal was associated with a marked increase in myelin deposition and a significant improvement in remyelination as compared to spontaneous recovery. Immunocytochemical studies of the oligodendroglial cell population at the time of cuprizone termination and at different times thereafter showed that there was a marked increase in the number of NG2-BrdU-positive precursor cells together with a marked decrease in MBP expression at the peak of cuprizone-induced demyelination. The amount of precursor cells decreased markedly during spontaneous remyelination and was accompanied by an increase in MBP reactivity. In the Tf-treated animals, these phenomena occurred much faster, and remyelination was much more efficient than in the untreated controls.

A critical question in the Tf studies is whether or not the Tf has acquired iron once it is injected \textit{in vivo} or from the cell culture media. The presence of iron is ubiquitous and compounded with the high affinity of Tf for iron is (association constant of $10^{22}$ M$^{-1}$) suggest that even small concentrations of iron would rapidly bind to injected Tf (Aisen and Listowski, 1980). However, most of the soluble iron in any biological system is in the ferrous form, and would have to be converted to ferric iron before it would bind to Tf. Regardless, in the last five decades of transferrin-TfR1 literature, to the best of our knowledge a single report convincingly demonstrating functional role of transferrin independent of iron delivery does not exist. Therefore, it is likely that the observed pro-myelinating effects of injected Tf \textit{in vivo} and \textit{in vitro} are due to increased iron availability to developing oligodendrocytes.
To determine if the Tf injections were effective in an iron-deficient environment in the brain, Tf was injected intracranially to study the possibility of reversing the hypomyelination processes produced by iron deprivation. At P24, all of the myelin components were markedly diminished in the iron deficient (ID) rats and the ICI of Tf in this case was only able to partially correct the effects of iron deprivation, since with the exception of proteins, none of the various constituents studied reached normal values. These data suggest that adequate iron in the brain, at least at some point during the intervention, are required for the full Tf effect.

Many factors could impact the results of the intracranial injections on the iron deficient rats. Therefore, OLGcs were isolated from control and ID animals. The cultures of OLGcs isolated from ID rats had fewer differentiated cells relative to controls as measured by the number of MBP-positive cells (from 39% to 17%) and compared with the undifferentiated PSA-NCAM positive cells (From 5% to 8%) (Badaracco et al. 2008). When OLGcs isolated from ID rats in culture were treated with Tf, there was an increase in the number of MBP (differentiated) positive cells (from 16.5% to 32%) (Badaracco et al. 2008). On the other hand, the number of PSA-NCAM (undifferentiated) positive cells dropped from 8% in the ID-OLGcs to 4% in the OLGcs isolated from ID rats that were treated with Tf. The in vitro treatment with Tf produced a correction in the differentiation of OLGcs isolated from ID rats consistent with in vivo experiments (Badaracco M.E in press; Badaracco et al. 2008; Ortiz et al. 2005).

Despite the apparent importance of transferrin for oligodendrocytes, mature oligodendrocytes in the adult white matter express much lower levels of transferrin receptor 1 than do other parts of the brain including cortex. Several studies have
independently failed to detect Tf receptor 1 (TfR1) in white matter tracts of adult animals with different technical approaches. Immunohistochemical studies assessing distribution of transferrin receptor 1 in the brain have shown strong staining of cortex and the neurons, but found at most faint staining of Tf receptors in the mature oligodendrocytes (Connor and Menzies 1995; Giometto et al. 1990). In-situ hybridization detected abundant transcripts of Tf receptor 1 mRNA in adult mouse cortex, but low levels in the white matter tracts of adult mouse brain (Han et al. 2003). The levels of transferrin receptor 1 mRNA in white matter did not increase even in conditions of severe iron deficiency (Han et al. 2003). Radiolabeled transferrin binds gray matter, but only minimally to the white matter tracts in adult mouse, rat and human brains (Mash et al. 1990) (Hill et al. 1985; Hulet et al. 1999a). In vitro oligodendrocyte progenitors express transferrin receptors, but if they are allowed to differentiate into mature oligodendrocytes, transferrin receptor protein expression decreases as detected by Western blot analysis (unpublished data). These data are consistent with studies that have shown that indices of myelination (MBP, CNPase activity, PLP) can be increased by injections of apotransferrin into mice at P3, but not at P20 (Escobar Cabrera et al. 1994; Marta et al. 2000). Therefore, it appears that there is a “critical temporal window” in the mouse brain development in which oligodendrocytes are responsive to apotransferrin injections that can be explained by presence of transferrin receptors on early OPCs. When oligodendrocytes reach maturation at P20, their downregulation of transferrin receptor makes them no longer responsive to exogenous transferrin (Hulet et al. 2002).

Despite their lack of transferrin receptor, mature oligodendrocytes continue to remain the principal cell in the brain that stain for iron (Benkovic and Connor 1993).
Beard et al. (2003) suggested that there is a continued need for iron uptake given that iron deficiency in the adult can lead to decrease in myelin indices. However, *in vivo* iron uptake data have provided a more direct evidence, suggesting that after iron import into brain peaks in second post-natal week (P15) in rat, it subsequently decreases as animal ages and is maintained at very low levels into adulthood (Taylor and Morgan 1990). These findings would suggest that majority (if not all) of the iron found within oligodendroglia in adult brain may have been acquired early at peak myelination, in the first postnatal month in rodents (an equivalent of first two postnatal years in humans).

Despite a lot of work in this field, the developmental time at which oligodendrocytes cease to have a requirement for iron is still not clear and should be further investigated.

Despite having transferrin levels of less than 1% of normal controls in the brain, hypotransferrinemic (Hpx) mice are not hypomyelinated (Dickinson and Connor 1995; Dickinson and Connor 1998). Paradoxically, these mice even have higher than normal levels of iron in the myelin (Ortiz et al. 2004). Because these mice require transferrin injections for survival; they cannot be used to argue that transferrin is not required for iron delivery to oligodendrocytes. Indeed the human transferrin used to replace the transferrin in these mice can be detected in the brain and in oligodendrocytes (Dickinson and Connor 1995). Nonetheless, the work on iron transport with the Hpx mice convincingly demonstrates non-transferrin-mediated iron uptake in the brain and uptake of iron into the oligodendrocytes, which continues in the absence of transferrin (Takeda et al. 1998). The observation that in oligodendrocytes from mixed glial cultures in media lacking transferrin (both normal and oligodendrocytes isolated from hypotransferrinemic mice) actually *increased* internalization of Fe-59 (Takeda et al. 1998) have prompted us
to consider alternatives to transferrin for delivery of iron into oligodendrocytes. We have proposed that this alternative iron transport to oligodendrocytes involved H-ferritin.

b. Ferritin—role in iron storage, transport and oligodendrocyte function

Ferritin is a high-capacity iron storage protein; theoretically capable of binding over 4500 iron atoms (Harrison and Arosio 1996). Ferritin has been classically thought of as a cytosolic iron buffer because of its ability to sequester excess iron thereby preventing iron-mediated, free radical toxicity. Existence of extracellular ferritin suggested that the function of this protein extends beyond its role as an intracellular iron buffer, and may provide the opportunity for ferritin to function as an iron delivery protein. Ferritin is a 24-subunit protein, composed of heavy (H) and light (L) chains which can associate to form either homo- or heteromers, the ratio of which is tissue and cell type-specific (Harrison and Arosio 1996; Harrison 1996). Although the brain has twice the amount of H-ferritin than L-ferritin, the exact ratio of H- and L-ferritins is dependent on the specific region of the brain, the age of the animal and specific cell type in question (Connor and Menzies 1996). For example, neurons express mainly H-ferritin, while microglia express mainly L-ferritin. Oligodendrocytes express a mixture of both L- and H-ferritins (Blissman et al. 1996; Connor and Menzies 1996). The distribution of ferritin subunits in these cells is consistent with their function and pattern of iron utilization. L-ferritin is the predominant type of ferritin in microglia, and is associated with iron storage, while neurons and oligodendrocytes, which are high in H-ferritin have a high metabolic rate and a high iron turnover (Connor and Menzies 1996). Blissman et al. (1996) showed that in the
developing pig brain, the accumulation of iron coincides with the appearance of immunostainable H-ferritin in CNPase-positive cells, a standard marker of mature oligodendrocytes. Even though only a subset of all CNPase-positive cells were also Fe/H-ferritin positive, only Fe/H-ferritin positive cells expressed MBP (Blissman et al. 1996). This latter in vivo study and a cell culture study by Sanyal et al. (1996) were the first to suggest that accumulation of H-ferritin in oligodendrocytes might be an important prerequisite for myelination.

The importance of H-ferritin for oligodendrocyte function was also explored using an H-ferritin knockout mouse model. The null mutation for H-ferritin is embryonically lethal between 3.5 and 10.5 days post-fertilization (Ferreira et al. 2000; Ferreira et al. 2001). The H-ferritin heterozygote (+/-) mice have approximately 20% of the normal levels of H-ferritin in the brain accompanied by significant decreases in galactolipids, cholesterol and phospholipids, and PLP in their myelin compared to +/+ controls (Ortiz et al. 2004). Therefore, the myelin profile in the H-ferritin-deficient mice is similar to the dietary iron deficiency models. The brain iron concentration of these animals is normal, however, and iron staining in the oligodendrocytes could be demonstrated, although the amount of iron is not known. At present, it is not known whether hypomyelination associated with H-ferritin knockdown in this mouse model is due to a compromised ability to handle the intracellular iron (oligodendrocytes express endogenous cytosolic H-ferritin) or due to decreased iron delivery to oligodendrocytes by suboptimal levels of extracellular H-ferritin.
The case for an H-ferritin receptor on oligodendrocytes:

In 1974 while studying liver uptake of injected Fe-59 in rats, Unger and Hershko first hinted at the possibility of existence of a plasma membrane receptor for ferritin in liver. Since then, ferritin receptors have been reported in binding studies in a variety of cell types including rat (Mack et al. 1985), pig (Adams et al. 1988a) and human hepatocytes (Adams et al. 1988b), guinea pig reticulocytes (Blight and Morgan 1987), Molt4 T lymphoid cells (Moss et al. 1992), K562 erythroleukemia cell line (Fargion et al. 1988), HL-60 promyelocytic cell line (Covell and Cook 1988), T lymphocytes (Fargion et al. 1991) and erythroid precursors (Gelvan et al. 1996). In a series of binding experiments, Hulet et al. showed that saturable and specific H-ferritin receptor(s) existed in white matter tracts of the rodent and human brain and demonstrated in cell culture studies that ferritin receptors were unique to oligodendrocytes (Hulet et al. 1999a; Hulet et al. 2000; Hulet et al. 2002; Hulet et al. 1999b). Developmentally, H-ferritin binding followed the spatial and temporal sequence associated with myelinogenesis (Hulet et al. 2002). The binding constant (Kd) for H-ferritin in the brain and on oligodendrocytes is 4.65 nM, comparable to those in non-CNS tissue suggesting that the putative receptor in the brain may be the same protein (Hulet et al. 1999a). These results suggest that at least two alternative iron delivery mechanisms may exist in the adult brain: transferrin and H-ferritin.

The possibility that H-ferritin could play a significant role as an iron delivery protein in the brain and specific to oligodendrocytes represents a paradigm shift from the traditional thinking that iron delivery to cells is exclusively transferrin mediated. A critical question that required immediate attention was to find the source of H-ferritin.
Currently, two potential sources have been identified (Figure 1.6). One potential source is direct transport of H-ferritin across the BBB (Fisher et al. 2007). The second potential source is from microglia (Zhang et al. 2006). Even before myelination begins, microglia begin to accumulate iron in white matter (Cheepsunthorn et al. 1998; Connor and Menzies 1996; Zhang et al. 2006). Indeed, the onset of microglial staining for iron and ferritin during development precedes that of oligodendrocytes (Cheepsunthorn et al. 1998) and ameboid microglia stain strongly for Tf receptors in corpus callosum of 1-10 day old rats, just prior to peak myelination (Kaur and Ling, 1995). The suggestion that microglia express Tf receptors should be viewed with caution because this study did not control for Fc receptor binding of the secondary antibody by microglia, even though the findings are consistent with the observed relatively high levels of iron and ferritin in these cells prior to onset of myelination. When myelination begins, microglia decrease the amount of stainable iron, while iron-positive oligodendrocytes and iron staining in the newly synthesized myelin begin to appear and increase over time (Cheepsunthorn et al. 1998). Collectively, these findings suggest that in early postnatal period, microglia first accumulate iron, serving as a sort of an iron capacitor, and releasing it to developing OPCs during myelination when the oligodendrocyte requirement for iron is the highest (Figure 1.6). This concept was further explored in-vitro showing that media from iron-loaded primary rat microglia contained factors that promoted the survival of developing primary rat OPCs in culture (Zhang et al. 2006). Of the iron related proteins, H-ferritin, but not L-ferritin or transferrin, was elevated in the media from the iron loaded microglia. Transfection of these cells with siRNA directed to H-ferritin abolished the trophic effect of microglia conditioned media on OPCs, strongly suggesting that the trophic factor is H-
ferritin (Zhang et al. 2006). Collectively, these studies suggest that microglia play an important role in supporting oligodendrocyte development and that microglia may contribute iron for the myelination process, mainly in form of H-ferritin.

If extracellular H-ferritin was an important iron source for developing oligodendrocytes, then the other major question to be answered was the identity of the receptor for H-ferritin endocytosis in oligodendrocytes. The identity of H-ferritin receptor in any cells was unknown until recently when Chen et al. (2005) demonstrated that overexpression of Tim-2 (T cell immunoglobulin mucin domain 2) protein in a T-cell line confers these cells the ability to endocytose H-ferritin. Tim-2 is expressed in the brain on oligodendrocytes and that blocking Tim-2 abates H-ferritin binding to primary rat oligodendrocytes (Todorich et al. 2008c) (chapter 2).

In conclusion, there is compelling evidence that iron is essential for normal myelin production and iron accumulation by oligodendrocytes is an early event in the development of oligodendrocytes. Iron delivery to oligodendrocytes appears to be accomplished by a combination of at least two proteins: transferrin and H-ferritin (Figure 2.6). The role of H-ferritin and transferrin in both iron delivery and intracellular iron handling further emphasizes the importance of these proteins and iron in oligodendrocyte function. Although transferrin-transferrin receptor 1 molecular mechanism of iron delivery is well characterized, by comparison, very little is known about H-ferritin-mediated iron delivery, its receptor on oligodendrocytes, in-vivo significance for iron delivery and myelination, and its potential contribution to dysmyelinating disorders. It is therefore the purpose of this thesis to address some of those unanswered questions. In Chapter 2 we demonstrate that Tim-2 is expressed on cells of oligodendrocyte lineage
and is iron-regulated receptor for H-ferritin in oligodendrocytes. In Chapter 3, we demonstrate that recombinant H-ferritin supports growth and survival of oligodendrocyte progenitors, and prevents depletion of intracellular iron in absence of transferrin. In Chapter 4, we show that vanadium metal displaces iron from ferritin and that this process contributes to oxidative stress and apoptosis of oligodendrocyte progenitors, which may be one of the main contributing factors of developmental vanadium-induced hypomyelination. In Chapter 5, we show that the other reported ligand of Tim-2, Sema4A is overexpressed in multiple sclerotic plaques and causes process collapse and apoptosis of oligodendrocyte progenitors in culture, findings that have implications for pathogenesis of remyelination failure in multiple sclerosis.
FIGURES:

Figure 1.1. Perl’s staining of normal adult mouse brain is confined mainly to oligodendrocytes in white matter tracts. This representative micrograph of iron staining in corpus callosum from a normal mouse brain demonstrates labeling of small cells aligned in rows (black arrows), which is characteristic of oligodendrocytes. Additional oligodendrocytes are visible that are not aligned in rows (green arrows). These cells have an eccentric nucleus and very few processes typical of oligodendrocytes morphology. The reaction product is brown and appears in one pole of the cell because of the eccentric nucleus.
Figure 1.2. Iron staining in adult mouse striatum. Another brain region that stains heavily for iron with the Perl’s is the striatum. In this brain region, the cells are also small and round with relatively scant cytoplasm and processes and an eccentric nucleus. The morphology is again typical of oligodendrocytes and the iron-positive cells are primarily associated with the striasomes of the striatum although scattered cells are found throughout the striatum. The iron reaction product is brown. This is a standard Perl’s reaction that was enhanced by the treatment in 3,3’ diaminobenzidine (Connor and Benkovic, 1992; Hill and Switzer, 1984).
Figure 1.3. Iron staining of 15-day-old rat subcortical white matter. This representative micrograph illustrates iron accumulation by developing oligodendrocyte progenitors (arrows) with multiple processes in corpus callosum of P15 rat.
Figure 1.4. Perl’s staining in normal human white matter. Higher power (40X) micrograph demonstrating iron staining (Perl’s reaction) in oligodendrocytes (e.g. large arrow) of normal human white matter. Staining is confined predominately to the cytoplasm, which surrounds larger eccentrically positioned nucleus. Diffuse background staining is observed with small rings (e.g. small arrows) or beads which are oligodendrocyte processes.
Figure 1.5. Perl staining of normal human white matter is distributed in patches.

Low power (A) and high power (B) micrographs of 30-micron sliding microtome frozen sections of normal human white matter stained with the Perl’s reaction for iron and counter-stained with cresyl violet to see the cell distribution. Alternating darkly stained with very lightly stained areas gives the white matter tracts a patchy appearance. Oligodendrocytes are found within the non-iron staining patches. The functional and epigenetic issues associated with this staining pattern are not known, but it does appear that the patches of iron staining are associated with blood vessels. The robust white matter staining has been a challenge for many laboratories to obtain despite the reports that there is more iron in the white matter than corresponding gray matter (Rajan, et al., 1976; Curnes, et al., 1988). We have found the best way to obtain strong white matter staining in the brain is to (1) use tissue (including human) that has not been fixed for more than 24 hours (2) the thicker the section the better. The optimal sections are 30-50 um from a freezing microtome. We have almost never observed the patchy staining on paraffin sections or thin cryostat sections.
Figure 1.6. Schematic of current understanding of iron sources for developing oligodendrocytes in CNS. Transferrin delivers iron to oligodendrocyte progenitor cells (OPCs). The source of transferrin for these cells is not known but is thought to come across the blood-brain-barrier (BBB) from serum. The transferrin made by oligodendrocytes is reportedly not secreted (see text) and the only other source of transferrin in the brain is the choroid plexus. In parallel, H-ferritin with its iron is transported across BBB (Fisher et al. 2007) and microglia also release this protein (Zhang et al. 2006) and delivers iron both to oligodendrocytes at different differentiation stages (Hulet et al. 2000).
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Chapter 2

Tim-2 is the receptor for H-Ferritin on oligodendrocytes

ABSTRACT:
Oligodendrocytes stain more strongly for iron than any other cell type in the CNS, and they require iron for the production of myelin. For most cell types, transferrin is the major iron delivery protein, yet oligodendrocytes continue to uptake iron in the absence of transferrin. Thus, an alternative iron delivery mechanism must exist. Given the significant long-term consequences of developmental iron deficiency and the iron requirements for normal myelination, identification of the iron delivery mechanism for oligodendrocytes is important. Previously, we have reported that oligodendrocytes bind H-ferritin and that H-ferritin binds to white matter tracts in vivo. Recently, T cell immunoglobulin and mucin domain-containing protein-2 (Tim-2) was shown to bind and internalize H-ferritin. In the present study, we show that Tim-2 is expressed on oligodendrocytes both in vivo and in vitro. Further, the onset of saturable H-ferritin binding in CG4 oligodendrocyte cell line is accompanied by Tim-2 expression. Application of a blocking antibody to the extracellular domain of Tim-2 significantly reduces H-ferritin binding to the differentiated CG4 cells and primary oligodendrocytes. Tim-2 expression on CG4 cells is responsive to iron; decreasing with iron loading and increasing with iron chelation. Sequence homology comparison demonstrates that Tim-2 3′ UTR contains stretch of sequence 71% homologous to the consensus sequence of TfnR IRE, which may represent a site of post-transcriptional regulation of Tim-2 mRNA.
stability by iron. To evaluate the importance and function of Tim-2 gene \textit{in vivo}, we used a mouse line deficient in Tim-2. We demonstrate that Tim-2 knockout mice have a normal myelin profile, but a 20\% decrease in myelin iron content relative to wild-type controls. Taken together, our data provide compelling evidence that Tim-2 is the iron-regulated receptor for extracellular H-ferritin on oligodendrocytes, and that Tim-2/H-ferritin function as one of the iron import systems in oligodendrocytes.
INTRODUCTION:

According to WHO, iron deficiency is the most common nutrient deficiency in the world, and it is present in 25% of children under the age of 2 (2001); (DeMaeyer and Adiels-Tegman 1985). The neurological sequelae of chronic, severe, childhood iron deficiency include poor school performance, decreased cognitive abilities, and behavior problems (Beard and Connor 2003; Lozoff et al. 2006; Oski and Honig 1978; Oski et al. 1983), most of which persist following iron supplementation (Felt et al. 2006; Lozoff et al. 2006). Iron-deficient individuals demonstrate increased latency of auditory brain stem responses and evoked visual responses (Cankaya et al. 2003; Roncagliolo et al. 1998), both of which have been accepted as surrogate markers of myelin status. Thus, the majority of neurological problems associated with iron deficiency have been attributed to hypomyelination (Oski and Honig 1978; Oski et al. 1983). In rats, developmental iron deficiency in either the early postnatal period or later, after weaning, results in a decrease of myelin-related proteins (MBP, CNPase, PLP), as well as in fatty acids and cholesterol (Ortiz et al. 2005); (Beard et al. 2003; Larkin 1990). Oligodendrocytes are rich in iron, and are the principal cells in the brain that stain for iron (Benkovic and Connor 1993; Connor and Menzies 1995; Connor and Menzies 1996; Dwork et al. 1988). Further, enzymes required for myelin synthesis, which are enriched in oligodendrocytes, require iron as a co-factor (Connor and Menzies 1996), and peak iron uptake in the brain coincides with peak myelination (Crowe and Morgan 1992; Taylor and Morgan 1990). Therefore, understanding the mechanisms by which oligodendrocytes acquire iron is
critical to the overall picture of oligodendrocyte biology and may provide strategies for the treatment of dysmyelinating disorders.

Traditionally, studies of cellular iron import have focused on the transferrin (Tf)-transferrin receptor (TfR-1) system. However, the expression of Tf receptors decreases as oligodendrocytes mature and in adult brain white matter and mature oligodendrocytes is low (Hill et al. 1985; Hulet et al. 1999a; Hulet et al. 1999b) (Han et al. 2003). In a myelin-deficient rat model in which mature oligodendrocytes fail to thrive, the lack of oligodendrocyte survival in the adult has no effect on transferrin receptor expression (Roskams and Connor 1992). Furthermore, hypotransferrinemic mice (Hpx) do not have myelin defects, and in fact, have elevated iron concentrations in the myelin (Ortiz et al. 2004). Iron uptake into oligodendrocytes derived from Hpx mice continues in absence of transferrin in vitro and in vivo (Takeda et al. 1998), suggesting that non-transferrin-mediated iron uptake must exist in oligodendrocytes. Given the strong iron staining in mature oligodendrocytes (Connor and Menzies 1996; Connor et al. 1995) and the clear role for iron in myelination, this evidence collectively points to the existence of an iron uptake system for iron in oligodendrocytes that is independent of Tf.

Prior studies have demonstrated that oligodendrocytes as well as other cells express a receptor for ferritin, but the identity of this receptor has not previously been identified. Ferritin consists of H and L subunits that aggregate to form a 24-mer protein. Ferritin is traditionally considered to be an intracellular iron storage protein; However, ferritin is also present in serum. In humans and rodents, circulating ferritin is rich in L-ferritin, but the level of H-ferritin rises in inflammation (Harrison 1996). We previously demonstrated that H-ferritin binds to white matter tracts in human and rodent brains.
(Hulet et al. 1999a; Hulet et al. 2000; Hulet et al. 1999b) and that the spatial and temporal pattern of H-ferritin binding in the white matter of the CNS during mouse development coincided with the onset of myelination (Hulet et al. 2002). We also have demonstrated that uptake of FITC-tagged H-ferritin is limited to rat oligodendrocytes, but not astrocytes in primary rat mixed glial cultures (Hulet et al. 2000). Thus, there is compelling evidence that oligodendrocytes appear to selectively bind and internalize H-ferritin.

Recently, Chen et al. demonstrated that Tim-2 is an endocytosing receptor for H-ferritin in the immune system (Chen et al. 2005). Tim-2 is a type 1 transmembrane protein expressed in the immune system on B cells and on Th2-differentiated T-lymphocytes, but it is also expressed in liver and kidney cells and in the brain (Chakravarti et al. 2005; Chen et al. 2005). Tim-2 reportedly also binds to semaphorin 4A (Sema4A) in T cells (Kikutani and Kumanogoh 2003; Kumanogoh et al. 2002). Sema4A is a member of a large family of semaphorins important in CNS development, axonal steering and apoptosis. Functionally, the emerging consensus is that Tim-2 is a negative regulator of T cell proliferation (Knickerbein et al. 2006) (Rennert et al. 2006). Similarly, H-ferritin has also been reported to inhibit proliferation of lymphocytes and myeloid cells (Recalcati et al. 2008). Tim-2 has been implicated in the pathogenesis of EAE (Chakravarti et al. 2005; Kumanogoh et al. 2002), murine allergic conjunctivitis (Fukushima et al. 2007), and a mouse model of asthma (Rennert et al. 2006).

In this chapter, we demonstrate that Tim-2 is expressed on oligodendrocytes and evaluate its function as a cellular receptor for H-ferritin in these cells.
METHODS:

Immunostaining

For Tim-2 immunostaining of the brain, 6-month-old C57BL/6 mice were anesthetized with pentobarbital and perfused with cold Ringer’s lactate, and then fresh 4% paraformaldehyde (Sigma, St. Luis, MO). Brains were removed and kept in 4% paraformaldehyde overnight, followed by dehydration in 30% sucrose solution. The tissue was cut into 10 µm thick, sagittal, paraffin-embedded sections. For immunostaining, the sections were deparaffinized in xylene/EtOH with antigen retrieval in citrate buffer. Endogenous peroxide activity was blocked with 0.3% H2O2 for 5 min. After general blocking in 10% normal goat serum, tissue was incubated with purified rabbit antiserum raised to the C-terminal domain of Tim-2 (Chen et al. 2005) at 1:1000 dilution for 16 hr. The specificity of this antiserum for murine Tim-2 has been previously shown (Chen et al. 2005) and the epitope for this antibody is the last 16 residues of the intracellular domain of Tim-2 (DQVYIIEDEPTYPEEES). Tim-2 staining was visualized by using secondary anti-rabbit-AlexaFluor 488 (Invitrogen) at 1:150 dilution for 2 hr. Images were acquired on a Leica confocal microscope.

Western blots

For Tim-2 western blots of white matter homogenates, 6-month-old mice were euthanized by cervical dislocation. The brain was quickly removed, and bisected in the mid-sagittal plane. Subcortical white matter was manually dissected away from the neighboring cortex and other structures using a dissecting microscope. The white matter isolates were homogenized in RIPA buffer containing 0.5% v/v Triton X and
supplemented with mammalian protease inhibitor cocktail (Sigma) on ice for 30 min, followed by sonication. Total protein was determined by using a DC assay (Biorad), and 50 ug of total protein was separated by 12% SDS PAGE (Biorad). The proteins were transferred to a nitrocellulose membrane following standard procedures. The membrane was probed with anti-Tim-2 rabbit antiserum (Chen et al. 2005) at 1:250 dilution for 16 h at 4°C and visualized with HRP-conjugated secondary antibody using ECL reagent (Perkin Elmer).

Primary Rat Cultures

Primary rat oligodendrocyte progenitor cells (OPCs) were prepared from mixed glial cultures, obtained by the method of McCarthy and DeVellis (McCarthy and DeVellis 1980) from newborn rats. All animals were treated in accordance with Institutional Animal Care and Use Committee (IACUC) of Penn State Hershey. Briefly, 1-2 day old Sprague-Dawley rat pups were sacrificed by cervical dislocation and then decapitated. The brain was removed and placed in CMF-HBSS solution (Gibco). The meninges were surgically removed, and cells were separated by dicing the tissue and treating it with 0.25% trypsin-EDTA (Gibco) for 30 min. Following incubation in DNase for 5 min, the brain homogenate was passed through 150 and 75 um Nitex screens (SEFAR America, Depew, NY) and resuspended in DMEM/10% FBS. Cells were plated in poly-D-lysine (Sigma)-coated flasks. Cells were allowed to grow for 2 weeks, and on day 14 microglia were removed by shaking at 265 rpm for 1 h. Subsequently, the oligodendrocyte-enriched fraction was obtained by shaking the remainder of cells at 265 rpm for 18 hr. This latter fraction was plated on poly-D-lysine-coated slides and used for staining, or it was
distributed into 6-well plates at a concentration of 1 million cells per well in 2 ml of N2S media. These cultures were used for binding studies within 2 days of plating. We routinely achieved cultures of approximately 90% oligodendrocytes and 10% astrocytes, as we have described previously (Zhang et al. 2005; Zhang et al. 2006). For immunostaining, OPCs were grown in N2S medium for 3 d (Zhang et al. 2005), and in N2B2 medium for additional 3-5 d (Zhang et al. 2005). The oligodendrocyte cells were identified by staining for CNPase (Abcam) and GFAP (Sigma) for oligodendrocytes and astrocytes, respectively. The slides were first washed in PBS 3 times for 5 min each, and then fixed in fresh ice-cold 4% PFA for 2 min. After washing, cells were stored at -80°C until use. Subsequently, the slides were dried at room temperature for 1 hr and were then blocked in 10% NGS for 3-4 h at 4°C followed by incubation in purified anti-Tim-2 rabbit antiserum at 1:250 dilution (Chen et al. 2005) for 16 hr at 4°C. (Although the Tim-2 antiserum was raised to mouse Tim-2, it also detects rat Tim-2 by both immunohistochemistry and Western blotting). Slides were costained with either the oligodendrocyte marker, CNPase (mouse mAb, Abcam, 1:100) for 16 h, or with GFAP (mouse mAb, Sigma; 1:200) for 16 hr. Secondary antibodies used were anti-rabbit-AlexaFluor488 at 1:150 dilution (Invitrogen) for Tim-2 and anti-mouse-AlexaFluor555 at 1:150 dilution (Invitrogen) for CNPase and GFAP, respectively for 2 h at room temperature. Additional antibodies used in this study were anti-A2B5 (mouse IgM; R&D Systems), marker of bipotential bipolar oligodendrocyte progenitors and O4 (mouse IgM; Neuromics), marker of branched pro-oligodendrocytes. The staining with primary antibodies was visualized by anti-mouse IgM AlexaFluor555, 1:150 (Invitrogen) for 2 hours at room temperature (22°C). Cross-reactivity between the secondary antibodies was
excluded by cross-staining with the opposite secondary antibody—anti-mouseAF555 did not detect staining with Tim-2 antibody, and anti-rabbitAF488 did not detect staining with GFAP and oligodendrocyte marker antibodies. Fluorescence images were acquired on Leica confocal microscope.

**CG4 cells**

The rat CG4 cell line, which can be differentiated into either oligodendrocytes or astrocytes, was the generous gift of Dr. Lynn Hudson (NIH, Bethesda, MD). The cells were maintained in N2S medium (containing PDGF and FGF) and used between passages 25-35. CG4 cells grown in N2S media were considered undifferentiated, while oligodendrocyte differentiation was induced by growing them in N2B2 medium (withdrawal of FGF and PDGF) for a minimum of 5 days. Withdrawal of FGF and PDGF growth factors in low-serum (0.5%) N2S media has been previously shown to differentiate CG4 cells into oligodendrocytes (Louis et al. 1992).

**a) Tim-2 expression on developing CG4 cells**

Differentiated and undifferentiated CG4 cells were collected and plated on poly-D-lysine-coated slides. Cells were stained with Tim-2-purified rabbit antiserum (1:250 for 16 h at 4°C), using procedures as described for the primary cultures. For the biochemical studies, differentiated and undifferentiated CG4s were lysed with RIPA buffer (supplemented with mammalian protease inhibitor cocktail (Sigma)). The lysates were centrifuged and supernatant discarded. The pellets, containing the cell membranes were washed and redigested with RIPA/0.5% Triton X supplemented with mammalian
protease inhibitor cocktail (Sigma) for 30 min on ice, and recentrifuged at 10,000 g for 10 minutes. The supernatant of this protein extraction step was collected and total protein was determined using the BioRad DC assay. Samples of 50 ug of total protein were then subjected to separation by SDS-PAGE, followed by transfer to nitrocellulose membrane using standard techniques. The blots were then probed with rat monoclonal anti-Tim-2 (R&D Systems) 1:250 for 16 h at 4°C. The secondary Ab used was HRP-conjugated, anti-rat-Ig 1:2500 for 4 hr at 4°C, subsequently incubated with ECL reagent (Perkin Elmer). Bands were visualized by using a FUJI system equipped with a digital camera. Unedited pictures were densitometrically analyzed using MultiGauge software.

b) Iron-loading and chelation:

CG4 cells were differentiated into oligodendrocyte by exposure to N2B2 medium for at least 5 days, and then plated in 6-well plates at a concentration of 1 million cells per well in 2 ml of N2B2 medium. After overnight incubation, the cells were treated either for 48 h with 100 uM desferroxamine (Sigma, St. Louis, MO), for 16 h with 40 uM TMH-ferrocene (Malecki et al. 2002) or for like periods with no reagent (control). The cells were then harvested and counted. Cells were continuously monitored by light microscopy and by MTT assay to verify that the treatments had no effect on cell viability. One million cells in each group were lysed in RIPA/0.5% Triton X, and the protein concentration was determined by using the Biorad DC assay. Tim-2 expression was detected by Western blotting using mouse monoclonal anti-Tim-2 antibody (R&D Systems) as described above, standardized to total protein.
**Ferritin binding analysis:**

a) *Saturation analysis:*

Recombinant human H-ferritin, tagged with six histidines, was produced in competent BL21 *E. coli* by transfecting a plasmid containing wild-type H-ferritin as we previously reported (Fisher et al. 2007). Following induction with isopropyl-β-D-thiogalactoside, the bacteria were lysed, and recombinant H-ferritin was purified over a nickel column (Pierce). The identity of recombinant H-ferritin was verified by Western blot by using HS-59 antibody (mouse monoclonal to H-ferritin, a generous gift from Dr. Paolo Arosio, Italy). The purity was >90% as verified by Comassie staining of a denaturing SDS-PAGE gel (data not shown). Endogenous LPS activity was less than 1.0 IU/mL as assessed by the limulus amoebocyte lysate (LAL) assay (Lonza, Switzerland). Iodination of H-ferritin was performed by using a standard chloramine-T method (Hunter and Greenwood 1962). Iodinated ferritin was separated from unreacted I\(^{125}\) by Sephadex G25 column (Pharmacia) and used within 2 weeks from iodination. The specific activity of labeled H-ferritin at the time of use ranged 100,000-240,000 cpm/ug of protein.

Total cell homogenates of differentiated and undifferentiated CG4 cells were used for H-ferritin binding studies, as we reported previously for oligodendrocytes (Hulet et al. 2000). Briefly, cells were collected mechanically and pelleted by centrifugation. The pellet was washed twice by resuspending it in 1ml of 1X PBS. Subsequently, the pellet was homogenized using a rotary dounce, and protein concentration was measured by using the Bradford assay. The binding of homogenates from oligodendrocytes and differentiated and undifferentiated CG4 cells was performed as described before (Hulet et al. 2000). In brief, to establish the total specific and non-specific binding, a range of
concentrations of H-ferritin-I\(^{125}\) was added with or without 1000-fold molar excess of unlabeled H-ferritin to 25µg total protein of the CG4 cell homogenate. The molarity of H-ferritin protein solution was calculated based on the MW of the single chain (22 KDa). The binding buffer consisted of 50 mM Tris-HCl (pH 7.4), 0.1% BSA. Incubations were carried out at 22°C for 2h. The binding was terminated by the addition of 3 ml ice-cold 50 mM Tris-HCl. Using a cell harvester, the bound radioactivity was isolated by rapid filtration and washing over Whatman glass fiber C filters, which were previously coated with 5% non-fat dry milk and 0.1 mg/ml horse spleen ferritin to minimize non-specific binding, as we described previously (Hulet et al. 2000).

\(b)\) Competition analysis

Competition analysis was done on oligodendrocyte differentiated CG4 cells (differentiated by exposure to N2B2 media for minimum of 5 days). CG4 cells were plated into 6 well plates at concentration of 1.5 million cells per well in 2 ml of N2B2 media. Two days after plating, cells were washed twice with HBSS-CM. Cells were then exposed to increasing concentrations of blocker (recombinant H-ferritin, spleen ferritin or apo-transferrin) ranging from 10-500 fold molar excess of binding concentration of H-ferritin-I\(^{125}\) (20 nM) for 3 hr at 4°C. Subsequently, cells were exposed to 20 nM H-ferritin-I\(^{125}\) in PBS for 1 hour at 22°C. Cells were subsequently washed 3x with excess PBS, and collected mechanically. Bound radioactivity was measured by using a gamma counter.
c) Blocking Ferritin binding with Tim-2 antibody

The blocking experiments with Tim-2 mAb were performed on both CG4 cells and primary rat OPCs. CG4 cells were differentiated for at least 5 days by exposure to N2B2 media. Approximately 24 hr before each experiment, CG4 cells were split and plated in 6-well plates at a concentration of $10^6$ cells per well. At the time of the experiment, the cells were 90% confluent. Alternatively, primary OPCs were prepared as described in methods section of this chapter and plated in 6-well plates at a concentration of $10^6$ cells per well. OPCs were maintained in N2S (N2B2 medium supplemented with 10ng/ml PDGF and bFGF) medium for less than 5 d until use for binding experiments. The cells were washed twice with HBSS-CM, and then exposed to increasing concentrations of anti-Tim-2 mAb or PBS or a 300 molar excess of unlabeled H-ferritin for 2 h at 4°C. Subsequently, 20 nM of H-ferritin-I$^{125}$ was added to the incubation medium containing either Tim-2 antibody or PBS or H-ferritin competitor and incubated for additional one hour at 22 °C. The cells were washed 4 times in excess volume of PBS, and harvested mechanically in PBS. Counts were measured in a gamma counter equipped to detect radiation from I$^{125}$. The blocking rat anti-Tim-2 mAb used in this experiment (Chen et al. 2005) was raised to the extracellular domain of Tim-2 and has been shown to block activation of a Tim-2 reporter T cell line by soluble recombinant H-ferritin (Chen et al. 2005). The specificity of this antibody for Tim-2 has been shown previously; the antibody binds to cells transfected with Tim-2, but not to cells transfected with either Tim-1 or Tim-3 (Chen et al. 2005).
Myelin isolation and analysis:

The Tim-2 knockout mouse line (Tim-2 -/-) was obtained from Biogen, Idec as a generous gift of Dr. Paul Rennert, PhD (Rennert et al. 2006). All male mice, age 16 months were used in the analysis. Controls were Balb/c +/- mice bread and raised under the identical conditions as knockouts. Unless indicated otherwise, all solutions were prepared with iron-free water, and tissue manipulated in iron-free tubes. Myelin isolation was performed using modified method of Norton and Poduslo (1973). Briefly, Tim-2 knockout (n=3) and age/sex matched wild-type controls (n=3) were sacrificed by cervical dislocation. The calvarium was opened and the brain including cerebellum was removed with curved forceps and flash-frozen in isopentane cooled with liquid nitrogen. Brains were stored at -80°C until use. Myelin extraction commenced with whole brains being thawed and homogenized in 8 ml of 0.32 M sucrose solution supplemented with mammalian protease inhibitors (1:100 dilution from manufacturer’s stock, Sigma). The homogenate was prepared with 10 strokes of a rotary dounce homogenizer on ice, and a 200 ul aliquot of the total brain homogenate from each brain was saved for whole brain analysis. The remainder of a whole brain homogenate was layered over 13 ml of 0.85 M sucrose solution in the ultracentrifuge (UCF) tube. The resulting setup was ultracentrifuged at 75,000 g for 30 minutes at 4°C. The myelin isolate formed as a whitish suspension at the interface of two sucrose solutions and was collected using serological pipette. That isolate was subsequently resuspended with ice-cold iron-free water, and re-ultracentrifuged at 75,000 g for 15 minutes. This time, myelin appeared as whitish pellet at the bottom of the UCF tube. The identical procedure (hypotonic shock) was repeated three more times and, at the end, purified myelin was collected mechanically into pre-
weighed Eppendorf tubes. Subsequently, myelin samples were dried under a vacuum rotavap for 4 hrs, and the weights of tubes determined after drying. Subtraction of weights both before and after gave total myelin yield.

\[ \text{a) Quantification of myelin proteins:} \]

To extract myelin proteins, the whole brain and purified myelin samples were resuspended in lysis buffer (RIPA supplemented with 1% Triton X and mammalian protease inhibitor cocktail) in a fixed volume to myelin weight ratio. The homogenate was passed 5 times through a 22 gauge needle and incubated on ice for 1.5 hrs. The homogenate was centrifuged at 10,000 g and the supernatant collected for analysis. The protein concentration of these samples was determined by using the BioRad DC protein assay, and total extracted protein per myelin sample calculated. Equal amounts (50 ug) of total protein were separated on SDS-PAGE gradient gel under denaturing conditions (for analysis of CNPase, MBP and MOG) and 5 ug total protein applied on slot blot (for analysis of PLP levels). The transferred membranes were blocked in 5% blotto/TBST overnight and subsequently probed with the following antibodies: anti-CNPase (1:250, mouse monoclonal Ab, Abcam); anti-MBP (1:1000, mouse monoclonal Ab, Chemicon); anti-MOG (1:500; rabbit polyclonal Ab, Abcam) and anti-PLP (1:1000, mouse monoclonal Ab, Chemicon). All antibodies were diluted from manufacturer’s stock solutions in 5% blotto/TBST and incubated for 24 hrs at 4°C. The bands were visualized via appropriate HRP-conjugated secondary antibodies and ECL reagent (Perkin Elmer) on Fuji system. The expression levels of individual proteins were determined by
densitometry using multi-Gauge software and were evaluated for statistical significance using GraphPad software.

b) Quantification of myelin and whole brain iron:
Aliquots of whole brain and myelin homogenates containing 200 ug total protein were frozen on dry ice and total iron levels in samples determined as described previously (Ortiz et al. 2004). Iron quantification was performed by atomic absorbance spectroscopy in a standard matrix following sample digestion in nitric acid. The raw values of iron measurements were expressed as ug of iron per mg total protein.

RESULTS:
To define the cellular distribution of Tim-2 protein expression in the adult mouse CNS, we stained brain slices of 6 month old C57BL/6 mice with purified rabbit antiserum to Tim-2. Tim-2 staining, visualized by secondary AlexaFluor488-conjugated antibody, was mainly confined to oligodendrocytes in white matter tracts (Figure 2.1 A and 2.1 B) throughout all areas of the brain, including oligodendrocytes in the subcortical white matter and striatum. Anti-Tim-2 did not stain neurons in any brain region, including the cortex, Purkinje cells of the cerebellum, or cellebellar nuclei. There was light staining in the choroid plexus (data not shown). To demonstrate that Tim-2 was indeed present in the white matter fractions, we performed Western blotting on homogenates of the corpus callosa of 6 month old mice and detected a single band at
approximately 50 KDa (Figure 2.1 C), consistent with the molecular mass previously reported for glycosylated Tim-2 (Chen et al. 2005; Kumanogoh et al. 2002).

To investigate the expression pattern of Tim-2 on isolated oligodendrocytes in vitro, we used purified anti-Tim-2 antiserum to stain primary rat oligodendrocyte-enriched cultures that had been grown on poly-D-lysine-coated slides and then lightly fixed and frozen, allowing the entry of antibody to intracellular proteins and to the Tim-2 cytosolic domain. Tim-2 positive cells were round with few branches, similar to cells identified by staining for CNPase, a marker in oligodendrocytes (Figure 2.2 A). Indeed, almost all cells expressing CNPase also expressed Tim-2, while cells expressing GFAP, a marker for astrocytes, did not (Figure 2.2 B). Because all CNPase-positive cells were also Tim-2 positive, but not all Tim-2 positive cells were also CNPase positive, we further investigated whether Tim-2 is expressed earlier in the oligodendrocyte lineage. Therefore, we performed co-localization staining with early markers of oligodendrocyte differentiation and Tim-2, and found that Tim-2 is expressed in all the O4 and A2B5-positive cells of oligodendrocyte lineage examined (Figure 2.3, A and B).

To specifically examine the relationship between oligodendrocyte maturation, H-ferritin binding, and Tim-2 expression, we used the rat CG4 cell line, which can be induced to differentiate into oligodendrocytes by growth in N2B2 medium. Membrane fractions from undifferentiated CG4 cells did not bind H-ferritin, but CG4 cells grown in N2B2 bound H-ferritin in a saturable manner (Figure 2.4 A). In addition, competition assays revealed that the binding is specific for H-ferritin, but not the L-subunit-rich horse spleen ferritin or transferrin (Figure 2.4 B). Immunostaining of the CG4 cells with Tim-2 coincided directly with the H-ferritin binding; anti-Tim-2 did not stain undifferentiated
CG4 cells, but it stained CG4 cells that had differentiated into oligodendrocytes (Figure 4C, D). The expression of Tim-2 on CG4 cells was confirmed by Western blotting with anti-Tim-2 (Figure 2.4 E), which demonstrated a band at approximately 50KDa following oligodendrocyte differentiation of CG4 cells but not in undifferentiated CG4 cells.

To directly demonstrate that H-ferritin binding in these cells is through interaction with Tim-2, we exposed differentiated CG4 cells to a blocking antibody directed to the extracellular domain of Tim-2. Exposure of oligodendrocyte-differentiated CG4 cells to anti-Tim-2 mAb decreased H-ferritin binding in a dose-dependent manner (Figure 2.5 A). At the highest concentration tested (200 ug/mL), the Tim-2 antibody almost completely eliminated specific H-ferritin binding by these cells. The same experiment performed with non-specific rat (isotype matched) IgG did not have any effect on the binding of H-ferritin to differentiated CG4 cells (Figure 2.5 B). Similar results were obtained with primary cultures enriched in oligodendrocyte progenitors; 200 ug/mL concentration of Tim-2 antibody significantly reduced specific binding of H-ferritin to primary OPCs (Figure 2.6 A). In contrast, the same concentration of rat IgG failed to decrease binding of H-ferritin to primary OPCs (Figure 2.6 B).

To evaluate if iron availability affects Tim-2 expression, we treated differentiated CG4 cells with 40 uM TMH-Fe for 16 hr or with 100 uM desferroxamine (DFO) for 48 h (Figure 2.7). The studies were performed on the CG4 cells because even small changes in iron availability are toxic to primary oligodendrocytes (Zhang et al. 2005; Zhang et al. 2006), and the relatively small changes in iron availability did not affect Tim-2 expression or H-ferritin uptake in OPCs (Hulet et al. 2000). In differentiated CG4 cells, however, which were able to tolerate higher iron loads, TMH-Fe mediated iron delivery
resulted in decreased Tim-2 expression, while DFO treatment increased Tim-2 expression (Figure 2.7). Thus, Tim-2 is upregulated when the availability of iron is low and downregulated when the iron status of the cell is increased. The CG4 cell viability was unaffected by the manipulation of iron availability in our experiment (Figure 2.7 C and D) as determined by MTT assay.

To begin to determine the mechanism of iron regulation of Tim-2 expression on oligodendrocytes, we investigated whether Tim-2 mRNA sequence contained a putative iron responsive element. We scanned the entire mRNA of Tim-2 against the consensus IRE sequence of TfnR1 and found a segment that is 71% homologous to the consensus IRE. The comparison between TfnR1 IRE sequence and the Tim-2 candidate sequence is shown, with homologous bases shown in red (Figure 2.8 A). The location of the candidate sequence within the 3’ untranslated region of Tim-2 mRNA suggests that if it indeed properly folded could function in a similar fashion to the TfnR IRE (Figure 2.8 B). The functionality of this sequence as an IRE is beyond the scope of this thesis project.

To begin to investigate Tim-2 function in vivo, we obtained a mouse line that is genetically deficient in Tim-2 (Rennert et al. 2006). We isolated the myelin from 16 month old Tim-2 knockout mice and age/sex matched wild-type controls and analyzed their myelin profile biochemically. Myelin yield from Tim-2 knockout and wild type mice are comparable both in quantity of isolated myelin and the amount of total extractable proteins (Figure 2.9 A, B). Western blot analysis for all the major myelin proteins (CNPase, MBP, PLP and MOG) demonstrates no significant difference in the expression of these proteins in Tim-2 KO as mice compared to controls at this age group (Figures 2.10 and 2.11). However, the atomic absorption measurements of iron levels in
myelin isolates and whole brain homogenates demonstrated a significant decrease in the iron content in myelin of Tim-2 KO mice relative to wild-type controls. Iron analysis in the whole brain homogenates demonstrated a similar trend, but the differences did not reach statistical significance (p=0.06). Overall, our results indicate that absence of Tim-2 in vivo results in partial disruption in iron delivery to oligodendrocytes and myelin, but not to the extent that affects the myelin status of 16-month-old mice.

DISCUSSION:

In the current study, we show that Tim-2 is expressed on oligodendrocytes in rodent CNS both in vivo and in vitro, and we present evidence that Tim-2 is the cell surface receptor for H-ferritin in these cells. Western blot analysis of white matter homogenates, primary oligodendrocytes and oligodendrocyte differentiated CG4 cells indicates a protein of approximately 50 KDa, which is consistent with what is reported in the literature for glycosylated Tim-2 (Chen et al. 2005; Kumanogoh et al. 2002). This molecular weight also compares favorably with previous reports attempting to isolate ferritin receptor of 53 KDa (Adams et al. 1988a; Adams et al. 1988b). Furthermore, we demonstrate that Tim-2 expression is selective for oligodendrocytes, but not astrocytes, which is consistent with our previous findings that oligodendrocytes, but not for astrocytes internalize H-ferritin in culture (Hulet et al. 2000). Differentiating CG4 cells into oligodendrocytes triggers the onset of saturable H-ferritin binding, which is accompanied by Tim-2 expression. In toto, we observed a consistent overlap between H-ferritin binding and Tim-2 expression in oligodendrocytes in vivo and in vitro.
Importantly, treating either primary oligodendrocytes or differentiated CG4 cells with a blocking monoclonal antibody to the extracellular domain of Tim-2 decreased the binding of H-ferritin to these cells. Previously, the same antibody was used to block activation of a Tim-2 reporter T cell line by soluble H-ferritin (Chen et al. 2005). We therefore conclude that Tim-2 is the cell surface receptor for H-ferritin on oligodendrocytes. These findings, coupled with our previous reports for ferritin binding in the brain, indicate that Tim-2 may provide an alternative to the TfR1 for transporting iron into oligodendrocytes.

A number of autoradiographic and immunostaining studies have demonstrated lower Tf binding and expression of TfR in mature oligodendrocytes of adult white matter in mice, rats, and humans (Han et al. 2003; Hill et al. 1985; Hulet et al. 1999a; Hulet et al. 1999b). The enigma, however, has been that adult oligodendrocytes have higher levels of stainable iron than any other cells in the CNS. We have previously shown that developing oligodendrocytes can nonetheless obtain iron by binding and internalizing extracellular H-ferritin (Hulet et al. 2000), and the results presented here demonstrate that Tim-2 is the receptor by which this is achieved. The potential advantages of this dual system are two fold. First, H-ferritin-mediated delivery of iron to oligodendrocytes prevents these cells from having to compete for iron with neurons and other cells that use the TfR. Second, ferritin is capable of delivering much higher amounts of iron per mole than transferrin. This function may be particularly significant in the times of increased iron requirements, such as during myelination. Indeed, peak iron import into the brain coincides with peak myelination (Taylor and Morgan 1990) and we have shown that \textit{in vivo} binding of H-ferritin in mouse white matter tracts spatially and temporally follows
the onset of myelination (Hulet et al. 1999a). We further demonstrate that Tim-2 expression occurs on oligodendrocyte progenitors, identified as A2B5- and O4-positive cells. The expression of Tim-2 on these cells is consistent with our earlier reports of H-ferritin uptake by oligodendrocyte precursors (Hulet et al. 2000). We are currently studying the steps in oligodendrocyte differentiation when downstream Tim-2 signaling is activated by H-ferritin. One possibility is that the presence Tim-2 and the subsequent binding of H-ferritin promotes inhibition of oligodendrocyte proliferation, a reported function of H-ferritin and Tim-2 in lymphocytes (Knickelbein et al. 2006), which would allow and even stimulate OPCs to differentiate. We explore this possibility further in Chapter 3.

Traditionally, transferrin has been considered an essential factor for myelination, and an indispensable component of chemically defined medium for oligodendrocytes (Bottenstein 1986; Espinosa de los Monteros et al. 1999). Oligodendrocyte progenitor cells (OPCs) express high levels of transferrin, but reportedly do not secrete this protein (de Arriba Zerpa et al. 2000). However, exogenously administered apotransferrin accelerates differentiation of OPCs into mature oligodendrocytes, and a single intracranial injection of apotransferrin increases myelin deposition and MBP/CNPase levels in-vivo (Escobar Cabrera et al. 1994; Escobar Cabrera et al. 1997; Paez et al. 2004; Paez et al. 2006b; Paez et al. 2002). This pro-myelinating effect of apotransferrin is only observed if transferrin is injected at P3, and is lost after P14, an effect that can be explained by either downregulation of the TfR or loss of requirement for iron import after the OPCs differentiated into mature oligodendrocytes (Han et al. 2003; Hill et al. 1985; Hulet et al. 1999a). In all, our data indicate that as oligodendrocytes mature, they are left
with the other proposed iron import system--H-ferritin. The possibility that H-ferritin can replace transferrin requirement, support growth of oligodendrocytes and maintain intracellular iron levels in absence of transferrin is examined in Chapter 3.

What is the source of H-ferritin for oligodendrocytes? Recent studies in our laboratory have provided evidence that H-ferritin can cross the blood-brain-barrier (Fisher et al. 2007). Furthermore, iron-loaded microglia release H-ferritin \textit{in vitro} (Zhang et al. 2006). We demonstrated that conditioned media from microglial cell cultures is trophic to oligodendrocyte progenitors, an effect that is abated by treating microglia with siRNA to H-ferritin (Zhang et al. 2006). These findings are in agreement with our earlier \textit{in vivo} observations that microglia in the vicinity of myelinogenic foci are iron-positive before oligodendrocytes start to myelinate (Cheepsunthorn et al. 1998). Subsequently, microglia release iron during the peak myelination period, at the same time myelinating oligodendrocytes become iron enriched. These data suggest a transfer of iron from microglia to oligodendrocytes during peak myelination; a concept currently under investigation. It is possible that during CNS development microglia serve as an “iron capacitor”, accumulating iron over time before myelination, repackaging iron and then releasing iron in the form of H-ferritin to myelinating oligodendrocytes. There may also be a continual supply of blood borne H-ferritin, because a transport mechanism for H-ferritin has been identified on the brain microvasculature (Fisher et al. 2007). The notion that H-ferritin may be important in myelination is further reinforced by our report that H-ferritin +/− (heterozygote) mice have significantly decreased myelin indices (Ortiz et al. 2004). Although these mice have traditionally been considered a model of compromised
iron storage, they also highlight the potential importance of extracellular H-ferritin for delivery of iron to oligodendrocytes.

The cell culture analyses in our current study did show, however, that levels of Tim-2 expression on oligodendrocytes are influenced by the labile iron pool in oligodendrocyte-differentiated CG4 cells. Iron chelation increased Tim-2 levels, whereas iron loading had the opposite effect. A similar effect is seen for transferrin receptors (Syed et al. 2006). Our data are in agreement with a similar experiment by Gelvan et al., who found that in erythroid precursor cells iron loading decreased, and iron chelation increased ferritin binding (Gelvan et al. 1996). We could not, however, perform these studies in primary oligodendrocytes, because the concentrations of DFO and TMH-Fe required to alter Tim-2 expression levels in CG4 cells were toxic for primary cells. We further demonstrate a candidate sequence within 3′ UTR of Tim-2 mRNA that is 71% homologous to the consensus IRE of TfnR. Although the full functionality of this sequence and its ability to bind iron regulatory proteins (IRPs) has yet to be determined, it illustrates a potential mechanism by which iron can regulate Tim-2 expression within oligodendrocytes.

So, what is the role of Tim-2 protein in oligodendrocyte in vivo? Our observation that Tim-2 knockout mice have a 20% decrease of iron in the myelin fraction is a compelling argument that one of the functions of H-ferritin/Tim-2 in oligodendrocytes is iron delivery. However, this decrease in iron was apparently insufficient to produce hypomyelination as our preliminary data demonstrate absence of Tim-2 gene had no effect on myelin yield, content and expression of major myelin proteins. So, why are the Tim-2 KO mice not hypomyelinated? There are several possibilities that explain this
somewhat surprising result. One possibility is that absence of ferritin receptor causes a
temporal delay of development and maturation of oligodendrocyte progenitor cells during
the period of peak myelination (first three post-natal weeks in rodents), but that
oligodendrogliogenesis and myelinogenesis in the Tim-2 KOs eventually catches up to
that of wild type control mice. Therefore, our studies in Tim-2 mice would benefit from a
more detailed analysis of developing oligodendrocyte (NG2+ cell) parameters, including
timing of migration, proliferation and maturation of NG2 cells during that early
developmental period. The other possibility is that mature oligodendrocytes in adult brain
have decreased requirement for iron import and that most (if not all) of their iron is
acquired in early development at which multiple complementary and alternative systems
of iron acquisition by oligodendrocytes may exist. A clear candidate is transferrin/TfnR1,
which has been up to now considered to be an essential factor for myelination
(Bottenstein 1986; Espinosa de los Monteros et al. 1999). We examine this possibility in
Chapter 3 and demonstrate clearly that transferrin or H-ferritin independently of each
other can support growth and iron requirements of oligodendrocyte progenitors in culture.
Finally, oligodendrocytes might have evolved yet another alternative mechanism of
ferritin uptake. Recently, an endocytosing receptor for L-ferritin, Scara5 has been
described in developing kidney (Li et al. 2009). It is presently unknown whether
oligodendrocytes express Scara5 or whether they have the capacity to bind and
endocytose L-ferritin, but if they did, it would allow for another mechanism of uptake of
H-ferritin into these cells (as H-ferritin is exclusively found as a heteropolymer with L-
ferritin in vivo) (Harrison 1996). All of the possible alternative mechanisms of iron
import to developing oligodendrocytes are discussed in more detail in Chapter 6.
In conclusion, we report that rodent Tim-2 expression in the CNS is limited to oligodendrocytes and parallels the pattern of H-ferritin binding. Further, binding of H-ferritin to oligodendrocytes is blocked by a mAb to Tim-2, providing direct evidence that Tim-2 is the receptor for H-ferritin on these cells. These findings provide novel insights into iron acquisition by oligodendrocytes. Because Tim-2 is expressed and functions in the lymphocytes in the immune system, this raises the interesting possibility of cross-talk between the immune system and oligodendrocytes, particularly in neuroinflammatory disorders resulting in demyelination. This possibility will be examined in Chapter 5.
FIGURES:

**Figure 2.1. In vivo, Tim-2 is expressed in the mouse brain oligodendrocytes.** Purified rabbit antiserum raised to Tim-2 stains primarily oligodendrocytes in corpus callosum and striatum. These confocal microscopic images (63X capture magnification) show Tim 2 staining in the striatal white matter tracts (A), and corpus callosum (B). In both images, the reaction product is confined to small round cells with eccentric nuclei. The traditional arrangement of oligodendrocytes in “tram-track rows” (yellow arrows) is seen in the striatal image (A). Some staining in myelin is indicated by red arrows in both the striatum and corpus callosum. The blue color in the micrographs identifies cell nuclei by staining with DAPI.
Figure 2.1 (C): Western blotting of white matter homogenates from adult mice probed with purified rabbit antiserum to Tim-2 shows a single band at approximately 50 KDa. This molecular mass is consistent with that reported for Tim-2 (Chen et al. 2005) (Kumanogoh et al. 2002).
Figure 2.2. In vitro, Tim-2 is expressed in oligodendrocytes, but not astrocytes.

Figure 2.2A and 2.2B: Purified rabbit antiserum to Tim-2 selectively stains oligodendrocytes, but not astrocytes in glial cultures. (A) Tim-2 staining colocalizes with CNPase positive oligodendrocytes (red). The yellow arrows in the overlay image indicate examples of CNPase positive cells strongly colocalizing with Tim-2 expression. (B) No Tim-2 staining can be detected on the GFAP-positive astrocytes (red) that are occasionally present in these cultures. This was true for all the GFAP-positive astrocytes examined across several different culture preparations. All CNPase-positive cells were also Tim-2-positive, but not all Tim-2-positive cells were also CNPase-positive suggesting that Tim-2 is expressed in oligodendrocyte progenitor cells at an earlier maturational state. Up to 30% of CNPase-negative oligodendrocyte progenitors stained for Tim-2. This panel is a representative picture of staining performed in triplicate over several different preparations of mixed glial culture.
Figure 2.3. In vitro, Tim-2 is expressed in oligodendrocyte progenitors. Confocal microscopic images show Tim-2 expression in cells of oligodendroglial lineage. (A) Tim-2-purified rabbit antiserum colocalizes with bipolar A2B5-positive oligodendrocytes (top set of panels) and (B) O4-positive oligodendrocytes (bottom set of panels) in mixed glial cultures enriched in oligodendrocytes. This panel is a representative picture of staining performed in triplicate over several different preparations of mixed glial cultures.
Figure 2.4. H-ferritin binding and Tim-2 expression are coincident in an oligodendrocyte cell line. (A) The CG4 rat oligodendrocyte/astrocyte cell line), when differentiated into oligodendrocytes by exposure to N2B2 media (withdrawal of FGF/PDGF) for minimum of 5 days binds H-ferritin saturably. In the undifferentiated state, CG4 cells do not bind H-ferritin. (B) Excess recombinant H-ferritin, but not horse spleen ferritin (L-rich ferritin) or transferrin effectively competes for with H-ferritin binding sites on differentiated CG4 cells. (C) (D) Immunostaining with Tim-2 rabbit antiserum and (E) Western blot analysis of membrane homogenates reveals that Tim-2 is expressed in oligodendrocyte differentiated CG4 cells, but not in undifferentiated CG4 cells.
Figure 2.5. Binding of H-ferritin to CG4 cells is dependent on Tim-2. (A) Pretreatment of oligodendrocyte differentiated CG4 cells with a blocking monoclonal antibody raised to the extracellular domain of Tim-2 significantly decreases specific binding of 20 nM H-ferritin-I\(^{125}\) to these cells. (B) The same experiment repeated with rat IgG isotype matched control antibody (non-specific antibody control) fails to influence H-ferritin binding to differentiated CG4s. Statistical significance was analyzed by one-way ANOVA with Dunnet’s post-test comparison (only the indicated group comparisons were significantly different).
Figure 2.6. Binding of H-ferritin to cultures of primary oligodendrocytes is dependent on Tim-2. (A) Pre-treatment of primary rat oligodendrocyte progenitors with blocking monoclonal antibody raised to the extracellular domain of Tim-2 (same antibody as in Figure 2.5) significantly decreases specific binding of 20 nM H-ferritin-I\(^{125}\) as compared to controls. (B) A parallel experiment performed in the presence of non-specific rat IgG failed to block binding of H-ferritin-I\(^{125}\). Statistical significance was evaluated by unpaired t test. Only the indicated comparison was significant (*, p=0.0139).
Figure 2.7. Iron status effects the expression of Tim-2 in CG4 cells. (A), iron loading with lipophilic iron compound (TMH-ferrocene) of differentiated CG4 cells results in decreased Tim-2 expression as shown by Western blot. Tim-2 expression was quantified densitometrically and the results shown in graphic form are pooled data from three independent experiments. Statistical significance determined using an unpaired t-test. (B), iron chelation with desferrioxamine (DFO) results in increased Tim-2 expression in differentiated CG4 cells. Representative Western blots are shown. The levels of Tim-2 in different treatment conditions were quantified densitometrically. The graphs represent pooled data from two independent experiments. Statistical significance was determined using an unpaired t-test. As a control, we monitored cell viability microscopically and also by MTT assays on TMH-Fe (C) and DFO (D)-treated differentiated CG4 cells after 16 and 48 hours, respectively. Neither treatment resulted in significant loss of cell viability (ns, no significance using unpaired t-test).
Figure 2.8. The 3′ untranslated region of Tim-2 mRNA contains putative consensus iron responsive element (IRE) sequence. The entire sequence of Tim-2 mRNA was compared to the consensus sequence of TfnR iron responsive element. A stretch of sequence with highest percent homology (71%, indicated in red) was identified in 3′ UTR of Tim-2 mRNA, starting at 805 position (A). Relative positions of the proposed Tim-2 IRE relative to the protein coding sequence and stop codon, as well as TfnR’s IREs are also shown in (A). In B, proposed mechanism of gene expression control by cytosolic iron for both Tim-2 and TfnR involves iron-dependent binding of IRP proteins and protection from 3′ exonuclease digestion.
Figure 2.9. Tim-2 knockout mice have normal myelin yield compared to wild type controls. The myelin was extracted from whole brain sucrose homogenates of 16-month old male mice (knockout N=3, and controls N=3) using step sucrose gradient. Myelin yield was determined by measuring myelin dry weight (A) and total myelin extracted protein (B).
Figure 2.10. Western blot analysis shows normal expression of major myelin proteins in Tim-2 KO mice. The myelin was extracted from whole brain sucrose homogenates of 16-month old male mice (knockout N=3, and controls N=3) using step sucrose gradient. Myelin protein expression in wild-type (WT) and knockout animals (KO) in purified myelin fraction (A) and whole brain homogenates (B) was determined by Western blotting, and the representative blots are shown below.
Figure 2.11. Tim-2 knockout mice have normal biochemical profile of myelin proteins in the brain. The myelin was extracted from whole brain sucrose homogenates of 16-month old male mice (knockout N=3, and controls N=3) using step sucrose gradient. Myelin protein expression in wild type and knockout animals in purified myelin fraction (A, C, E, G) and whole brain homogenates (B, D, F, H) was determined by Western blotting (Figure 2.9). Bands were scanned and analyzed densitometrically, and statistical significance determined using unpaired t-test at significance level of \( p=0.05 \) (ns, not significant).
Figure 2.12. Tim-2 KO mice have decreased iron content in the myelin. Aliquots of myelin and whole brain homogenates from 16-month-old male mice (Tim-2 knockout N=3, and controls N=3) containing 200 ug total protein were analyzed for iron content using atomic absorbance spectroscopy. Raw absorbance values obtained were fitted to a standard curve and normalized to total protein to obtain iron levels in myelin fractions (A) and whole brain homogenates (B). Means evaluated for significance using unpaired t-test show statistically significant decrease in the iron content of myelin of Tim-2 KOs compared to controls (A) (p=0.0029), with the similar trend observed in the whole brain homogenates that did not reach statistical significance (B) (p=0.06).
REFERENCES:


Hulet, S. W., S. Menzies, et al. (2002). "Ferritin binding in the developing mouse brain follows a pattern similar to myelination and is unaffected by the jimpy mutation." Dev Neurosci 24(2-3): 208-13.


Chapter 3

H-ferritin and transferrin are complementary iron delivery systems in oligodendrocytes

ABSTRACT:

There is a critical relationship between oligodendrocyte development, myelin production, and iron bioavailability. Iron deficiency leads to hypomyelination both in humans and animal model, and the neurological sequelae of hypomyelination are significant. Therefore, understanding the molecular mechanisms of iron import into oligodendrocytes is necessary for devising effective strategies for iron supplementation. Although transferrin has been considered an essential component of oligodendrocyte media in culture, we have established that receptors for H-ferritin exist on cells of oligodendroglial lineage and that uptake of extracellular H-ferritin by oligodendrocyte progenitors in culture is via receptor mediated endocytosis. These data strongly argue that extracellular ferritin is a major source of iron for oligodendrocytes. In this study, we demonstrate that transferrin deprivation in oligodendrocyte media resulted in loss of cell viability of oligodendrocyte progenitors in culture. The loss of cell viability can be prevented by supplementing media with membrane-permeable TMH-ferrocene, but not membrane-impermeable ferric ammonium citrate. Similarly to ferrocene, H-ferritin supported the growth of oligodendroglial cells and prevented the loss of intracellular iron in OPCs produced by transferrin deprivation. Finally, we demonstrate that extracellular
H-ferritin stimulates differentiation of OPCs by increasing expression of MBP and olig2 proteins. Overall, our results demonstrate that extracellular H-ferritin is an alternative and complementary transferrin system for iron delivery in oligodendrocytes.

INTRODUCTION:

Oligodendrocytes are the only cells in the brain involved in the production of myelin (Barres 2008) (Franklin and Ffrench-Constant 2008), and a principal cell in the brain that stains for iron (Benkovic and Connor 1993; Connor and Menzies 1995; Connor and Menzies 1996; Todorich et al. 2008b). Peak iron uptake in the brain during second post-natal week of development in rodents coincides temporally with the onset of myelination (Crowe and Morgan 1992) suggesting an important role of iron in the process of myelin production. Most definitive evidence comes from human and animal studies, which demonstrated that severe chronic iron deficiency leads to hypomyelination in humans (Beard 2008) and animal model (Ortiz et al. 2004; Wu et al. 2008), suggesting that iron is essential for oligodendrocyte development and myelination. Hence, a thorough and complete understanding of the molecular mechanisms conducting iron import into oligodendrocytes is an important prerequisite for devising effective therapeutic strategies for iron delivery and treatment of iron deficiency-associated hypomyelination.

Traditionally, studies on iron import into cells have focused on transferrin-transferrin receptor 1 system, which arguably is the most important iron import
mechanism in vertebrates (Donovan and Andrews 2004). Transferrin receptors have been reported in oligodendrocyte progenitors, and transferrin has been considered an essential component of chemically-defined oligodendrocyte media (Bottenstein 1986; Espinosa de los Monteros et al. 1999) (Paez et al. 2004; Paez et al. 2006b). Furthermore, injections of transferrin have been shown to improve myelination in variety of animal models (Adamo et al. 2006; Badaracco et al. 2008; Escobar Cabrera et al. 1997), and adding Tf in purified cultures of oligodendrocyte progenitors results in increased oligodendrocyte differentiation (Paez et al. 2006b; Paez et al. 2002). Nonetheless, transferrin receptors appear to downregulate as oligodendrocytes mature and are altogether not detected in corpus callosum and mature oligodendrocytes in the adult brain (Han et al. 2003; Hulet et al. 1999b). Furthermore, oligodendrocytes in mixed glial cultures deprived of transferrin continue to uptake iron, which suggested non-transferrin-mediated iron uptake mechanism in cells of oligodendrocyte lineage (Takeda et al. 1998).

Because extracellular H-ferritin has been shown to participate in non-transferrin mediated iron delivery and it can store/deliver large quantities of iron (Harrison and Arosio 1996), we investigated whether extracellular H-ferritin can provide an alternative source of iron to oligodendrocytes. Previously, we have shown that binding sites for H-ferritin exist in both adult and developing brain and that the binding of H-ferritin in the brain as well as oligodendrocyte progenitor cells is both saturable and specific (Hulet et al. 1999a; Hulet et al. 2000; Hulet et al. 2002; Hulet et al. 1999b). In culture, oligodendrocyte progenitor cells uptake H-ferritin in a manner consistent with receptor-mediated endocytosis (Hulet et al. 2000), but the receptor for H-ferritin has remained unknown until recently. Chen et al. demonstrated that Tim-2 (T cell immunoglobulin
mucin domain 2 protein) specifically binds H-ferritin, but not L-ferritin, and is sufficient to permit endocytosis of extracellular H-ferritin in the immune system (2005). Recently, we demonstrated that Tim-2 is expressed in the brain of rodents on cells of oligodendrocyte lineage and blocking it abates binding of H-ferritin to these cells (Todorich et al. 2008c) (Chapter 2), suggesting that Tim-2-mediated endocytosis of extracellular H-ferritin is responsible for H-ferritin-mediated iron delivery to developing oligodendrocytes.

In the current study, we demonstrate that transferrin deprivation of oligodendrocyte progenitor cells results in loss of cell viability after 48 hours in culture, which can be prevented by treatment with recombinant H-ferritin and membrane permeant TMH-ferrocene, but not membrane-impermeant ferric ammonium citrate. Furthermore, we show that ferritin treatment prevents the loss of cellular iron associated with TfN deprivation. Finally, we demonstrate that rH-ferritin was strongly pro-myelinating to oligodendrocyte progenitors accelerating the spontaneous differentiation of OPCs in vitro. Collectively, our results demonstrate complementary roles of transferrin and H-ferritin in iron delivery to developing oligodendrocytes.
METHODS:

**Primary Cultures:** Primary rat oligodendrocyte progenitors were obtained from newborn rat pups using methods of McCarthy and DeVellis (McCarthy and de Vellis 1980). Briefly, pregnant Sprague-Dawley rat dams were obtained from Charles River Laboratories and housed at Penn State Animal Facility in accordance with the protocols prescribed by animal care and use committee (IACUC no. 98-077). The newborn rat pups were decapitated, and brains removed and washed in Hanks buffer. The meninges were surgically removed and brain diced and homogenized in Trypsin/EDTA for 30 minutes at 37°C. After DNAsE I digestion, homogenized tissue mix was passed through a 150 then 75 micron mesh, and cells cultured in DMEM/10% FBS for 7-8 days. At that time, microglia were removed by orbital shaking at 265 rpm for 1 hour at 37°C. After brief recovery, OPC cells were isolated by orbital shake at 265 rpm for 18 hrs. The OPCs cells were briefly purified by differential adhesion, and then plated on poly-d-lysine coated plates or slides in N2S complete media (DMEM/F12 base supplemented with BSA 0.66 g/L; biotin 20 mg/L; putrescine 32 mg/L; apo-transferrin 80 mg/L; Progesterone 0.63 ug/L, 1X ITSS, 1X antibiotic/antimycotic and 0.5% FBS, 10 ng/ml PDGF, 10 ng/ml FGF) and used for experiments.

**Recombinant H-ferritin preparation:** cDNA encoding wild-type human H-ferritin tagged with poly-histidine was subcloned in pET30a(+), and subsequently verified by sequencing (Todorich, et al, 2008). Recombinant H-ferritin produced in BL21 E. coli, was purified on nickel column using standard techniques. The identity of rH-ferritin was verified by SDS-PAGE followed by Western blotting with anti-H-ferritin mouse
monoclonal antibody (HS59, generous gift of Dr. Paolo Arosio, Milan, Italy; data not shown). The purity was verified by Comassie staining of the denaturing SDS-PAGE gel loaded with 10 ug of rH-ferritin (data not shown).

**Cell viability analysis:** MTT assay was purchased from Roche and used in accordance with the manufacturer’s protocol. The MTT reagent was added directly to the OPC media for the last 4 hr of treatment. After overnight solubilization in SDS-based detergent, the absorbance was measured at 595 nm. LDH release was measured in cell-free cell culture supernatants which were collected 48 hr after treatment with Tf withdrawal and H-ferritin supplementation, and LDH content determined using a commercial kit (Roche) according to the manufacturer’s protocol. LDH determinations were carried out either immediately after the experiment, or after several days (with samples kept at 4°C) without affecting the results.

**Intracellular iron measurements:** Primary rat OPCs were incubated in either complete N2S media, N2S media with low transferrin, or low transferrin N2S supplemented with 55 ug/ml recombinant H-ferritin for 6 hrs. At that time point, 0.25 uM calcein AM dye (Invitrogen) was added to the media of all the groups, and allowed to uptake for 45 minutes at 37°C under photoprotection. Subsequently, cells were washed in ice-cold Hanks buffer and mechanically harvested in RIPA buffer. The fluorescence of 100 uL aliquots of cell homogenate was determined on Spectramax Gemini fluorescence plate reader with excitation and emission spectra of 486 nm and 517 nm, respectively.
**Immunostaining:** H-ferritin treated oligodendrocyte cultures were fixed in freshly prepared ice-cold 4% PFA for 5 minutes. After wash in PBS, cells were air-dried and frozen at -70°C until use. Following the thaw, cells were washed in PBS and then blocked in 10% NGS overnight. Subsequently, the cells were incubated with anti-MBP at 1:200 dilution for 16 hrs, and after that exposed to anti-mouse-AF555 (Invitrogen) for 2 hrs at 4°C. Nuclear staining was detected by DAPI at 1:750 dilution for 2 hrs concurrently with the secondary antibody.

**Western blotting:** H-ferritin-treated and control cells were washed with PBS and mechanically collected in RIPA/1% TritonX buffer. Cells were homogenized by passing through 22 gauge needle five times, and incubated on ice for 90 minutes. Total protein extract was subsequently obtained by centrifuging at 10,000g for 10 minutes and harvesting the supernatant. Protein concentration was determined using Biorad DC protein assay and 20 ug of total protein separated using denaturing SDS-PAGE 4-20% Criterion (Biorad). Separation and subsequent transfer to nitrocellulose membrane was done using standard techniques. After blocking in 5% blotto, membrane was probed with either anti-myelin basic protein mouse monoclonal antibody (1:1000, Upstate), anti-olig 2 rabbit polyclonal antibody (1:1000, Chemicon) and anti-actin rabbit polyclonal antibody (1:2000, Sigma) for 16 hours at 4°C. Bands were visualized using appropriate HRP-conjugated secondary antibodies using the ECL reagent, and detected using FUJI system.
RESULTS:

To begin investigating role of iron delivery systems in oligodendrocyte survival, we developed the cell culture model. Purified rat primary oligodendrocyte progenitors were incubated in complete N2S medium (0.5% FBS, supplemented with Apo-Tf, PDGF and bFGF) for 2 days before the experiment. On the third day post-plating, the cells were exposed for 48 hr to either complete N2S medium or N2S where Tf had not been added (N2S low Tf), and the cell viability was determined using LDH release and MTT assays. Results in Figure 3.1 demonstrate loss of cell viability of OPCs after 48 hrs in culture deprived of Tf. Specifically, Tf-deprived cultures exhibited four-fold increase in measurable LDH content in the incubating media (Figure 3.1 A), while producing a 5-fold decrease in MTT absorbance (Figure 3.1 B).

To demonstrate that loss of cell viability observed in response to transferrin deprivation is due to iron deprivation, we performed an identical Tf deprivation experiment while adding either increasing concentrations of lipophillic compound TMH-ferrocene or ionic ferric ammonium citrate to the cultures at the time of transferrin withdrawal (structures of these two compounds in Figure 7.4). Results in Figure 3.2 demonstrate that in the absence of transferrin (and ferritin) in culture media, membrane-permeable TMH-ferrocene, but not membrane impermeable ferric ammonium citrate was able to prevent loss of cell viability after 48 hrs of exposure. Specifically, TMH-ferrocene treated cells showed a dose-dependent decrease in LDH release and MTT absorbance increase with the addition of TMH-ferrocene, with the highest concentration restoring respective values to almost control values. On the other hand, equimolar concentrations of FAC in identical experimental conditions had minimal effect on LDH release and no
effect on MTT values (Figure 3.2 C, D; respectively). These results demonstrate that TMH-ferrocene, but not ferric ammonium citrate (FAC) is able to support growth and survival of OPCs in absence of transferrin.

To investigate whether H-ferritin was sufficient to replace the transferrin requirement for iron delivery to oligodendrocyte progenitors, we performed a similar experiment in the presence of recombinant human H-ferritin. Transferrin-deprived cultures OPCs showed loss of cell viability as demonstrated by LDH release and MTT assays after 48 hrs, which was prevented in a dose-dependent fashion with recombinant H-ferritin being added to the media at the onset of Tfn deprivation (Figure 3.3). Specifically, the cells in low Tf media co-treated with the highest concentration of rH-ferritin used in the experiment (55 ug/ml) demonstrate LDH release (Figure 3.3 A) and MTT values (Figure 3.3B) almost completely identical to the controls (N2S complete). As an additional proof, we include brightfield images of the parallel experiment with OPC cultures in N2S complete (Figure 3.3 C), N2S low Tf (Figure 3.3 D) and low Tf supplemented with 55 ug/ml recombinant H-ferritin taken at 48 hrs post-treatment (Figure 3.3 E). The transferrin-deprived cells appear few in number with collapsed processes and numerous cells detached from the surface of the culture dish (Figure 3.3 D). At the same time, transferrin deprived, but rH-ferritin supplemented OPCs (N2S-Tf+ 55 ug/ml rH-ferritin) appear viable and firmly adherent to the dish (Figure 3.3 E), with morphology and cell number comparable to that of the controls (N2S complete medium).

To investigate whether the mechanism of the observed H-ferritin rescue is via iron delivery, we measured intracellular iron content using calcein AM method previously described (Lee et al. 2007; Tenopoulou et al. 2007). After 6 hrs of incubation, OPCs in Tf
deprived N2S media showed approximately 50% increase in the calcein fluorescence reading, which is consistent with iron deprivation of these cells. At that time point, no visible cell death is observed due to Tf deprivation (data not shown), so this was not a confounder in this experiment. Conversely, concurrent treatment of Tf deprived OPCs with 55 ug/ml H-ferritin under identical conditions resulted in quenching of the fluorescence signal to the baseline control (even slightly beyond, which consistent with increased intracellular iron) (Figure 3.4).

To evaluate the pro-myelinating potential of extracellular H-ferritin-mediated iron delivery, we treated purified cultures of oligodendrocyte progenitors in N2S complete media (containing Apo-Tf, PDGF and bFGF) with increasing concentrations of recombinant H-ferritin and allowed them to spontaneously differentiate over a 6-day period. The untreated cells served as control. At 6 days post-treatment, cells were evaluated for expression of myelin basic protein (MBP) and olig2 by immunohistochemistry and Western blot. The results in Figure 3.4 A demonstrate dose-dependent increase in MBP immunoreactivity in rH-ferritin treated cultures compared to untreated controls, while apparent cell number (as demonstrated by DAPI staining, Figure 3.4 A) was comparable between the treatment groups. The increase in MBP expression in response to rH-ferritin treatment was confirmed by Western blotting, which also demonstrated an increase in expression of MBP in H-ferritin treated cells compared to control. In addition, we observed an increase in total cellular levels of olig2 in response to rH-ferritin treatment, consistent with increased differentiation of these cells. Actin expression served as a loading control, and the levels are consistent between the treatment groups.
DISCUSSION:

In the current study, we investigated the role of H-ferritin and transferrin in iron delivery to developing oligodendrocyte progenitors in vitro. We demonstrate that Tf deprivation of primary OPCs results in loss of cell viability, which was reversed by TMH-ferrocene and H-ferritin treatment, but not ferric ammonium citrate. We demonstrate that Tf deprivation results in decreased iron content of OPCs, an effect which was prevented by treatment with recombinant H-ferritin. Finally, we demonstrate that rH-ferritin stimulates differentiation of oligodendrocyte progenitors, as evidenced by increased expression of MBP and olig2 proteins.

For almost three decades, transferrin has been considered essential for survival, growth and maturation of oligodendrocytes. Indeed numerous studies have demonstrated the importance of this protein in myelination and oligodendrocyte development both in vivo (Adamo et al. 2006; Badaracco et al. 2008; Escobar Cabrera et al. 1997; Espinosa-Jeffrey et al. 2002; Saleh et al. 2003) and in vitro (Espinosa de los Monteros and Foucaud 1987; Paez et al. 2006b). As early as 1986, Bottenstein defined transferrin as one of the essential component of chemically defined medium for oligodendrocytes in culture (Bottenstein 1986). In agreement with these studies, our results demonstrating loss of cell viability of primary rat oligodendrocyte progenitors upon transferrin deprivation after 24 hrs in culture (Figure 3.1), which was confirmed both by MTT and LDH release assays.

Furthermore, in Figure 3.2 we demonstrate that the effect of Tf deprivation can be prevented in dose-dependent manner by treating OPCs with TMH-ferrocene, but not the same molar concentrations of ferric ammonium citrate, suggesting that the loss of cell viability due to transferrin deprivation is due to iron deprivation. TMH ferrocene is a
synthetic iron compound that delocalizes the positive charge of iron ion through the conjugated double bond system of two cyclopentadiene ring complexes (Cable and Isom 1999; Malecki et al. 2002). As such, this compound is hydrophobic, able to cross the plasma membrane, and deliver iron directly to cells without involving iron transport proteins. On the other hand, FAC is unable to do so without a transport system such as Tf/Tf receptor or an ionic metal transport system such as d-cytb/DMT1. Our findings reinforce the notion that the functional role of transferrin in oligodendrocytes is iron delivery as membrane permeant TMH-Fe, but not FAC can support growth of OPCs in absence of transferrin in media of these cells.

Because H-ferritin receptors are expressed on developing oligodendrocytes (Hulet et al. 2000; Todorich et al. 2009; Todorich et al. 2008c) and because ferritin can deliver as much as 2000-fold the amount of iron per mole compared to Tf (Harrison 1996), H-ferritin is a candidate to be the major biological iron delivery system for oligodendrocytes. Previously we demonstrated indirectly that treatment of OPCs with recombinant H-ferritin was able to increase iron in labile iron pool by monitoring the response of iron regulatory protein activity in the cells to ferritin exposure (Hulet et al. 2000). In this study, we extended these findings by demonstrating that similar to TMH-Fe, H-ferritin treatment in dose-dependent manner was able to prevent loss of cell viability of OPCs in culture following transferrin deprivation. To further document that the mechanism responsible for these observations is iron delivered by H-ferritin, we measured the intracellular iron pool using calcein AM fluorescent dye. Transferrin deprivation resulted in increase in calcein fluorescence compared to control, consistent with a decrease in content of iron in the labile iron pool (inverse calcein quenching).
Adding 55 ug/ml H-ferritin to low Tfβ media at the onset of Tfβ deprivation quenched the calcein fluorescence back to the baseline control values (even slightly beyond), confirming that H-ferritin treatment was able to prevent loss of cellular iron induced by transferrin deprivation. These observations suggest that indeed H-ferritin and transferrin have a redundant and complementary role in oligodendrocyte progenitors, which is iron delivery. Neither of them alone therefore is essential per se, but rather what is essential is an adequate source of bioavailable iron to the developing oligodendrocyte cell. Our observations are very similar to finding by Gelvan et al (2006) showing that extracellular ferritin delivers iron to developing erythroid precursors, which can be used for synthesis of heme. Furthermore, evidence from a different group showed recently that extracellular ferritin iron delivery can partially replace the transferrin requirement of developing erythroid precursors, which were previously thought as having absolute essential requirement for transferrin (Leimberg et al. 2003; Leimberg et al. 2005; Leimberg et al. 2008). Together, our finding supports the concept that in at least two organ systems where iron is essential for normal development and function of those tissues (brain oligodendrocytes and bone marrow erythroid progenitors), multiple complementary and alternative iron import systems (transferrin and non-transferrin (ie ferritin)) have evolved to deliver iron to the cells.

Because the ultimate function of oligodendrocytes is to produce myelin, we evaluated the pro-myelinating potential of extracellular H-ferritin-mediated iron delivery in oligodendrocytes by treating OPCs with increasing concentrations of recombinant H-ferritin. Incubating primary rat OPCs in complete N2S media for 6 days with increasing concentrations of rH-ferritin resulted in a dose dependent increase in MBP
immunoreactivity, without changing the cell number (DAPI staining). This pro-
differentiating effect of H-ferritin was confirmed by Western blotting, which show
increase in expression of both MBP and olig2 proteins, consistent with increased
differentiation state of rH-ferritin treated cells. These observations are in agreement with
several studies, which have shown that increasing iron availability to differentiating
oligodendrocytes by treatment with transferrin was able to accelerate their differentiation
(Paez et al. 2004; Paez et al. 2006a; Paez et al. 2006b; Paez et al. 2002). It is very likely
in lieu of our collective findings in this study that the increased iron delivery to
differentiating oligodendrocytes is the common mechanism for the pro-myelinating
effects of H-ferritin and transferrin on these cells.

Our results have significant implications for oligodendrogliogenesis during
normal development and remyelination, and beg the question regarding the source of
ferritin for oligodendrocytes in the brain. Throughout first and second post-natal weeks,
microglia appear to serve as an iron capacitor—they are the predominant cell expressing
ferritin in distinct myelinogenic foci at P5 and P15, but as animal ages to P30 the H-
ferritin immunoreactivity shifts from microglia to oligodendrocytes (Cheepsunthorn et al.
1998), suggesting the possibility of a direct transfer of iron from microglia to
oligodendrocytes via secreted H-ferritin. In vitro, we have demonstrated that microglia
release H-ferritin and that there is a trophic effect on oligodendrocytes involving secreted
H-ferritin protein from microglia (Zhang et al. 2006). Recently, Schonberg, et al showed
that LPS-activated oligodendrogliogenesis in the rat spinal cord, is preceded by robust
infiltration of H-ferritin-positive microglia. In this model, there was also a gradual shift
of H-ferritin immunoreactivity from microglia to oligodendrocytes (Schonberg and
McTigue 2009; Schonberg et al. 2007). The results in the normal development model and the spinal cord injury model make it tempting to speculate that extracellular H-ferritin released by microglia is a major source of iron for both the developing and remyelinating brain.
FIGURES:

Figure 3.1. Transferrin deprivation of primary rat OPCs in chemically defined medium for oligodendrocytes (N2S) results in loss of cell viability after 48 hrs.

Primary rat OPCs were incubated either in complete N2S or the same without addition of apo-transferrin (N2S low Tfn) for 48 hrs. At that time point, cell viability was measured by LDH release and MTT assay. An aliquot of cell-free media was taken and LDH levels determined as demonstrated in (A) and subsequently adding MTT reagent for 4 hrs, and solubilizing in SDS detergent with absorbance measured at 595 nm (B) In both, statistical significance was evaluated using unpaired t-test (***, p<0.001), and results are representative of data obtained in three independent experiments. Both assays show the loss of cell viability of oligodendrocyte progenitors after 48 hrs in media deprived of Tfn.
Figure 3.2. Membrane-permeable TMH-Fe, but not ferric ammonium citrate (FAC), prevent loss of cell viability in OPCs cultures deprived of Tfn in chemically-defined medium (N2S). Primary rat OPCs were incubated either in complete N2S medium or the same without the addition of apo-transferrin (N2S low Tfn) for 48 hrs or N2S low Tfn supplemented with increasing concentrations of TMH-ferrocene (0.25, 1.0, 2.5, and 5 μM) or ferric ammonium citrate (FAC) at the same molar concentrations (0.25, 1.0, 2.5, and 5 μM) for 48 hrs. At the 48 hr time point, cell viability was measured by LDH release and MTT assay. An aliquot of cell-free media was taken and released LDH levels determined using Roche LDH kit (A, C) and subsequently adding MTT reagent to the cells for 4 hrs, and solubilizing in SDS detergent with absorbance measured at 595 nm (B, D). In both, statistical significance was evaluated using a one-way ANOVA with Dunnett’s post test comparison to N2S/low Tfn group (white column) (**, p<0.01). The results are representative of data obtained in three independent experiments. Collectively, these results demonstrate the loss of cell viability of oligodendrocyte progenitors after 48 hrs in media deprived of Tfn, which is reversed in dose-dependent manner by increasing concentrations of TMH-ferrocene, but not FAC.
Figure 3.3. Adding rH-ferritin replaces transferrin requirement in chemically-defined medium (N2S) of oligodendrocyte progenitors. Primary rat OPCs were incubated either in complete N2S medium or the same without addition of Apo-transferrin (N2S low Tfn) for 48 hrs or N2S low Tfn supplemented with increasing concentrations of rH-ferritin (0.11, 1.1, 11, and 55 ug/ml) for 48 hrs. At 48 hr time point, cell viability was measured by LDH release and MTT assay. An aliquot of cell-free media was taken and released LDH levels determined using Roche LDH kit (A) and subsequently adding MTT reagent to the cells for 4 hrs, and solubilizing in SDS detergent with absorbance measured at 595 nm (B). In both, statistical significance was evaluated using one-way ANOVA with Dunnett’s post test comparison to the N2S/low Tfn group (white column) (**, p<0.01). The results are representative of data obtained in three independent experiments. Representative images show OPCs in control N2S media (C), N2S low Tfn media (D) and N2S low Tfn media supplemented with 55 ug/ml rH-ferritin. Collectively, the results demonstrate the loss of cell viability of oligodendrocyte progenitors after 48 hrs in media deprived of Tfn, which is prevented in dose-dependent manner by increasing concentrations of rH-ferritin.
Figure 3.4. Recombinant H-ferritin prevents loss of cellular iron in cultures of primary oligodendrocyte progenitors deprived of transferrin.

Primary rat oligodendrocyte progenitors were incubated either in chemically defined, complete N2S medium supplemented with apo-transferrin (N2S), N2S media with no additional transferrin added (N2S-Tf) or N2S-Tf media supplemented with 55 ug/ml recombinant H-ferritin at the time of transferrin withdrawal (N2S-Tf+55 ug/ml rH-ferritin). The cells were incubated for 6 hrs, and subsequently exposed to 0.25 uM calcein AM dye for 45 minutes. Cells were homogenized in RIPA buffer, and relative fluorescence (RFUs) normalized to total protein. The group means were evaluated for significance using one-way ANOVA followed by Bonferonni multiple post-test comparisons. Indicated comparisons were statistically significant (*, p<0.05; **, p<0.01).
Figure 3.5. H-ferritin increases differentiation of oligodendrocyte progenitors in culture. Purified OPCs in N2S complete media were treated with increasing concentrations of H-ferritin for 6 days in culture. Untreated cells served as control. At that time point, cells were fixed and stained for myelin basic protein (MBP) and DAPI (nuclei). Representative images of 4 independent experiments are shown in (A) and suggest that rH-ferritin treatment increases quantity of MBP produced by oligodendrocytes without altering cell number (DAPI staining). (B) Western blot analysis of rH-ferritin treated OPCs demonstrates a strong increase in MBP and increase in olig2 expression in response to rH-ferritin treatment, which is consistent with increased differentiation of oligodendrocytes. Actin was used as a loading control.
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Chapter 4

Vanadium-induced destruction of oligodendrocyte progenitors reveals novel connections to ferritin and iron: the mechanism of vanadium-mediated developmental demyelination?

ABSTRACT:

The second post-natal week in rats is the period of most intense oligodendrocyte development and myelin production and coincides with peak iron import into the CNS and oligodendrocytes. During that time oligodendrocyte progenitors are sensitive to agents that may disturb normal iron homeostasis and assimilation into these cells. One mechanism by which iron homeostasis can be disrupted is by environmental exposure to other metals. Vanadium is a transition metal, and exposure to vanadium during early brain development produces hypomyelination with variety of related neuro-behavioral phenotypes. In the current study we investigated cellular and molecular mechanisms of hypomyelination induced by vanadium exposure in developing rat brain. We demonstrate that both \textit{in vivo} and \textit{in vitro}, oligodendrocyte progenitors (OPCs) are more sensitive to vanadium exposure than astrocytes or mature oligodendrocytes. Vanadium exposure in OPCs resulted in increased measurable ROS generation and increased annexinV labeling of OPCs suggestive of apoptosis. Exposure to recombinant ferritin and iron independently exacerbated vanadium cytotoxicity, while iron chelator desferroxamine (DFO) prevented cytotoxic/apoptotic effects of vanadium. To definitively illustrate a relationship between ferritin and vanadium, we demonstrate that vanadium induced
release of highly red-ox active iron from ferritin and exacerbated DNA nicking produced by iron-rich spleen ferritin, but not iron-poor apoferritin, resulting in single and double strand breaks, in a DNA relaxation assay. As oligodendrocyte progenitors express endogenous H-ferritin and assimilate extracellular ferritin during development, we propose that developmental exposure to vanadium interferes with normal iron assimilation into intra- and extra-cellular ferritin in oligodendrocytes resulting in elevated oxidative stress and their apoptosis. Therefore, depletion of OPCs due to vanadium exposure in early post-natal period may be an important mechanism contributing to vanadium-induced hypomyelination.
INTRODUCTION:

Vanadium is a metal in the first transition series and it is widely distributed in nature (Nriagu and Pacyna 1988). While some derivatives of vanadium have been found useful in medicine and the industry (Ray et al. 2006), acute environmental and occupational exposure to this metal continues to be a health risk to humans and animals (Shrivastava S 2007). Vanadium emission owing to human industrial activities has been estimated to comprise about 53% of total atmospheric vanadium (Hope 1994). A major source of vanadium toxicity remains the burning of fossil fuels as seen in the Arabian Gulf (Haider et al. 1998) and the Niger Delta region of Nigeria (Igado 2008) where massive incidental and or intentional burning of vanadium containing fuels results in pollution of confined ecosystems.

Little is known about tissue and cellular mechanisms of vanadium toxicity. Vanadium induces oxidative stress caused by free radical generation and this action has been strongly linked to the majority of the toxic and molecular effects of vanadium compounds on biological systems (Evangelou 2002). Antioxidants such as vitamin E (α-tocopherol) and its derivatives, ascorbic acid and catalase are substances that have been shown to directly or indirectly protect cells against adverse effects of toxic radical reactions resulting from vanadium exposure (Mates 2000). Vanadium at the +5 oxidative state (metavanadate) entering the biological system is reduced to vanadyl (IV) by glutathione in erythrocytes or by ascorbic acid and other reducing substances in plasma and then transported by albumin and by transferrin, one of the iron transport proteins with which vanadium associates with selectively (Sabbioni and Marafante 1981) (Degani et al. 1981). At the cellular level, vanadium (V) enters the cell through anion transport...
mechanisms (Heinz et al. 1982) and/or the transferrin/transferrin receptor system (Nagaoka et al. 2004), and becomes reduced to vanadyl mainly by intracellular glutathione (Sabbioni et al. 1993). Although there is little data in relation to vanadium interactions with iron within biological systems, it is possible to speculate based on the fact that absorbed vanadium is bound in part to transferrin that vanadium could interfere with the metabolism and storage of iron and vice-versa (EVM 2002).

One of the major pathologic effects of vanadium on the CNS appears to be myelin disruption (Garcia GB 2004). Treating adult rats with vanadium results in histological disruption of myelin, which is accompanied by increased in lipid peroxidation in discrete brain areas (Garcia et al. 2004; Sasi et al. 1994). The developing brain was also found to be extremely sensitive to hypomyelinating effects of vanadium toxicity. Nursing pups from dams, which were treated with 3mg/kg sodium metavanadate during the early postnatal period showed a variety of behavioral defects with motor impairments (Soazo and Garcia 2007). The behavioral alterations were accompanied by and attributed to significant myelin pathology—decreased thickness of corpus callosum and decreased staining of MBP in corpus callosum and cerebellum of litters of vanadium treated dams compared to controls (Soazo and Garcia 2007). Although this study clearly demonstrated causal connection between vanadium toxicity and disruption of myelin morphology, the relevant question that remains unanswered is the role of oligodendrocytes, the myelin producing cells in the mechanism of vanadium-induced myelin pathogenesis.

Oligodendrocytes are the cells in CNS capable of producing myelin (Trapp 2004). They develop from neural stem cells, and originate in the subventricular zone and subsequently proliferate and migrate throughout the developing brain (Nishiyama et al.
The second post-natal week in the rat represents the period of highest proliferation, differentiation and myelin production by oligodendrocyte cells (Cammer 1984). Coincidentally, this period is also associated with the highest iron import into oligodendrocytes (Crowe and Morgan 1992; Taylor and Morgan 1990). Oligodendrocytes are the principal cells in the brain that stain for iron (Benkovic and Connor 1993; Connor and Menzies 1996; Todorich et al. 2008a); and developmental iron deficiency leads to hypomyelination (Larkin 1990; Ortiz et al. 2004); (Beard et al. 2003). A comprehensive review on the role of iron and iron transport proteins in oligodendrocyte development and myelination has recently been published (Todorich et al. 2008a) (chapter 1). In brief, oligodendrocytes have evolved two independent iron import mechanisms—transferrin and ferritin. The classical transferrin/transferrin receptor system operates in oligodendrocyte progenitors, but appears to be downregulated as oligodendrocytes mature (Todorich et al. 2008b). Ferritin is an alternative iron import system in OPCs, which may persist through different states of oligodendrocyte differentiation (Hulet et al. 1999a; Hulet et al. 2000; Hulet et al. 2002; Hulet et al. 1999b). In addition to assimilating iron through endocytosis of extracellular ferritin, oligodendrocytes also express high levels of intracellular ferritin (Connor et al. 1994; Connor and Menzies 1996; Connor et al. 1990)(Cheepsunthorn et al. 1998). Increase in H-ferritin protein and mRNA expression has been noted in oligodendrocyte adhesion in vitro (Sanyal et al. 1996) suggesting that onset of intracellular expression of ferritin by OPCs is somehow important for their development, myelination and axonal engagement. Although the precise role of intracellular ferritin in oligodendrocyte physiology, development and myelination is unknown, the possibilities include iron mobilization within the cell during
peak myelination and cellular protection against oxidative injury (Sanyal et al. 1996; Todorich et al. 2008a) and thus any factor that would perturb fine balance between iron assimilation from extracellular transferrin and ferritin into intracellular ferritin and myelin producing proteins during oligodendrocyte development may cause increase in the oxidative stress and oligodendrocyte death with consequent hypomyelination. We hypothesized that developmental exposure to vanadium causes hypomyelination via destruction of oligodendrocyte progenitors by virtue of interfering with iron assimilation and increasing iron release from ferritin, resulting in oxidative stress and apoptosis of OPCs.

In the current study we show that vanadium exposure in early postnatal period results in destruction of oligodendrocyte progenitors, but not astrocytes both in vivo and in vitro. We also demonstrate that vanadium exposure produces a cytotoxic effect on OPCs resulting from increase in oxidative stress and apoptosis of OPCs. Finally we show that vanadium induces release of iron from ferritin and that ferritin and iron exposure exacerbate the cytotoxic effects of vanadium on OPCs. Considering the established role of ferritin in iron homeostasis of developing oligodendrocytes, our findings suggest that high content of iron and ferritin in developing oligodendrocytes make them uniquely vulnerable to vanadium exposure, and that the destruction of OPCs may be the key mechanism contributing to vanadium-induced developmental hypomyelination.
MATERIALS AND METHODS:

Reagents--Sodium metavanadate (Sigma) was obtained as powder and diluted with PBS to a stock solution of 2.5 mM, which was kept in aliquots at -20°C, and used as needed. Desferroxamine (Sigma) was dissolved in water and frozen 100 mM aliquots of stock solution were used for individual experiments.

Animals—Pregnant Sprague-Dawley rat dams at two weeks of gestation were purchased from Charles River Laboratories, and housed at Pennsylvania State University College of Medicine Animal Core facilities. All experiments on animals were performed in accordance with ethical standards and Institutional animal care and use committee (IACUC, protocol numbers: 98-007; 2008-022). Starting at P1, rat pups (N=5) were injected with 3 mg/kg Na-metavanadate IP once daily for 14 days as in (Garcia et al. 2004b; Soazo and Garcia 2007). These concentrations were previously shown to induce hypomyelination in both adult and developing rat pups (Garcia et al. 2004; Soazo and Garcia 2007). Littermate controls (N=5) receiving the same volume of saline were used as controls. The pups were housed with their dams with ad-libitum diet during the study and their weights were measured daily on a benchtop scale. At P15, the rotarod testing of motor function was performed on the vanadium-treated and control rats. Briefly, all animals were taken through a training period on the rotarod, and subsequently tested in three independent trials with extensive rest in between the trials. The outcome measured was time it took for the rat to fall off the rotarod (at a speed of 5 rpms). The rotorod performance was scored by two investigators blinded to the experimental condition. The times of three trials were averaged across each group and evaluated for significance using unpaired t-test. Subsequently, animals were anesthetized and perfused with ice-cold PBS.
(pH=7.4) for 10 minutes. Brains were removed and placed in 4% paraformaldehyde for 24 hours. Subsequently after a brief rinse in PBS, brains were taken through the sucrose gradient (10%, 20%, and 30% sucrose) and kept frozen at -80°C. The brains were sectioned in cryostat starting at mid-sagittal plane laterally at 20 um thickness, with every other section used for immunohistochemical study.

**Immunohistochemistry**—frozen sections were thawed, and immersed in PBS. Antigen retrieval was performed in 10 mM citrate buffer for 25 minutes, with subsequent peroxidase quenching in 3% H2O2/methanol. All the sections were blocked in 2% milk overnight and probed either with anti-NG2 rabbit polyclonal antibody, 1:500 (Millipore) or anti-GFAP mouse monoclonal antibody 1:400 (Sigma) diluted in 2% milk for 16 hours at 4°C. The detection of bound antibody was via HRP-conjugated secondary antibodies (VECTASTAIN, (Vector Labs) according to the manufacturer’s protocol. The reaction product was enhanced with DAB for 6-10 minutes, with subsequent dehydration in ethanol. Images were acquired on Nikon brightfield scope equipped with a digital camera. To quantitate the number of oligodendrocyte progenitors and astrocytes in each treatment group, random sections (four per animal, total of N=5 animals per treatment group) of genu corpus callosum stained for NG2 and GFAP markers were counted by two blinded investigators at 40X magnification. The cell counts were expressed as number of cells per 40X field and represent pooled data from two independent investigators. The data were evaluated for statistical significance using unpaired t-test.

**Primary cultures**—were prepared from newborn rat pups of CRL SD rats according to standard protocol of McCarthy and DeVellis (1980). Briefly, newborn rat pups were decapitated and heads immersed in 70% ethanol, following by extensive wash with
Hank’s balanced salt solution. The brain was extracted and meninges carefully removed. The cerebral tissue was diced and trypsinized for 30 minutes. Following digestion with DNAse I, the tissue homogenate was passed through 150 and 75 um Nitex screens. Filtered cells were resuspended in plating medium (DMEM/10% FBS) and cultured for 8 days. To purify different types of glia, mixed glial cultures obtained in this way were subjected to a serial shaking purification protocol (McCarthy and de Vellis 1980). First, microglia were removed by shaking for 1 hour at 265 RPMs. Subsequently, oligodendrocyte progenitor cells were removed by shaking for 18 hour at 265 RPMs. The remaining astrocytes were allowed to recover for one day, and then collected by trypsinization, counted and plated for experiments in poly-d-lysine coated 24-well plates. Oligodendrocyte progenitor fraction was subsequently further purified by differential adhesion through incubation in Petri dish for 30 min to remove more adherent astrocytic and microglial contamination. This procedure routinely yielded >90% pure population of OPCs (verified by A2B5 staining, not shown), which was then re-plated in poly-d-lysine coated 24 well plates or 4-well chamber slides and used for experiments.

**Immunostaining of OPCs for H-ferritin**—Primary rat OPCs plated in chamber slides were washed in PBS fixed for 5 minutes in ice-cold 4% paraformaldehyde for 5 minutes. After 2 washes in PBS, cells were air dried and frozen at -80°C. The day of the experiment, cells were thawed and permeabilized in 0.1% TritonX for 5 minutes. After 3 washes in PBS, cells were blocked in 10% NGS for 16 hours, with exposure to primary antibodies diluted in 1.5% normal goat serum for 16 hours. The primary antibodies used were anti-H-ferritin rabbit polyclonal Ab 1:100 (homemade, (Thompson et al. 2003)) and anti-A2B5 mouse IgM 1:100 (R&D Systems). Nuclei were stained with DAPI at 1:750.
dilution (Invitrogen). Staining was visualised with anti-rabbit-AF488 and anti-mouse IgM-AF555 antibodies (Molecular probes) at 1:150 dilution for 1.5 hrs. Images were acquired at Leica confocal microscope.

**Cytotoxicity assays and apoptosis analysis**—Primary cells (oligodendrocytes or astrocytes) were treated with Na-metavanadate or Na-metavanadate plus DFO at different concentrations. Untreated cells (with vehicle) served as control. In all experiments, cells were exposed to these experimental conditions for 48 hours, unless indicated otherwise. The cell viability was determined using MTT assay according to manufacturer’s protocol, and absorbance measured on plate reader at 595 nm. All absorbance values were normalized to control to obtain percent survival. Alternatively, for apoptosis detection cells were washed with PBS (pH=7.4) for 5 minutes and then exposed to Annexin V-AlexaFluor488 (Molecular probes) in Annexin binding buffer at (manufacturer recommended) 1:25 dilution for 40 minutes. Cells were washed in binding buffer and visualized under Nikon fluorescence microscope. Representative images of Annexin V binding to treated OPCs in random fields were reproduced, and annexin V-labeled cells counted in five random fields for each experimental condition in triplicates by an experimenter blinded to the experimental condition. The counts in the different experimental groups were averaged and evaluated for statistical significance using one-way ANOVA.

**Intracellular ROS measurements**—Primary rat OPCs were plated at a concentration of 0.5 million cells per well in 6 well plates. The cells were treated with 100 uM Na-metavanadate in N2S media for 6 hrs. Subsequently, cells were washed in warm Hank’s buffer and incubated with 10 uM 2’-7’-dichlorofluorescein (DCF; Calbiochem) diacetate
dye for 1 hour as previously described (Mawatari et al. 1996). After incubation, the cells were collected mechanically, resuspended in PBS buffer and aliquoted in 96 well plates. Fluorescence was measured on Gemini SpectraMAX plate reader with 495 nm excitation and 515 nm emission spectra. The obtained fluorescence values were averaged and evaluated for statistical significance using unpaired t-test.

**Recombinant ferritins**—Purified horse spleen ferritin (SF) and Horse spleen apoferritin (Apo-SF) were obtained from Calbiochem. Recombinant human H-ferritin cDNA tagged with poly-histidine was subcloned into pET plasmid, amplified, verified by sequencing. The plasmid was transformed into competent BL21 E. Coli and protein overexpression induced with IPTG (isopropyl-β-D-thio-galactoside) when the OD value of bacterial culture reached 0.7. The recombinant H-ferritin was purified over Nickel column (Pierce) according to the manufacturer’s protocol and identity and purity verified by Western blot and Comassie staining of denaturing SDS PAGE gel, respectively. We verified presence of ferroxidase activity of this recombinant protein by measuring rate of oxidation of ferrous into ferric iron at 310 nm absorbance (Figure 4.7 B).

**Ferritin-iron release and DNA relaxation assay**—Supercoiled DNA relaxation assay was used to determine mechanism of vanadium-induced iron release from ferritin as we have described previously (Surguladze et al. 2004). The pUC19 supercoiled DNA plasmid (New England Biolabs) was incubated with vanadate alone or various recombinant ferritins either alone or in presence of metavanadate. Reaction mixtures (20-30 uL) contained DNA (0.5 or 1 ug), dissolved in 10 mM HEPES (pH 7.5), 50 mM NaCl, 2.5 mM MgCl₂, 2.5 mM DTT. Reactions with ferritins, DNA and vanadium were incubated for increasing time duration and quenched by addition of 10 µL of 50%
glycerol, 50 mM EDTA, 50 mM EGTA and 0.1% bromophenol blue. The samples are separated using 1.5% agarose gel run in 40 mM Tris-acetate, 1 mM EDTA (TAE), pH 8.0. The DNA bands were visualized using SYBR green staining (Invitrogen) according to manufacturer’s protocol.

RESULTS:

To investigate the *in vivo* effects of vanadium exposure on myelin producing cells in CNS, we exposed newborn rats to daily injections of 3 mg/kg sodium metavanadate IP during first two post-natal weeks of development while littermate rats of same sex receiving same volume of PBS IP served as controls. The vanadium treated rat pups had a comparable, although slightly decreased, rate of weight gain in the first two weeks of development (Figure 4.1 A). However, the pups receiving vanadium injections had a substantial impairment in motor function as demonstrated by rotarod testing at P15 (Figure 4.1 B). Upon histological examination of the brains of the P15 rat pups, we observed a 2.5 fold decrease in the number of NG2-positive oligodendrocyte progenitors in genu of corpus callosum of vanadium treated rats compared to controls (Figure 4.2 A; B, E). On the other hand, the number of astrocytes in the same region of corpus callosum was increased in the vanadium treated group (Figure 4.2 C, D, F). Astrocytes of the vanadium treated group had more extensive branching and more “aggressive” appearance suggestive of activation (Figure 4.2 C, D).

The *in vivo* data suggested that vanadium was cytotoxic to oligodendrocytes, but stimulated astrogliosis. To investigate relative sensitivity of different glia to cytotoxic
effect of vanadium compounds, we obtained primary rat cultures enriched in oligodendrocyte progenitor cells, mature oligodendrocytes and astrocytes. The purified cultures of different glia (OPCs, mature oligodendrocytes and astrocytes) were exposed to increasing concentrations of Na-metavanadate for 48 hours. The cell viability was determined using MTT assay, and absorbance normalized to control (untreated) to obtain percent survival. By comparing the same concentration of metavanadate treatment across different cells types, we found that oligodendrocyte progenitor cells were most vulnerable to vanadium, while mature oligodendrocytes and primary astrocytes were relatively resistant. Primary OPCs had a LD50 of approximately 75 uM, while the LD50 was approximately 200 uM for primary astrocytes and mature oligodendrocytes (Figure 4.3, A-C).

To begin to examine the mechanism of cell stress and cytotoxicity induced by vanadium exposure in OPCs, we measured ROS generation in OPCs using dichlorofluorescein dye (DCF). At 6 hours post-treatment, exposure to Na-metavanadate in OPCs resulted in nearly 3 fold increase in DCF fluorescence compared to baseline in control (untreated cells) (Figure 4.4 A). To determine whether vanadium-induced cell death is through apoptosis, we measured extent of phosphatidyl serine inversion at the membrane by AnnexinV labeling. At 12 hrs post-treatment, 100 uM Na-metavanadate produced 10 fold increase in number of annexinV-positive cells compared to the untreated control. These data suggests that cytotoxic mechanism of vanadium exposure is apoptotic cell death of OPCs.

To investigate a possible relationship between iron and vanadium toxicity in oligodendrocyte progenitors, we exposed primary rat OPCs to 100 uM Na-metavanadate
with or without concurrent treatment with increasing concentrations of iron chelator desferoxamine (DFO) for 48 hours, and then analyzed morphology and cell viability using brightfield microscopy and MTT assay, respectively. Vanadium treated oligodendrocyte progenitor cells showed process retraction, membrane blebbing, fragmentation, and detachment from the surface of the dish at 48 hr post-treatment compared to control (untreated) cells, which appeared adherent and with extended and ramified processes (Figure 4.5 A, B). The cell morphology including branching was restored when 100 uM DFO was co-incubated at the time of vanadium treatment (Figure 4.5 C). In Figure 4.5 D, we confirm these findings and demonstrate that 100 uM vanadium kills 70% of primary OPCs, an effect that was partially reversed by different concentration of iron chelator DFO as demonstrated by MTT assay. To determine the effect of DFO on apoptotic indices of vanadium in OPCs, we exposed these cells to 100 uM Na-metavanadate with or without 100 uM DFO for 12 hours. Vanadium treatment resulted in increased annexinV green fluorescence label compared to control (Figure 4.5 E). Concurrent treatment with iron chelator DFO significantly decreased annexin V staining to almost baseline levels (Figure 4.5 E).

Because of the well-established role of H-ferritin in iron delivery and homeostasis of developing oligodendrocytes, we decided to further explore the relationship between ferritin and ferritin-delivered iron in the cytotoxic mechanisms of vanadium on oligodendrocytes. We prepared His-tagged recombinant human H-ferritin in BL21 E. Coli and purified it over Nickel column as described previously (Todorich et al. 2008b). The identity of recombinant protein was verified by Western blot (anti-human H-ferritin HS-59 monoclonal antibody, generous gift from Dr. Paolo Arosio) and purity by
Comassie staining of SDS PAGE gel with 10 ug of protein loaded per well (Figure 4.6 A). We tested whether the purified recombinant H-ferritin possessed intact ferroxidase activity using method previously described (Levi S et al. 1988). The results in Figure 4.6 B show that presence of recombinant H-ferritin increase the rate and the amount of ferric iron formation compared to baseline auto-oxidation of the same in PBS buffer alone, suggesting that recombinant H-ferritin produced had significant ferroxidase activity and ability to incorporate iron in the solution. We then used this recombinant H-ferritin in the vanadium cytotoxicity experiments. Primary rat OPCs were treated either with 100 uM Na-metavanadate alone or in the presence of increasing concentration of recombinant human H-ferritin for 48 hours, and the cell viability at the time measured using MTT assay. The results summarized in Figure 4.6 C show that increasing concentrations of recombinant H-ferritin exacerbated cytotoxic effect of Na-metavanadate on primary rat OPCs as measured by MTT assay. The same concentrations of recombinant H-ferritin without vanadium had no significant effect on viability of OPCs as evaluated by MTT assay. Similarly, when we incubated primary OPCs with increasing concentrations of TMH-ferrocene (lypophyllic compound that can deliver iron into cells independent of iron-import proteins), we obtained similar dose-dependent synergistic effect to the vanadium toxicity in OPCs (Figure 4.6 D). Again, the same concentrations of TMH-ferrocene alone in this experiment did not decrease cell viability of OPCs. Collectively, these data suggested an important connection and interaction between iron and iron storage/delivery protein H-ferritin in cytotoxic mechanism of vanadium.

Because of their high iron content and high iron utilization, mature oligodendrocytes express high levels of endogenous H-ferritin (Cheepsunthorn et al.
1998; Connor et al. 1994; Connor and Menzies 1996; Connor et al. 1990). To show that this is also true in oligodendrocyte progenitors, we immunostained OPCs for H-ferritin and colocalized it with A2B5 marker of OPCs. In Figure 4.7 we demonstrate that H-ferritin immunoreactivity within A2B5-positive bipolar oligodendrocyte progenitor cells in culture is primarily localized in perinuclear region of the cell body, which is consistent with the cytosolic distribution of endogenous cellular H-ferritin. There was minimal staining of the cell processes and no staining in cells where primary antibody to H-ferritin was omitted (data not shown). These results raise the possibility that vanadium may interfere with iron homeostasis by endogenous (cytosolic) ferritins in addition to iron assimilation through extracellular ferritins in oligodendrocyte progenitors. To characterize the mechanism by which that may happen, and specifically to test whether the exacerbating effect of ferritin on vanadium mediated cytotoxicity is due to iron release, we used a DNA relaxation/nicking assay previously characterized in our lab (Surguladze et al. 2004). This assay uses supercoiled DNA that runs as a low-MW ladder in the control group. However, agents (such as free iron) that produce free radicals can result in single strand breaks (resulting in relaxed form, high MW band) or double strand breaks (resulting in linear form, intermediate MW band) (Surguladze et al. 2004).

Incubating supercoiled DNA plasmid (puc19) with increasing concentration of vanadium (25-200 uM) alone failed to produce either relaxed or linearized DNA (Figure 4.8, a-d). Incubating puc19 plasmid with horse spleen ferritin (iron-rich) alone resulted in time-dependent conversion to relaxed form (single strand nicking), but no visible linearization (double strand nicking (Figure 4.8, e1-e7)). However, this effect was greatly augmented when iron-rich spleen ferritin was coincubated with 200 uM Na-metavanadate, increasing
amounts of relaxed DNA (arrow 3) and linear DNA from double nicks (arrow 2) (red box). No DNA nicking was produced in apo-spleen ferritin alone or in combination with 200 uM Na-metavanadate (Figure 8, g1-7; h1-7). These data suggested that vanadium produced synergistic effect on ferritin, resulting in increased iron release to produce free-radical mediated DNA strand breaks in this assay.

**DISCUSSION:**

In this study, we investigated cellular and molecular mechanisms of hypomyelination due to developmental vanadium exposure. We demonstrate that oligodendrocyte progenitors are most vulnerable to environmental vanadium exposure both in vivo and in vitro, suggesting that injury to OPCs during early postnatal period represents a contributory cellular mechanism to vanadium-induced hypomyelination. Furthermore, we show that vanadium destruction of OPCs involves increased oxidative stress and apoptosis of these cells. The apoptotic indices and cytotoxicity of vanadium on OPCs were significantly decreased by concurrent exposure to iron chelator DFO, but increased with exposure to recombinant H-ferritin and lipophillic iron delivery compound TMH-ferrocene, which suggested a role for iron and ferritin iron delivery in cytotoxic mechanisms of vanadium. Finally, in the DNA relaxation assay, we demonstrate that vanadium causes release of highly red-ox active iron from ferritin, resulting in DNA relaxation and nicking. Because of the well established relationship of oligodendrocyte progenitors to iron and ferritin during development (they import iron
through ferritin in early development, and express significant levels of endogenous ferritin), we propose that this relationship makes OPCs uniquely vulnerable to pro-apoptotic effects of vanadium exposure.

*In vivo* experiments on vanadium neurotoxicity have suggested that hypomyelination is one of the main pathological outcomes in the brain of toxic vanadium exposure (Garcia GB 2004). It appears that vanadium exposure produced hypomyelination both in adult and developing brain, although the mechanism by which each of those are produced may be distinct. Relevant to developmental hypomyelination, Soazo and Garcia (2007) showed that pups consuming milk from vanadate treated dams for the first three weeks of development showed evidence of demyelination/hypomyelination in discrete brain regions by postnatal day 21. We hypothesized that hypomyelinating effects of vanadium exposure during this early period would be due to destruction of oligodendrocytes. To test our hypothesis *in vivo*, we injected newborn rat pups with 3mg/kg of sodium metavanadate IP once daily in for the first two post-natal weeks of development. Rat pups receiving vanadium injections all survived and had comparable, albeit slightly reduced, rate of weight gain during this period. However, the significant motor performance deficits in vanadium treated pups when compared to vehicle-treated controls as early as P15 suggested severe neurological pathology. In support of our hypothesis, cell count data indicate that metavanadate treatment in the early post-natal period results in 2.5 fold decrease in number of NG2 positive oligodendrocyte progenitors in genu corpus callosum. In contrast, the astrocyte population in the same region (genu corpus callosum) was increased. This astrogliosis observed in the vanadium treated rats may have been a result of astrocyte activation in
response to parenchymal (oligodendroglial) injury and/or initiated inflammatory response, which could have contributed to further destruction and depletion of oligodendroglial population. In toto, our in vivo observations suggest that oligodendrocyte progenitors, but not astrocytes are more susceptible to vanadium exposure in early post-natal period, suggesting that loss of OPCs may contribute to hypomyelination due to developmental vanadium exposure.

To explore the vanadium mediated mechanisms of injury to oligodendrocytes we developed a cell culture approach. Our findings in vitro reinforced our observations in vivo. In culture, vanadium was toxic to the glial cells in dose-dependent manner. Primary oligodendrocyte progenitors were more sensitive to vanadium exposure than other glia (mature oligodendrocytes or astrocytes). Greater sensitivity of primary oligodendrocyte progenitors compared to mature oligodendrocytes and astrocytes may be attributed to a much lower expression of antioxidant defense systems (particularly glutathione) (Hemdan and Almazan 2007; Husain and Juurlink 1995; Juurlink et al. 1998; Thorburne and Juurlink 1996) in OPCs relative to astrocytes and mature oligodendrocytes. Indeed, we observed that vanadium exposure to OPCs resulted in 2.5 fold increase in generation of reactive oxygen species. It is likely that vanadium induced oxidative stress because it can generate ROS either directly via the Fenton-like reaction (Evangelou 2002) or by displacing/releasing red-ox active iron from ferritin stores (Figure 4.8) as this was also suggested previously (Monteiro et al. 1991). Furthermore, the exposure of OPCs to vanadium resulted in increased in annexinV labeling of these cells, suggestive of phosphatidylserine inversion at the plasma membrane and apoptosis of oligodendrocytes. Concurrent treatment of OPCs with the iron chelator, DFO during vanadium exposure
reversed both cytotoxicity, cell morphology and process branching (Figure 4.5 A-D), and apoptotic indexes of vanadium injury to OPCs (Figure 4.5 E). Although a direct chelating effect of DFO on vanadium could not be ruled out, the remarkable protection of DFO from the sodium metavanadate induced apoptotic death on OPCs suggested the possibility of involvement of iron and iron-associated proteins in cytotoxic mechanisms of vanadium in OPCs. Indeed, adding iron in form of TMH-ferrocene (lyophillic iron delivery compound) to OPCs exacerbated the cytotoxic effect of vanadium (Figure 4.6). Adding recombinant H-ferritin to the OPCs, however, had a similar effect of exacerbating vanadium toxicity in OPCs. These data suggest that exposure to vanadium at developmental time points when developing oligodendrocytes assimilate iron through ferritin may be detrimental to these cells, as vanadium appears to synergistically interact with ferritin in this model.

We previously demonstrated that extracellular ferritin can deliver iron to OPCs (Hulet et al. 2000; chapter 3), that ferritin receptors are selectively present on oligodendroglial cells (Hulet et al. 2000; Todorich et al. 2008a; Todorich et al. 2008c) and that distribution of ferritin receptors temporally and spatially overlaps with onset of myelination (Hulet et al. 2002). In addition, oligodendrocytes also express endogenous cytosolic H-ferritin (Connor et al. 1994; Connor and Menzies 1996; Connor et al. 1990), and onset of expression of this protein during the development in oligodendrocytes is P15 (peak myelination and iron import into the brain) (Cheepsunthorn et al. 1998). Therefore, our finding that endogenous H-ferritin is also expressed in A2B5-positive oligodendrocyte progenitors in culture (Figure 4.7) suggested a possibility that vanadium may interfere and directly interact with cytosolic ferritin iron stores, resulting in release
of highly red-ox active iron. To examine this possibility, we used supercoiled DNA relaxation assay in which ferritin-dependent DNA nicking and relaxation is highly dependent on free radical generation by red-ox active iron released from ferritin (Surguladze et al. 2004). In this experiment, we show that vanadium stimulated time-dependent DNA nicking and generation of single and double stranded DNA breaks when incubated with iron-rich spleen ferritin, but not in iron-poor apo-ferritin. Vanadium alone at those concentrations did not have any effect on either single stranded or double stranded DNA breaks. As DNA nicking in this assay is highly dependent on iron-mediated free radical production (Surguladze et al. 2004), these data strongly suggest that vanadium induces release of iron from ferritin supporting the notion that this effect may underlie the observed synergy between ferritin, iron and vanadium cytotoxicity in oligodendrocytes.

It is still unclear ultrastructurally how vanadium promotes iron release and/or inhibits iron assimilation into ferritin in the oligodendrocyte cell. One possibility is that the larger ionic radius of vanadium (relative to iron) results in structural changes of the ferritin 24-mer as ferritin attempts to sequester vanadium from solution. This effect would impair ferritin function in iron uptake and promotes iron release and/or target ferritin for degradation with subsequent iron release. This possibility is supported by findings that various metals indeed can inhibit ferroxidase activity (Huber and Frieden 1970). Another possibility is that vanadium (as vanadyl) would release ferrous iron (Fe²⁺) directly through direct redox interaction with the ferric hydrite crystals at the ferritin core (Boyer et al. 1988) or by oxidative modification and alteration of the ferritin shell (Kang 2009). Nonetheless, it is clear that vanadium interacts with ferritin to promote release of
red-ox active iron, which exacerbates oxidative stress leading to increased cellular toxicity. High levels of ferritin within OPCs as well their assimilation of iron through endocytosis of extracellular ferritin at developmentally critical periods may uniquely predispose their destruction due to vanadium exposure at peak myelination.

In toto, we report novel in vivo and in vitro evidence of cellular and molecular mechanisms that may contribute to demyelination caused by vanadium exposure in early post-natal period. Our findings suggest that vanadium mediated injury to oligodendrocyte progenitors in early postnatal period and their unique relationship to ferritin may contribute to if not be the mechanism of vanadium-mediated hypomyelination.
FIGURES:

Figure 4.1. Vanadium exposure during early post-natal period leads to impaired motor functioning. Newborn rat pups were injected with 3 mg/kg body weight of Na-metavanadate solution IP once per day for 14 days (N=5). Injection of equal volume of PBS to littermate siblings served as control (N=5). Each day, weight of the pups was measured and plotted (A) and the means of the weights compared for significance using t-tests at each time point (ns, not significant; *, p<0.05; **, p<0.01; ***, p<0.001). At PND 15, vanadium-treated and control rats were subjected to rotarod testing of motor function. Each rat was evaluated in 3 independent trials with rest periods in between. The time from start of rotarod to the moment that the rat fell off the rotarod was recorded by two blinded investigators. The values were averaged and evaluated for significance using one-sided t-test (**, p<0.01)
Figure 4.2. *In vivo*, vanadium exposure during early post-natal period results in a decreased number of NG2 positive oligodendrocyte progenitors and increased GFAP-positive astrocytes in genu corpus callosum of PND15 rat. Vanadium-treated (3 mg/kg IP once daily) and control (PBS) rats were sacrificed and sagittal sections of the brain stained for NG2 and GFAP markers. Representative images taken from random fields showing NG2-positive OPCs (A, C), and GFAP-positive astrocytes (B, D) in the genu corpus callosum of metavanadate treated and control (PBS) rats. The number of NG2-positive (black arrows) and GFAP-positive cells were counted by two blinded investigators and averages of N=5 per group counts are shown per high powered field (HPF, 40X) in (C) and (D) respectively. The groups were evaluated for significance using unpaired t-test (*, p<0.05; **, <0.01).
Figure 4.3. *In vitro*, primary oligodendrocyte progenitors (OPCs) are more vulnerable to cytotoxic effects of vanadium than mature oligodendrocytes or astrocytes. Primary astrocytes, OPCs and mature oligodendrocytes exposed to increased concentration of Na-metavanadate for 48 hours. MTT reagent was added for last 4 hours of treatment, the cells were solubilized and absorbances measured at 595 nm. All absorbance values were normalized to controls to obtain % survival. The means were evaluated for significance using one-way ANOVA with Dunnett’s post-test comparison to the control group. Only the indicated groups are statistically significant (* p<0.05; ** p<0.01). The data are representative of at least 3 different independent experiments.
Figure 4.4. Vanadium increases ROS generation and apoptosis of primary oligodendrocyte progenitors in culture. Primary rat OPCs were exposed to 100 uM Na-metavanadate or vehicle (PBS) as control for 6 hours (ROS measurement) and 12 hours (apoptosis determination). Intracellular ROS generation was detected by incubating cells with dichlorofluorescein dye (DCF) for 1 hour at 37°C, and measuring relative fluorescence in equal number of cells in vanadium treated and control groups. Panel (A) demonstrates 2.5-fold increases in measurable ROS production in response to vanadium exposure 6 hours post treatment. The average fluorescence of three independent experiments (subtracted for background) was plotted and analyzed for significance using unpaired t-test (***, p<0.001). (B) At 12 hr time point, vanadium-treated and control cells were exposed to alexa-fluor 488 conjugated annexinV dye, and bound fluorescence detected on Nikon fluorescence scope at 10X magnification. Random images captured were counted for annexinV-positive cells in 4 quadrants by an investigator blinded to the experimental condition. Plotted averages were analyzed for significance using unpaired t-test (***, p<0.001).
**Figure 4.5. Iron chelator desferroxamine (DFO) protects oligodendrocyte progenitors from vanadium-induced cytotoxicity and apoptosis.** Primary rat oligodendrocyte progenitors were treated with 100 uM Na-metavanadate alone or with increasing concentrations of DFO. The untreated cells served as control. (A) Representative brightfield images of OPCs, control (untreated), 100 uM vanadium treated without and with 100 uM DFO at 48 hours post-treatment. Images demonstrate loss of cell viability, process collapse and detachment from the surface of the dish in vanadium treated cells, which is completely prevented by co-administration with 100 uM DFO. We confirm this finding in (B), which shows that increasing concentrations of DFO reverse 48 hour cytotoxicity of 100 uM Na-metavanadate on primary rat OPCs as measured by MTT assay. The absorbance obtained at 595 nm was normalized to the control group to obtain the percentage survival for each experimental group. The significance was determined using one-way ANOVA with Dunnett’s post-test comparisons with the Na-metavanadate-only group (*** p<0.001). All data in the figure are representative of at least 3 independent experiments. (E) At 12 hours post-treatment, 100 uM DFO reduces extent of apoptotic phosphatidylin serine inversion of Ne-metavanadate treated OPCs as detected by Annexin-AF488 labeling. Random images obtained on Nikon fluorescent microscope were counted by an investigator blinded to the experimental condition. The means were analyzed for significance using one-way ANOVA with Tukey’s post-test comparisons between multiple groups. The indicated group comparisons were significant (**, p<0.01, *** p<0.001). The data are representative of three independent experiments.
Figure 4.6. Adding recombinant H-ferritin or lypophilic iron delivery compound, TMH-ferrocene increase vulnerability of primary rat OPCs to vanadium cytotoxicity. (A) Recombinant His-tagged human ferritin heavy chain (rHF) was prepared in BL21 E. Coli and purified over Nickel column, with identity and purity verified by Western blot and Comassie staining of denaturing gel. Both show characteristic bands at 22 KDa consistent with ferritin monomers. M is the lane denoting molecular weight markers. (B) The enzymatic activity of the ferroxidase of recombinant H-ferritin was verified by its ability to oxidize ferrous iron in solution. The solution of 0.4 ug/ul rH-ferritin in PBS (pH=7.4) was incubated with a single injection of ferrous sulfate, and formation of ferric iron was monitored by measuring absorbance at 310 nm (detects amber color associated with ferric iron formation). Presence of recombinant H-ferritin in solution significantly increases rate and the amount of ferric iron formed compared to auto-oxidation of ferrous iron in PBS alone, suggesting that recombinant H-ferritin possesses significant ferroxidase activity. (C)(D) Primary rat OPCs were exposed to 100 uM Na-metavanadate either alone or in presence of increasing concentrations of recombinant H-ferritin (C) or TMH-ferrocene (D). The dose-dependent exacerbation of the cytotoxic effect of vanadium was quantified by MTT assay. All absorbance values were normalized to control and expressed as percent survival. Statistical significance was determined by one-way ANOVA with Bonferroni’s post-test comparisons between multiple groups. Comparisons between control (untreated) and vanadium or H-ferritin/TMH-Fe only groups are indicated, as well as comparisons between vanadium only and vanadium with rHF/TMH-Fe groups (* p<0.05, ** p<0.01, ***p<0.001; ns not significant). The data are representative of at least two independent experiments. (D)
Figure 4.7. Oligodendrocyte progenitors in culture express endogenous cytosolic H-ferritin. Rat primary oligodendrocyte progenitors were stained with antibodies for H-ferritin (A) and A2B5 marker (B), and were visualized with Alexa-flour 488 and 555 secondary antibodies, respectively. DAPI was used as a nuclear stain (C) and overlay projection is shown in (D). Representative confocal images of two independent experiments demonstrate primarily perinuclear localization of H-ferritin expression (yellow arrows) in A2B5-positive oligodendrocyte progenitors with minimal staining of oligodendrocyte processes.
Figure 4.8. Vanadium interacts with iron-rich ferritins to induce single and double strand breaks in supercoiled DNA relaxation assay. (A) Increasing concentration of vanadium (a 25 uM; b 50uM; c 100 uM; and d 200 uM) were co-incubated with puc19 plasmid DNA for various duration of time (7.5-120 min). Lane with puc19 without vanadium served as a control. Vanadium alone did not have any effect on DNA (a, b, c and d). Incubating iron rich horse spleen ferritin (e) alone with DNA caused time-dependent DNA relaxation/ss nicking (arrow 3), but not linearization/ds nicking (arrow 2). The effect was significantly augmented in same experiment with addition of 200 uM Na-metavanadate, producing both single (arrow 3) and double stranded nicks (arrow 2) in puc19 DNA (red box, f6, f7). None of these effects were observed if iron-poor horse spleen apo-ferritin is used, even in the presence of 200 uM Na-metavanadate. (B) Shows absorbance of horse spleen ferritin (SF) and horse spleen apo-ferritin (apo-SF) at 310 nm as a surrogate marker of iron content. The PBS alone was used as a baseline measurement (blank).
A

25 nM NaVO₃ alone

25 nM NaVO₃ alone

50 nM NaVO₃ alone

100 nM NaVO₃ alone

200 nM NaVO₃ alone

SF alone

SF + 200 nM NaVO₃

Apo-SF

Apo-SF + 200 nM NaVO₃

B

Relative Iron content of ferritins

OD at 310 nm

PBS

SF

Apo-SF

1-7.5 min  
2-15 min  
3-30 min  
4-45 min  
5-60 min  
6-90 min  
7-120 min

a-25 nM NaVO₃ alone
b-50 nM NaVO₃ alone
c-100 nM NaVO₃ alone
d-200 nM NaVO₃ alone
e-horse spleen ferritin alone
f-horse spleen ferritin

g-horse spleen apoferritin
h-horse spleen apoferritin
i-horse spleen apoferritin

plus 200 nM NaVO₃
Figure 4.9. Our current understanding of mechanisms of vanadium-mediated oligodendrocyte cell injury. As a transition metal, vanadium participates in redox reactions, contributing to the generation of ROS (toxic hydroxyl radical) through Fenton-like mechanism. In addition, our findings suggest that vanadium can promote release of redox active iron from the stores in ferritin, which further exacerbates ROS generation, cellular toxicity leading to apoptosis and destruction of oligodendrocyte progenitors.
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Chapter 5

Sema4A is overexpressed in multiple sclerosis plaques, collapses processes and causes apoptosis of oligodendrocyte progenitors—role in MS remyelination failure?

ABSTRACT:

Multiple sclerosis is the most common autoimmune demyelinating disorder in which activated lymphocytes infiltrate the white matter and in concert with local inflammatory cells (microglia and astrocytes) produce myelin and oligodendrocyte destruction leading to demyelination. Although myelin repair is initiated by endogenous oligodendrocyte progenitor cells, it nonetheless fails in majority of MS patients. Therefore, identifying molecular signals leading to oligodendrocyte destruction may not only lead to myelin protection strategies, but may also increase remyelination. Semaphorins are a family of transmembrane and secreted proteins originally described as axonal chemorepellants in the developing CNS, which have recently gained attention as immune regulators. Sema4A is a class IV semaphorin and reported ligand of Tim-2 in lymphocytes. Sema4A is expressed on B cells, activated T cells and dendritic cells, and functions in activation of CD4+ T cells and autoimmunity. Although the role for Sema4A in immune activation is known, its role in oligodendrocytes in the context of MS has not been studied. In this study, we report that Sema4A protein is overexpressed within MS plaques compared to normal white matter, and that the chief cellular source of Sema4A
within the MS plaques appear to be infiltrating lymphocytes and microglia. Incubating cells with recombinant Sema4A results in dose-dependent cytotoxicity to OPCs, but not astrocytes, an effect accompanied by process collapse, membrane blebbing and phosphatidylserine inversion suggestive of apoptosis in OPCs. We further demonstrate that the recombinant Sema4A protein preferentially binds to primary oligodendrocyte progenitors over astrocytes, and that Sema4A binding to OPCs can be competed off by cold Sema4A, but not cold H-ferritin or apo-transferrin recombinant proteins. In toto, we propose a novel role for Sema4A as a mediator of immune crosstalk with the remyelinating oligodendrocytes within MS plaque and a regulator of their apoptosis.
INTRODUCTION:

Multiple sclerosis is the most common autoimmune demyelinating disease, and a leading cause of neurological disability among young adults (Compston and Coles 2008). The pathogenesis of this disease involves infiltration of the CNS by activated, myelin-reactive lymphocytes, macrophages and microglia with resultant destruction of myelin and myelin-producing oligodendrocytes (Kieseier et al. 2008; Lucchinetti et al. 2000). With the advent of immunomodulatory drugs, MS treatment has been improved considerably, yet the long-term progression and reversal of neurological disability associated with MS still remains a challenge (MacLean and Freedman 2009).

One way of reversing MS-associated neuropathology and preventing secondary axonal loss in MS is by promoting endogenous remyelination of existing MS lesions (Franklin 2002; Franklin and Ffrench-Constant 2008). Despite the fact that in many (particularly toxin-based) animal models of demyelination, the process of remyelination is activated and is either complete or substantial (Johnson and Ludwin 1981; Matsushima and Morell 2001; Pavelko et al. 1998; Rodriguez and Miller 1994; Woodruff and Franklin 1999), in multiple sclerosis patients endogenous remyelination occurs in only small subset of plaques (shadow plaques) and is almost never complete (Chari 2007). The reasons for remyelination failure in MS are unknown, but could involve inappropriate signaling to remyelinating oligodendrocyte progenitor cells (OPCs). It is now clear from multitude of experimental evidence that endogenous OPCs are chief remyelinating cells both in human and animal models of demyelinating disease (Franklin 2002; Franklin and Ffrench-Constant 2008). During remyelination process, OPCs recapitulate their normal
developmental program. OPCs are recruited, and infiltrate the lesion shortly after demyelinating event, and subsequently have to survive, proliferate, differentiate and engage the axon while producing myelin (Franklin and Ffrench-Constant 2008). Interference with any of these steps may significantly compromise the remyelination process. Although OPCs (NG2 positive cells) can be observed within some MS plaques, their number and density is generally less in majority of the plaques compared to normal white matter, and in some MS plaques they are completely missing (Chang et al. 2000; Chang et al. 2002; Wolswijk 1998; Wolswijk 2000).

As MS plaque is a very hostile environment, one possibility that could contribute to oligodendrocyte demise within the plaque is that the untimely and inappropriately regulated expression of soluble signaling molecules by inflammatory and immune cells could lead to inappropriate cross-talk to the remyelinating OPCs with their consequent destruction and apoptosis. Many neuro-immune molecules, such as cytokines, chemokines, Fas/FasL signaling systems, with defined functions in the immune system have also been implicated in oligodendrocyte destruction and apoptosis in demyelinating disorders (Boulanger 2001.). Semaphorins are an example of a shared signaling system between immune and nervous systems. Class III and class IV semaphorins, in particular have been shown to be highly expressed in the immune system, but they also function as positional cues for cells during CNS development (Mizui et al. 2009). A class IV semaphorin, Sema4A has been shown to be highly expressed in antigen presenting cells (dendritic cells and B cells) and activated T cells (Kumanogoh et al. 2002), and is involved in stimulating helper T-cell proliferation and cytokine production. Its receptor on helper T-cells has been identified as Tim-2 (Kumanogoh et al. 2002) and interaction
between Sema4A is reportedly high affinity interaction that leads to phosphorilation of conserved tyrosines in cytoplasmic domain of Tim-2 (Kumanogoh et al. 2002). Mice deficient in Sema4A have dendritic cells with poor allostimulatory activities and impaired Th1 differentiation of T-cells with consequent deficient Th1 responses (Kumanogoh et al. 2005). Blocking antibodies to Sema4A result in decreased clinical and histopathological severity of EAE, mouse model of Multiple Sclerosis, which is prototypical Th1-mediated autoimmune disorder. Although, the role of Sema4A in the immune function and activation of T-cells in mouse model of MS is defined, little is known about the function of this protein at the effector end, particularly its role in oligodendrocytes in the context of Multiple Sclerosis.

In this chapter we demonstrate that class IV neuroimmune semaphorin, Sema4A is highly expressed within the MS plaque (compared to normal white matter), primarily immunolocalizing in infiltrating lymphocytes and activated microglia. We demonstrate that Sema4A treatment results in dose dependent cytotoxicity to purified cultures of oligodendrocyte progenitors, but not astrocytes. Sema4A-mediated death of OPCs is preceded by process collapse and associated with phosphatidylserine inversion, suggestive of apoptosis. In culture, recombinant Sema4A preferentially binds to OPCs over astrocytes, and we show that Sema4A binding to OPCs can be competed off by cold Sema4A, but not cold recombinant H-ferritin or apo-transferrin or blocking antibodies to Tim-2. In toto, our results highlight an important role of Sema4A in regulating survival of OPCs, and identify Sema4A-mediated signaling as potential contributor to oligodendrocyte demise in pathogenesis and remyelination failure of Multiple Sclerosis.
METHODS:

Recombinant proteins and antibodies: Recombinant Sema4A-Fc and Sema3A-Fc proteins were obtained from R&D systems. The extracellular domains of these proteins were produced and purified as Fc-chimeras in a mammalian cell line. Apo-transferrin was purchased from Sigma. Recombinant H-ferritin was prepared in competent BL21 E. Coli as described before (Todorich et al. 2008c). Anti-Sema4A (rabbit pAb) was purchased from Abcam, and anti-MBP (mouse mAb) was purchased from Upstate.

Immunostaining of the MS plaques: Paraffin embedded blocks of MS plaques’ tissue were obtained from the Human Brain and Spinal Fluid Resource Center, (VA West Los Angeles Healthcare Center, 11301 Wilshire Blvd. Los Angeles, CA 90073, sponsored by NINDS/NIMH, National Multiple Sclerosis Society, Department of Veterans Affairs). Normal tissue controls age/sex matched were obtained from Harvard Brain and Tissue Resource Center (115 Mill Street, Belmont, MA 02478, sponsored by grant from National Institute of Health, R-24-MH 068855). All sections were cut at 10 um thickness, and subsequently deparaffinized in xylenes and hydrated in ethanol. After a brief rinse in dH2O, antigen retrieval was performed in 10 mM citrate buffer (pH=6.0) for 20 minutes. Subsequently, endogenous peroxidase activity was blocked in 3% H2O2/methanol, and after that blocked in 5% blotto/PBS (pH=7.4). The sections were incubated with either anti-Sema4A 1:100 for 16 hrs at 4°C or anti-MBP 1:200 for 16 hrs at 4°C. After several washes in PBS, sections were incubated in secondary antibodies (ABC Vectastain kit) in accordance to the manufacturer’s protocol. The staining was visualized using DAB (diaminobenzidine) using standard procedure. All images were acquired on brightfield microscope equipped with digital camera.
**Primary cultures:** Primary cultures enriched in oligodendrocyte progenitors and astrocytes were performed according to the protocol of McCarthy and DeVellis (1980) with minor modifications. Newborn rat pups were decapitated and heads immersed in 70% EtOH. After a thorough rinse in Hank’s buffer, the brains were removed and separated from meninges. Cerebral hemispheres were diced and homogenized in trypsin-EDTA, with subsequent digestion in DNase I. Resulting brain homogenate was passed through size exclusion mesh and single cell suspension plated in poly-d-lysine coated T150 flasks. Mixed glia were subcultured for 8-9 days before sequential purification using the shaking method. First, microglia were removed by short shake of 265 rpm for 30 minutes. Subsequently, primary oligodendrocyte progenitors were collected by shake of 265 rpm for 18 hrs. The remaining cells still adherent to the dish were astrocytes. Oligodendrocyte progenitors were collected and plated in poly-d-lysine coated 6 well plates in N2S media (Todorich et al. 2008c). Astrocytes were trypsinized and plated in 6 well plates in DMEM/10% FBS media. Both sets of cells were used for experiments within 2-3 days after plating.

**Cytotoxicity and apoptosis assays:** Increasing concentrations of recombinant Sema4A-Fc chimera protein were added directly to the media of either oligodendrocyte progenitors or astrocytes. For MTT assay, cells were exposed to recombinant Sema4A for 20 hrs and the MTT reagent was added for last 4 hrs of treatment. Cells were solubilized in SDS-based buffer overnight at 37°C, and subsequently absorbance measured at 595 nm using Spectramax Gemini plate reader.
For apoptosis analysis, Sema4A-treated OPCs were washed in ice-cold PBS (pH=7.4) and then subsequently exposed to annexinV-conjugated to AlexaFluor488 dye (Invitrogen) at 1:5 dilution in annexin binding buffer (pH=7.4) for 15 minutes (Invitrogen). The cells were subsequently washed two times in annexinV binding buffer and then visualized on Nikon inverted fluorescence microscope equipped with digital camera. Images in random fields were acquired and cells counted in 4 quadrants by an experimenter blinded to the experimental condition. The experimental means were evaluated for statistical significance using the unpaired t-test.

**Binding studies:** a) To evaluate binding of Sema4A, recombinant Sema4A-Fc protein was labeled with I\(^{125}\), using the method of Hunter and Greenwood (Hunter and Greenwood 1962) as previously described (Todorich et al. 2008c). Primary rat OPCs and astrocytes were plated in 6-well plates at concentration of 1 million cell per well. Media was removed and cells washed with Hank’s balanced salt solution (HBSS) supplemented with MgCl\(_2\) and CaCl\(_2\). Increasing concentrations of Sema4A-Fc-I\(^{125}\) protein were dissolved in phosphate buffered saline buffer (pH=7.4) and were incubated with primary OPCs or astrocytes for 2 hrs at 22\(^\circ\)C. After three washes in PBS buffer, cells were harvested mechanically in RIPA buffer and counts measured using gamma counter. Subsequently, the protein concentration of the counted sample was determined using BioRad DC assay, and all counts normalized to total protein in each of the samples.

b) **Competition study**—Media was removed from primary rat OPCs plated in 6-well plates at density of 1 million cells per, and subsequently washed with Hank’s balanced salt solution (HBSS) supplemented with MgCl\(_2\) and CaCl\(_2\). The cells were subsequently preincubated with increasing concentrations of Sema4A-Fc recombinant protein,
recombinant H-ferritin or apo-transferrin for 2 hours at 4°C in phosphate-buffered saline (PBS) buffer (pH=7.4). Subsequently, Sema4A-Fc- $^{125}$I was added to the final concentration of 0.1 ug/ml and incubated for 1.5 hrs at 22°C. Binding suspension was subsequently removed and cells washed 3 times with PBS buffer. Cells were harvested mechanically and counts measured on the gamma counter.

**RESULTS:**

To investigate expression of Sema4A protein in MS plaque, we obtained commercially available antibody to this protein. We demonstrate in Figure 5.1 A that this antibody recognizes Sema4A recombinant protein on Western blot and that it is specific for Sema4A as it cross-reacts with native (undenatured) recombinant Sema4A-Fc, but not recombinant Sema3A-Fc proteins on a slot blot (Figure 5.1 B). Subsequently, we used that antibody to evaluate expression and cellular distribution of Sema4A in 5 MS patient plaques and 4 control specimens containing normal human white matter. Table 5.1 summarizes histopathological parameters of the specimens used in the study. The MS and control patient population were matched well by age (average age 67 and 68, respectively) and gender (M:F ratio was 20%/80% for MS and 25%/75% for controls). In Figure 5.2, the representative image of immunostaining for Sema4A demonstrates much higher levels of Sema4A immunoreactivity within the MS plaque compared to normal white matter. Sema4A immunoreactivity was primarily localized in small cells cuffing around the blood vessels, which is pathognomonic for infiltrating lymphocytes within the MS plaque (green arrows). A second group of Sema4A-positive cells were large bloated
cells infiltrated within the myelin, which were morphologically characteristic of microglia/macrophages (red arrows). We defined the extent of demyelination and distinguished plaque area (pq) from normal appearing white matter by staining for myelin basic protein (MBP; Figure 5.2, C, D) for each plaque used in the study. Collectively, these data demonstrate that the levels of Sema4A protein are strongly upregulated within the MS plaque compared to normal white matter and that chief cellular source of this protein within the plaque are lymphocytes and activated macrophages/microglia.

To investigate the biological effects of Sema4A on oligodendrocytes, we developed a cell culture model. First, we incubated purified cultures of primary rat OPCs and astrocytes with increasing concentrations of recombinant Sema4A-Fc for 24 hrs in order to determine whether Sema4A had any cytotoxic effects. Data in Figure 5.3 demonstrate that Sema4A resulted in dose-dependent cytotoxicity to OPCs, but not to astrocytes, which paralleled the binding data in Figure 5.5. Morphologically, compared to the untreated controls in Figure 5.4 A, Sema4A treated OPCs demonstrated collapsed processes as early as 8 hrs post treatment (Figure 5.4 B, yellow arrows). Furthermore, many of the cells in the treated groups demonstrated grape-like membrane blebbing suggestive of apoptosis. To confirm that Sema4A-induced cytotoxicity is induction of apoptosis, we performed annexinV labeling of Sema4A treated and control OPCs (Figure 5.4; C, D). AnnexinV dye binds with high avidity phosphatidylserine, an inversion of which to the outer leaflet of plasma membrane is an early event at the initiation of apoptosis. At the same (8 hr time point), Sema4A treated OPCs demonstrated 2.5-fold increase in AnnexinV labeling compared to control, suggesting that Sema4A-induced cytotoxicity is indeed apoptosis.
To define whether specific binding sites existed for Sema4A on oligodendrocytes, we labeled the Sema4A-Fc recombinant protein with \(^{125}\text{I}\). In Figure 5.5 A, we demonstrate that Sema4A binds to purified cultures of OPCs with approximately five fold higher affinity than to primary rat astrocytes, suggesting that binding sites for Sema4A are enriched in oligodendrocytes compared to astrocytes. This binding preference may explain why Sema4A is pro-apoptotic to oligodendrocytes, but not to astrocytes. To demonstrate that this binding is specific, we performed a competition experiment. Figure 5.5 B demonstrates that increasing concentrations of cold Sema4A-Fc recombinant protein, but not of rH-ferritin or apo-transferrin were able to compete off Sema4A-Fc binding to OPCs, suggesting that binding is specific for Sema4A.

**DISCUSSION:**

In this study we demonstrate that class IV neuroimmune semaphorin, Sema4A is overexpressed in MS plaques compared to normal white matter and that within the plaques Sema4A expression primarily immunolocalizes in infiltrating lymphocytes and microglia/macrophages. The significance of the presence of Sema4A in MS lesions was shown in our cell culture models where recombinant Sema4A treatment results in dose-dependent cytotoxicity of primary oligodendrocyte progenitors (OPCs), but not astrocytes in culture. Sema4A-induced death of OPCs is accompanied by process collapse, membrane blebbing and phosphatidylserine inversion, which is highly suggestive of apoptosis. Recombinant Sema4A demonstrates binding preference for primary OPCs.
compared to astrocytes, and that binding can be competed off by cold Sema4A, but not H-ferritin or transferrin recombinant proteins. *In toto*, our findings establish a novel role for Sema4A in regulating survival of oligodendrocytes within MS plaques.

The clinical approach to repairing Multiple Sclerosis lesions appears to hinge on gaining complete understanding of signaling networks within MS plaques that interfere with infiltration, survival and differentiation of oligodendrocyte progenitors attempting to remyelinate the demyelinated axons. In this study we report that Sema4A is highly expressed within active MS plaques compared to normal human white matter. Our finding is in agreement with those of Williams et al, who recently reported that two class III semaphorins, Sema3A and Sema3F are also overexpressed within the actively demyelinating MS plaques, but failed to detect any semaphorin transcripts in normal white matter (Williams et al. 2007). Although the cellular source of Sema3A and Sema3F proteins within the MS plaques are reportedly activated microglia, astrocytes and to a small extent oligodendrocytes, we show that Sema4A immunoreactivity within the active plaques is primarily localized in infiltrated lymphocytes and microglia. Collectively, our results contribute to the evolving concept that various semaphorins are active participants of the inflammatory signalplex within MS plaque and that local immune cells (microglia and astrocytes) as well as infiltrating lymphocytes may participate in the pathological signaling involving these molecules.

Although originally described as axonal chemorepellants in developing CNS, semaphorins have been shown recently to regulate a variety of physiological and pathological functions within and outside of the CNS. We have shown in this study that at least one of the biological functions of Sema4A in OPCs is regulating their survival
through induction of apoptosis. The role of various semaphorins in regulating apoptosis in variety of cell types has been well documented. For example, Sema3A promotes apoptosis of leukemic T-cells (Moretti et al. 2008), neurons through Plexin A3 (Ben-Zvi et al. 2008) and activated microglia (Majed et al. 2006). Sema3F acts synergistically with Sema3A in promoting apoptosis of endothelial cells (Guttmann-Raviv et al. 2007). Sema3B induces apoptosis of breast and lung cancer cells (Castro-Rivera et al. 2008; Castro-Rivera et al. 2004). Finally, Sema4D (CD100) has been shown to collapse processes and cause apoptosis of neural precursors and immature oligodendrocytes (Giraudon et al. 2004). Giraudon et al. further showed that Sema4D is overexpressed within the demyelinating lesions of TSP/HAM patients (viral demyelinating disease) and that recombinant Sema4D as well as Sema4D-overexpressing T cells resulted in process collapse and destruction of immature oligodendrocytes, but not astrocytes (Giraudon et al. 2004). Therefore, our results taken in concert with those of Giraudon et al. suggest that both class IV semaphorins, Sema4A and Sema4D have very similar biological functions on oligodendrocytes.

So what is the Sema4A receptor in oligodendrocytes? Sema4A reportedly binds Tim-2 in the immune system (Kumanogoh et al. 2002), although several recent reports failed to confirm that interaction (Chen et al. 2005; Wilker et al. 2007). We have previously demonstrated that Tim-2 (T cell immunoglobulin mucin domain 2 protein) is selectively expressed on cells of oligodendrocyte lineage and that it acts as a cellular receptor for H-ferritin, an iron delivery protein in developing oligodendrocytes (Todorich et al. 2008c) (chapter 2). In that study we showed that Tim-2 is expressed in CNPase, O4 and A2B5-positive oligodendrocytes, but not GFAP-positive astrocytes. In this study, Sema4A
binding follows the similar trend: it is much higher in oligodendrocytes compared to astrocytes. However, we could neither compete off Sema4A binding on primary rat OPCs by recombinant H-ferritin (Figure 5) nor by blocking antibodies to Tim-2 that block H-ferritin binding to these cells (data not shown). Therefore, our results point out that Sema4A binds to a site on rat oligodendrocyte cell distinct from H-ferritin binding site on Tim-2. A recent paper by Toyofuku and colleagues demonstrated that Sema4A was capable of binding B-type plexins and Plexin D1 in the endothelial cells (2007). The anti-angiogenic functions of Sema4A reported in this study were dependent on presence of plexin D1, but not B plexins, suggesting that the functional receptor for Sema4A in endothelial cells is plexin D1 (Toyofuku et al. 2007). These studies raise possibility that one of either B or D-type plexins may also be the cellular receptor of Sema4A on oligodendrocytes as well, which should be investigated further. Although the membrane receptor for Sema4A on oligodendrocytes remains to be determined, it is clear that Sema4A has cytopathic effects on developing oligodendrocytes and may be important contributor to pathogenetic mechanisms of remyelination failure in MS.
FIGURES:

Figure 5.1. Anti-Sema4A antibody recognizes Sema4A, but not closely related Sema3A. (A) Recombinant Sema4A-Fc chimera protein was loaded to denaturing SDS-PAGE and either stained with Comassie or probed with anti-Sema4A antibody on Western blot (WB). The antibody recognizes the Sema4A-Fc chimera recombinant protein (A), which is of appropriate molecular size (115-120 KDa) for glycosilated protein. (B) Native (non-denatured) rSema4A-Fc and rSema3A were immobilized onto nitrocellulose membrane and probed with anti-Sema4A antibody via slot blot. This representative immunoblot demonstrates that antibody cross-reacts with rSema4A, but not rSema3A.
Table 5.1. Characteristics of MS patients and normal controls used in the study.

The tissue specimens of total of nine patients were used in the study, of which 5 were MS patients and 4 were normal controls. The average age of MS patients and controls were 67 and 68 years, respectively.

<table>
<thead>
<tr>
<th>MS patient</th>
<th>Primary diagnosis</th>
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<th>Gender</th>
<th>Plaque</th>
<th>Path. Classification</th>
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</tr>
<tr>
<td>3</td>
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<td>65</td>
<td>M</td>
<td>Active</td>
<td>demyelination, lymphocytic infiltrate (P, E), microglia present</td>
</tr>
<tr>
<td>4</td>
<td>Multiple Sclerosis</td>
<td>62</td>
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<td>Active</td>
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</tr>
<tr>
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<td>Multiple Sclerosis</td>
<td>48</td>
<td>F</td>
<td>Active</td>
<td>demyelination, lymphocytic infiltrate (P, E, D), microglia present</td>
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</table>

<table>
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<td>F</td>
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<td>Normal white matter</td>
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</table>
Figure 5.2. Sema4A is strongly upregulated in MS plaques. Sections of normal white matter and those of patients with MS (active plaques) were immunostained for Sema4A. Representative images are presented here that demonstrate strong immunoreactivity with Sema4A antibodies in the MS plaque compared to normal white matter. The reaction product was mainly localized in lymphocytes (perivenular cuff, green arrows) and scattered macrophages/microglia (red arrows). The size and location of the plaque was determined by immunostaining of the neighboring sections for myelin basic protein (MBP). These low powered images demonstrate representative results of MBP immunoreactivity in both normal white matter and MS plaques. Within the given specimen, the demarcation between region of strong demyelination/plaque (pq) and normal appearing white matter (nawm), was observed and is indicated by the red dotted line.
Figure 5.3. Sema4A is cytotoxic to primary OPCs, but not astrocytes in culture.

Purified cultures of primary rat oligodendrocyte progenitors (OPCs) and astrocytes were treated with increasing concentrations of recombinant Sema4A-Fc protein for 24 hrs. Untreated cells served as a control. The cell viability was determined using MTT assay. Individual means were evaluated for statistical significance using one-way ANOVA with Dunnett’s post-test comparisons. Only the indicated groups were significantly different from controls (**, p<0.01). The results, which are representative of three independent experiments, demonstrate dose-dependent cytotoxicity of primary OPCs (A), but not astrocytes (B) to Sema4A protein.
Figure 5.4. Sema4A collapses processes and causes apoptosis of oligodendrocyte progenitors. (A, B) Primary rat OPCs were incubated with 10ug/ml Sema4A-Fc protein for 8 hrs. The untreated cells served as control. Sema4A-treated OPCs show process collapse (red arrows), while others appear apoptotic with membrane blebbing (yellow arrows) suggestive of apoptosis. This was confirmed by AnnexinV labeling in the same experiment, which demonstrates significant increase in the number of annexinV-positive cells in Sema4A-treated group (D) compared control (C). Cell counts demonstrate 2.5-fold increase in annexinV labeled as a result of Sema4A treatment. Means were analyzed for significance using unpaired t-test (*, p<0.05).
Figure 5.5. Sema4A binds to oligodendrocyte progenitors, but not astrocytes in culture. (A) Purified cultures of primary rat OPCs and astrocytes were incubated with increasing concentrations of Sema4A-Fc-I\textsuperscript{125}, and after thorough washes, the cells were harvested and counts measured, normalized to total protein per sample. The means were evaluated for significance by pair-wise comparisons using one-sided t-test (p<0.05). Data are representative of two independent experiments. (B) Preincubating primary OPCs with increasing concentrations of cold Sema4A-Fc, but not rH-ferritin or transferrin effectively competes Sema4A-Fc-I\textsuperscript{125} binding. Data are representative of three independent experiments.
REFERENCES:


Chapter 6

Summary and future directions

The main objective of this dissertation project was to elucidate a novel mechanism of iron import into developing oligodendrocytes, and in doing so investigate how these processes may play a role in pathogenesis of oligodendrocyte destruction and dysfunction in dysmyelinating disorders. In this work, therefore, we presented evidence that Tim-2 is an iron regulated cellular receptor for extracellular H-ferritin in oligodendrocytes. In the CNS of rodents, Tim-2 appears to be expressed selectively on cells of oligodendrocyte lineage and blocking it abated specific binding of radiolabeled H-ferritin to primary oligodendrocyte progenitors and oligodendrocyte differentiated CG4 cell line. In vivo, however, knockout of Tim-2 ferritin receptor appears not to affect normal myelination status in 16 month old mice even though Tim-2 knockouts at that age group displayed a 20% decrease of myelin iron levels. Collectively these data establish the role of H-ferritin/Tim-2 in iron acquisition by oligodendrocytes. The fact that Tim-2 KO mice still acquired significant iron levels to myelinate is likely related to redundant mechanisms of ferritin and iron import that exist on oligodendrocytes, such as transferrin. In support of that argument, we demonstrate that extracellular H-ferritin indeed delivers iron in absence of transferrin and that H-ferritin and transferrin can replace each other as iron source to OPCs, supporting their growth and survival equally well independent of each other. Therefore, it appears that Tf and H-ferritin, with their respective receptors, represent complementary iron delivery systems in developing oligodendrocytes. We
further showed that H-ferritin delivered iron through this pathway is redox active (Fenton chemistry) and demonstrate that another transition metal, vanadium, results in release of red-ox active iron from ferritin, which results in increased oxidative stress and apoptosis of oligodendrocyte progenitors *in vivo* and *in vitro*. These effects may contribute to vanadium-induced hypomyelination, which is a chief neurological consequence of developmental exposure to vanadium compounds; a significant environmental concern in countries like Nigeria. Lastly, we demonstrate that the other reported ligand of Tim-2 in the immune system, Sema4A is overexpressed within active MS plaques compared to normal white matter. We demonstrate that recombinant Sema4A-Fc chimera collapses processes and causes apoptosis of oligodendrocyte progenitors in culture, which carries significant implications for pathogenesis of remyelination failure within MS plaque. We also found that Sema4A binds specifically to OPCs, but not astrocytes, however, we find no evidence of Sema4A binding dependence on Tim-2, suggesting that Sema4A on oligodendrocytes acts through an alternative receptor, most likely of the Plexin family. Collectively, our data are summarized in Figure 6.1.
Figure 6.1. Our current understanding on the role of H-ferritin receptor in oligodendrocyte iron delivery, myelination and apoptosis.

As the results in Figure 6.1 demonstrate, the findings in this thesis project have made several important contributions to oligodendrocyte biology, and yet many important questions that logically follow from these findings remain unanswered and should be subject of further research. Some of those questions (outlined in bold) are discussed below.
What is the molecular mechanics of iron release from endocytosed ferritin?

As mentioned, the significant contribution of this project is that Tim-2 is the binding receptor for extracellular H-ferritin in developing oligodendrocytes and that main function of that extracellular H-ferritin is non-transferrin mediated iron delivery to developing oligodendrocyte cell. The intracellular fate of ferritin associated iron in oligodendrocytes is unclear and an important future direction that stems from this thesis work. Specifically, how does iron contained within the endocytosed ferritin become available for use in the oligodendrocyte cytosolic iron pool? There are at least two possibilities. First, a direct release of iron from ferritin within the endosomal vesicular system may be possible by a mechanism analogous to that of transferrin iron release in the same compartment. If existing, the direct release of iron would likely be conducted through hydrophilic channels on the 3 fold axis of ferritin that have been reported to exist and function in shuttling iron in and out of the ferric hydrite core of ferritin in vitro (Jin et al. 2001; Theil et al. 2008). It is still unclear as to how the cells may regulate that release of iron physiologically, and this possibility should be investigated further.

The second and more plausible possibility is that endocytosed ferritin is shuttled to the lysosome of oligodendrocyte cell and there proteolytically degraded by lysosomal hydrolases. Such pathway was described in other cell types that have capacity to endocytose extracellular H-ferritin (Meyron-Holtz et al. 1999). Lysosomal degradation mechanism would allow for coupling of iron release for both cytosolic (endogenous) and extracellular ferritins (Arosio et al. 2008; Harrison and Arosio 1996) as at least some of the cytosolic ferritin iron stores are mobilized in this fashion. Degradation of the ferritin protein shell would subsequently release free iron that could make its way to the cytosol.
via ionic metal transporters (such as DMT1), which are known to function in such capacity (Andrews 1999) and could be the final common pathway for iron release from both intracellular and endocytosed ferritins. Which of these mechanisms is utilized by oligodendrocytes needs to be further investigated.

What is the source of H-ferritin to developing oligodendrocytes?

Oligodendrocytes are the only cell in CNS capable of producing myelin, and they originate from neural stem cells in the subventricular zone (SVZ), from where they proliferate and migrate into the brain parenchyma in early post-natal period. That period (roughly first three post-natal weeks in rodents and first two post-natal years in humans) is the peak period of oligodendrogliogenesis and myelination, but is also the peak period of iron import into brain (Crowe and Morgan 1992; Taylor and Morgan 1990). Recent evidence suggests that developing OPCs proliferate and migrate on the tracts established by microvasculature and that they develop an intimate relationship with the endothelial cells, which serve as a source of trophic factors that promote survival and proliferation of OPCs (ie. endothelins) (Arai and Lo 2009). This framework of oligodendrocyte development in early post-natal period fits nicely with the work presented herein as recent paper from our laboratory demonstrated that H-ferritin protein was able to be transported across blood brain barrier, and was able to deliver iron to the brain (Fisher et al. 2007). Therefore, it is likely that BBB transport of ferritin is one of the sources of H-ferritin and ferritin-bound iron to oligodendrocytes. Although serum ferritin is almost exclusively L-ferritin in the adult, H-ferritin within the serum in the early post-natal period of an infant may be increased. Mother’s milk is rich in H-ferritin (Dempster et al.
1986; Kavukcu and Taneli 1994), which could be transported to the blood of the newborn by paracellular route through the gut or by direct transcytosis through the enterocytes (Kalgaonkar and Lonnerdal 2009; San Martin et al. 2008) and serve as natural iron source through H-ferritin to developing oligodendrocytes. If this is the case, it is unlikely that H-ferritin and iron flux across BBB to OPCs is constitutive and unregulated. Developing oligodendrocytes may themselves control iron flux across blood brain barrier (BBB), including ferritin and transferrin transport. They may do that either directly or more likely through secreted molecular signals. Although this obviously requires further characterization, proof of concept for this hypothesis is presented in preliminary data (Figure 7.1, appendix) and suggests that conditioned media from iron deficient oligodendrocytes can alter iron status and expression of iron transport protein TfnR1 of BRECs, an in vitro model of blood brain barrier. The possibility of reciprocal chemical communication between endothelial cells at the BBB and myelinating oligodendrocytes as a means of regulating iron transport across BBB should be further investigated.

The other source of H-ferritin could come from microglia. In normal development, H-ferritin expression in microglia at P5 in distinct myelinogenic foci of subcortical white matter gradually shifts to oligodendrocytes by P30 (Cheepsunthorn et al. 1998). In cell culture, our group has previously demonstrated that iron loaded microglia conditioned media when placed on oligodendrocyte progenitors increase mitochondrial function and mitochondrial reduction potential of OPCs (Zhang et al. 2006). Treating microglia with siRNA directed to H-ferritin abated this effect suggesting that soluble trophic factor released by microglia to oligodendrocytes is H-ferritin (Zhang et al. 2006). Finally, recent work by Schonberg and McTigue showed that LPS injections
result in robust oligodendrogliogenesis that is preceded by accumulation of ferritin in microglia, which subsequently shifts to CC1+, BrdU+, Tim-2+ oligodendrocyte progenitors migrating from SVZ in adult rat spinal cord (Schonberg and McTigue 2009; Schonberg et al. 2007) (personal communication with Schonberg and McTigue). The oligodendrocyte generation and ferritin/iron expression was attenuated with application of iron chelator Exjade (deferrasirox) in this model (Schonberg and McTigue 2009). As extracellular H-ferritin levels have been known to increase in inflammation (Chen et al. 2005; Miller et al. 1991; Recalcati et al. 1998; Scaccabarozzi et al. 2000; Tsuji et al. 1991), collectively these data suggest that H-ferritin may be an important source of iron to remyelinating OPCs within the inflammatory MS plaques and also may be one of the key molecular determinants driving remyelination attempts by endogenous OPCs within MS plaques. This possibility should be a subject of further research.

**How to make sense out of some unexpected and confusing in vivo data?**

As demonstrated earlier, we believe that the main function of extracellular H-ferritin is non-transferrin mediated uptake of iron in developing oligodendrocytes, and that Tim-2 is the receptor for extracellular H-ferritin in oligodendrocytes. The atomic absorbance measurements show significant decrease of 20% in the myelin iron levels of Tim-2 deficient mice, which establishes the role of Tim-2/H-ferritin axis in iron transport to oligodendrocytes in vivo. However, the observed decrease of iron levels in the Tim-2 knockout mice was not sufficient to cause myelin effect as these mice display no gross hypomyelination (at least in the 16 month old mice used in this study) and appear grossly
neurologically normal. Therefore, the analysis of Tim-2 KO mice also points to a functional complementary iron acquisition system that maintained iron in oligodendrocytes and myelin within non-hypomyelinating range, allowing normal (or near normal) myelination in the absence of H-ferritin receptor. We propose that at minimum that complementary system involves transferrin. In support of that argument, we have demonstrated that extracellular H-ferritin and transferrin can replace each other in supporting growth and iron requirements of developing oligodendrocytes in culture (Chapter 4). Therefore, it appears that transferrin and H-ferritin, with their respective receptors, are complimentary and alternative iron delivery mechanisms in oligodendrocytes. Evidence that transferrin delivers iron, and that transferrin delivered iron is important in oligodendrocyte survival, growth and maturation in vivo and in vitro is compelling (Adamo et al. 2006; Badaracco et al. 2008; Bottenstein 1986; Cabrera et al. 2000; Connor and Menzies 1996; Escobar Cabrera et al. 1994; Escobar Cabrera et al. 1997; Espinosa de los Monteros and Foucaud 1987; Espinosa de los Monteros et al. 1999; Garcia et al. 2004a; Garcia et al. 2003; Marta et al. 2000; Ortiz et al. 2004; Ortiz et al. 2005; Paez et al. 2005; Paez et al. 2004; Paez et al. 2006b; Paez et al. 2002; Saleh et al. 2003; Sow et al. 2006) and was discussed at length in the introductory chapter. Yet, mice deficient in transferrin (Hpx mice) are also not hypomyelinated (Ortiz et al. 2004). On the contrary, Hpx mice develop iron overload in many tissues (liver, heart, pancreas), including the brain, myelin and oligodendrocytes (Ortiz et al. 2004; Simpson et al. 1993). Hpx mice have approximately twice the normal concentration of iron in the whole brain and seven fold increased iron concentrations in purified myelin (Ortiz et al. 2004). This contraintuitive result may be considered paradoxical to the well established view for
transferrin/TfR1 as solely iron delivery system to cells including oligodendrocytes. However, a documented increase in non-transferrin mediated iron import can explain the iron overload phenotype of these mice (Simpson et al. 1993). Supporting that argument is the finding that despite significantly lower serum transferrin levels, the rate of transport of injected Fe-59 into the brain of Hpx/+ heterozygotes is equivalent to that of wild type controls (Dickinson et al. 1996), and may be even substantially increased in Hpx/hpx homozygotes compared to wt controls (Ueda, et al. 1993). Ferritin may be responsible for the bulk of that effect as Hpx/hpx mice have been shown to have serum ferritin levels 15 times higher than normal wild type controls (Simpson et al. 1993), suggesting that ferritin may play a significant role in the non-transferrin mediated iron delivery, iron overload and hypermyelination observed in this model. It should be determined whether upregulation of H- or L- ferritins, or their combination is truly responsible for the effects observed in Hpx mice, and this should be subject of future research as this mouse model can still provide many novel insights into the various mechanisms of non-transferrin mediated iron uptake.

To make the story even more complicated, just recently Scara5 has been identified as necessary and sufficient receptor for endocytosis of L-ferritin in developing kidney (Li et al. 2009). Li, et al. presented convincing data showing that in TfnR1-negative kidney cells, Scara5 allows for non-transferrin mediated iron uptake by endocytosis of L-ferritin (2009). Although Scara5 transcripts have been detected in the brain, it is presently unknown whether developing oligodendrocytes express Scara5 protein or whether they have the capacity to uptake L-ferritin. However if they did, Scara5/L-ferritin delivery to oligodendrocytes would allow for yet another conduit for
iron into these cells in absence of transferrin or functional H-ferritin receptors. This exciting possibility should be further investigated.

As this elaborate discussion on transferrin- and non-transferrin mediated iron delivery demonstrates, there are multiple ways of delivering iron to developing oligodendrocyte cells. Some or all of those may be responsible for lack of myelin phenotype observed in Tim-2 KOs. Regardless, in this thesis project we have identified a novel pathway for delivering safe, relative red-ox free iron for supplementation in the iron deficiency, which is H-ferritin protein and its receptor on oligodendrocytes, Tim-2. Therefore, the future research should also be aimed at determining whether recombinant H-ferritin (delivered orally, parenterally or intraventricularly) has the capacity to correct hypomyelination produced by iron deficiency. Our unpublished data suggest that feeding iron-deficient rats with yeast overexpressing H-ferritin partially corrects iron-deficient brain iron levels, which is first and prerequisite step towards demonstrating possible therapeutic effects of ferritin as an iron delivery vehicle to CNS and oligodendrocytes. This is a fertile area for future research.
How does iron availability modulate oligodendrocyte development and myelin production?

In the introductory chapter we discussed the importance of iron in bioenergetics of oligodendrocyte cell as well as iron as a cofactor for necessary enzymes involved in myelin production and maintenance. Iron deficiency would interfere with those processes, which may contribute to the iron-deficiency associated hypomyelination. Another intriguing possibility is that limiting iron availability to oligodendrocytes alters oligodendrocyte development by interfering with lipid raft formation/stability. Lipid rafts (detergent resistant microdomains) have recently gained attention in their important role in signal transduction (Foster and Chan 2007; Patra 2008). Two critical plasma membrane receptors controlling proliferation and survival of OPCs—PDGF-R and FGFR2 have been reported to localize in lipid rafts (Baron et al. 2003; Bryant et al. 2008; Decker and ffrench-Constant 2004). For two integral myelin proteins, including oligodendrocyte-myelin glycoprotein (Boyanapalli et al. 2005) and myelin associated glycoprotein (Vinson et al. 2003) association with lipid rafts appears critical for proper function and association with their molecular targets on axons. Therefore, emerging evidence suggests that lipid rafts may be critical in regulating oligodendrocyte development and myelination. Cholesterol is an essential component of lipid rafts and removing cholesterol results in disruption of lipid raft structure/formation (Brown 2002), which consequently leads to impaired signal transduction through the receptor complexes which assemble at the rafts. Because the brain lacks the capacity to import cholesterol from the blood after closure of BBB (P0 in rat) it exclusively relies on endogenous synthesis of cholesterol, anything that interferes with that cholesterol metabolism may
interfere with lipid raft formation and stability with consequent downstream effects. In fact, cholesterol has been one component of myelin that is most robustly and most consistently decreased by iron deficiency (Ortiz et al. 2004). This is due to the fact that HMG-CoA reductase (rate limiting enzyme in cholesterol synthesis pathway) and squalene epoxidase (later part of cholesterol synthesis pathway) are iron dependent enzymes, and limiting iron may interfere with lipid raft formation and stability by virtue of inhibiting cholesterol synthesis and availability at critical times of oligodendrocyte differentiation (Cammer 1984). Preliminary results in Figure 7.2 demonstrate proof of concept for this hypothesis, and demonstrate that limiting iron availability over 24 hr period results in partial disruption of lipid rafts in OPCs.

Why is too much of a good thing, a bad thing?

Clearly, iron delivered by transferrin and ferritin is necessary as an essential factor for oligodendrocytes and myelination. Yet, too much of free, redox-active iron can lead to oxidative stress by formation of free radicals through Fenton mechanism (Todorich and Connor 2004), resulting to destruction of many cellular components and apoptosis. Oligodendrocyte progenitors are especially sensitive to ROS-mediated injury as they express much lower levels of cellular antioxidant defenses (such as glutathione) (Hemdan and Almazan 2007; Husain and Juurlink 1995; Juurlink et al. 1998; Thorburne and Juurlink 1996). Apoptosis and destruction of OPCs has been proposed as a chief mechanism of hypoxic hypomyelinating injury in premature infants (periventricular leukomalacia, PVL) (Volpe 2001), and extreme vulnerability of OPCs to oxidative injury
has been proposed as one of the key mechanisms contributing to their apoptosis in PVL. Vanadium is a metal of the transition series capable of inducing iron release from ferritin (Chapter 4). Because oligodendrocytes acquire iron from extracellular H-ferritin and also express intracellular H-ferritin, we hypothesized that vanadium interference with normal iron homeostasis with both intracellular and extracellular ferritin would result in OPCs destruction, which in fact we demonstrated happens in Chapter 4. Vanadium interaction with ferritin and its effect on oligodendrocytes as presented in this thesis, illustrates point that other heavy metals may act in similar fashion and that the unique physiological relationship of oligodendrocytes with the ferritin pathway of iron acquisition makes them potentially very vulnerable to the toxicity of these heavy metals at the critical periods during the development. Currently, it is unknown which heavy metals may have a similar effect on developing oligodendrocytes through H-ferritin, which should be further investigated, especially for those heavy metals that have confirmed neurological effects, such as lead (Pb), mercury (Hg), and cadmium (Cd) (Rice and Barone 2000). For example, in addition to well established neuron-toxic effects, chronic lead exposure leads to demyelination and hypomyelination in the CNS (Coria et al. 1984; Coria et al. 1985). Oligodendrocytes have been reported to be more vulnerable to lead exposure than astrocytes, with OPCs being more sensitive than mature oligodendrocytes (Deng et al. 2001), a cytotoxic pattern very similar to what we observed for vanadium exposure (chapter 4). Although lead is reportedly absorbed at least in part through the iron transport machinery, it is not clear whether lead interacts/interferes with the ferritin-iron homeostasis in the developing oligodendrocytes and whether this contributes to lead-induced demyelination. These represent important questions for future research.
Does the presence of Tim-2 on oligodendrocytes make them vulnerable for cross-talk with the immune system through interaction with Sema4A?

Before H-ferritin was determined as a ligand of Tim-2, class IV semaphorin Sema4A has been the only reported ligand of Tim-2 (Kumanogoh et al. 2002). We thought that Tim-2 on oligodendrocytes may engage Sema4A on lymphocytes during neuroinflammatory attack in CNS, such as EAE. In 2002 in *Nature*, Kumanogoh, et al. demonstrated that Sema4A is expressed in T and B lymphocytes and dendritic cells, and that its receptor on CD4+ helper T cells is Tim-2. Furthermore, inactivation of Sema4A by inactivating antibodies resulted in significant decrease in clinical and histopathological severity of EAE, mouse model of Multiple Sclerosis. Although, semaphorins have been shown to be important in neural development as axonal guidance molecules, little was known about function of Sema4A in CNS and virtually nothing about its function in oligodendrocytes. A close relative of Sema4A, Sema4D protein (CD100), which is also highly expressed in the immune system, has been shown to collapse processes and cause apoptosis of oligodendrocyte progenitors (Giraudon et al. 2004). Because blocking Sema4A and Tim-2 independently resulted in protection from EAE (Chakravarti et al. 2005; Kumanogoh et al. 2002), we simply had to ask the question whether Sema4A interacted with Tim-2 on oligodendrocytes and whether it had any cytopathic roles on oligodendrocytes that could contribute to demise of these cells in EAE and be relevant to Multiple Sclerosis.

Indeed, we have found very high expression levels of Sema4A within the MS plaque, while immunoreactivity for this protein was very low in the normal white matter. The Sema4A immunoreactivity in the MS plaque was mainly confined to lymphocytes.
(perivenular cuffs) and foamy macrophages within the plaque. *In vitro* experiments demonstrated that Sema4A is cytotoxic and pro-apoptotic to OPCs, but not to astrocytes, and that there is specific binding preference of Sema4A for OPCs over astrocytes, which was not blocked by either cold H-ferritin or Tim-2 blocking antibodies that effectively compete H-ferritin binding to these cells. Collectively our results demonstrated that the receptor/cell surface binding site of Sema4A on oligodendrocytes is distinct from H-ferritin and likely not Tim-2. These data reinforce several recent papers (Chen et al. 2005; Wilker et al. 2007) reporting inability to demonstrate Tim-2/Sema4A interaction and inability to reproduce Kumanogoh’s finding even in the immune system, suggesting that the original proposal about the interaction between these proteins should be re-examined and confirmed.

Although the effects of Sema4A in oligodendrocytes appear to be independent of Tim-2, Sema4A protein clearly may play a very important role in neuro-immune crosstalk that may contribute to the oligodendrocyte demise in Multiple Sclerosis. So, this represents an exciting, but separate area for future research. An obvious future question to pursue is to determine what is the binding receptor for Sema4A in oligodendrocytes that mediates some of the biological effects of Sema4A that we observed. We predict that this receptor will be of Plexin family, as those have been demonstrated as cellular receptors for wide array of other semaphorins (Mann and Rougon 2007; Mizui et al. 2009; Moretti et al. 2006; Tessier-Lavigne and Goodman 1996). This hypothesis is reinforced with recent report demonstrating that Sema4A protein is capable of binding B-type plexins and Plexin D1 in endothelial cells (Toyofuku et al. 2007). Which of those
plexin receptors (if any) is binding partner of Sema4A that transduces its effects on developing oligodendrocytes should be further determined.

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APPENDIX:

Figure 7.1 Cellular iron status influences functional relationship between oligodendrocyte progenitor cells (OPCs) and Bovine Retinal Epithelial Cell (BREC) model of Blood Brain Barrier (BBB). Primary rat OPCs were treated with 10 μM TMH-Fe or 80 μM DFO for 24 hrs in N2S complete media. The untreated OPCs served as a control. At that time, cells were washed thoroughly x3 in excess warm Hank’s buffer and incubated in DMEM/10% FBS media for 16 hrs. This conditioned medium was collected and placed on bovine retinal epithelial cells (BRECs) for 24 hrs. At that time, BRECs were collected and lysed in RIPA buffer and TfnR expression assayed using slot blot (A), which was quantified densitometrically (B). The data representative of two independent experiments demonstrate that cellular iron status of OPCs can affect TfnR expression (and consequently IRP activity) within the BREC model of BBB. The data suggest that iron transport across the BBB may be regulated directly by developing oligodendrocytes in accordance to their cellular need for iron.
Figure 7.2. Iron deprivation results in partial disruption of lipid rafts of primary oligodendrocyte progenitor cells (OPCs). Primary rat OPCs, were treated with 100 uM DFO for 16 hrs in complete N2S media (chemically defined medium for OPCs, supplemented with PDGF and FGF2). Untreated cells served as control. Subsequently, lipid rafts or detergent resistant microdomains (DRMs) were isolated using a cold non-ionic detergent (Triton X). Cells were lysed in 0.5ml of 1% Triton X-100 supplemented with mammalian protease inhibitor cocktail. After cell lysis, cells were homogenized and were put at the bottom of the gradient and then overlayed with 42.5%-35%-5% sucrose gradient. Following centrifugation for 19 hours at 4°C, 1 ml fractions were collected from top to bottom. Proteins recovered in equivalent volumes (55µl) of each fraction were separated by SDS-PAGE on a 10% gel. Protein bands were transferred to a PVDF membrane and flotillin (lipid raft marker) was visualized using anti-flotillin primary antibody (1:250). The data, representative of two independent experiments, demonstrate flotillin in fractions 3 and 4 for both treatment groups, but significant delocalization and shifting of flotillin into the non-raft fractions. These data suggest that limiting iron availability by DFO treatment results in partial disruption of lipid raft stability in OPCs in this model.
**Figure 7.3. Recombinant H-ferritin delivered iron is redox active.** Primary rat OPCs were treated with subtoxic concentrations of tert-butyl hydroperoxide and recombinant H-ferritin loaded with iron. After 48 hr of treatment, cell viability was determined by MTT assay and absorbance averages plotted and evaluated for significance using one-way ANOVA with Dunnett’s post-test comparisons (***, p<0.001; ns, not significant). Data representative of two independent experiments demonstrate synergistic effect of t-booh and H-ferritin exposure on cell viability of OPCs (A) and proposed Fenton mechanism by which ferritin-delivered iron enhances cytotoxicity of peroxide by catalytic formation of hydroxyl radical (B).

### A

<table>
<thead>
<tr>
<th>Condition</th>
<th>MTT (OD at 595 nm)</th>
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<tbody>
<tr>
<td>0.5 nM H-ferritin-Fe</td>
<td>ns</td>
</tr>
<tr>
<td>200 nM t-booh</td>
<td>ns</td>
</tr>
<tr>
<td>0.5 nM H-ferritin-Fe + 200 nM t-booh</td>
<td>ns</td>
</tr>
</tbody>
</table>

**p<0.001***

### B

**Fenton reaction:**

\[
Fe^{2+} + R\text{-}OOH \rightarrow Fe^{3+} + OH + R\text{-}OH
\]

**Hydroxyl radical (ROS)**
Figure 7.4. Structures of TMH-ferocene (A) and ferric ammonium citrate (B).
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